EFFECT OF FERMENTATION AND NUTRITIONAL CONDITIONS ON THE PROFILE OF FLAVOUR ACTIVE ESTER COMPOUNDS IN BEER

BY

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Submitted in fulfilment of the academic requirements for the degree of Master of Science (MSc) in the Discipline of Microbiology, School of Biochemistry, Genetics and Microbiology, Faculty of Science and Agriculture at the University of KwaZulu-Natal (Westville Campus).

As the candidate's supervisor's, I have approved this dissertation for submission.

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PREFACE

The experimental work described in this dissertation was carried out in the School of Biochemistry, Genetics and Microbiology; University of KwaZulu-Natal (Westville Campus), Durban, South Africa from July 2009 to December 2011, under the supervision of Professor B. Pillay and the co-supervision of Dr. A. O. Olaniran.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

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ACKNOWLEDGEMENTS

The author wishes to extend her sincere gratitude to the following person(s) and organizations:

Dr. A. O. Olaniran, Discipline of Microbiology, UKZN (Westville campus) for his time, supervision and contribution during the course of this study;

Prof. B. Pillay, Discipline of Microbiology, UKZN (Westville campus) for project supervision;

Mr. and Mrs. Hiralal (parents of the author) for their support and assistance;

FoodBev SETA for financial support;

Mr. B. Lamaletie (SAB) for supplying raw materials for this project;

The staff and postgraduate students of the Discipline of Microbiology, UKZN (Westville campus) with special reference to Ms. N. S. Abbai, Mr. B. Naidoo, Ms. A. Arjoon, Ms. L. Govender and Ms. F. Malick;

Her brother, Mr. C. Hiralal, and all friends with special reference to Mr. S. Boodhoo for their support, encouragement and love;

God, for guiding and granting her the strength to complete the study.

ABSTRACT

During fermentation, the yeast *Saccharomyces cerevisiae* produces a broad range of aroma-active esters that are important for the desirable complex flavour of beer. The sensory threshold levels of these esters in beer are low, ranging from 0.2 ppm for isoamyl acetate to 15-20 ppm for ethyl acetate. Although esters are only present in trace amounts in beer, they are extremely important as minor changes in their concentration may have dramatic effects on beer flavour. Therefore, optimization of the concentrations of these aroma-active esters in beer is of interest in beer brewing. The number and concentration of esters in beer may be influenced by the fermentation parameters, nutritional composition of fermentation medium and yeast strain type. Therefore, this study investigated the influence of fermentation temperature, pH, and wort nutritional supplements (amino acids and zinc) on the production of yeast-derived ester compounds. In addition, the overall fermentation performance was evaluated based on the reducing sugar and Free Amino Nitrogen (FAN) utilization, ethanol production and yeast cell density. These parameters were analysed using the Dinitrosalicyclic acid method, Ninhydrin assay, Gas Chromatography and standard spread plate technique. The concentration and stability of ethyl acetate, isoamyl acetate, phenyl ethyl acetate, ethyl hexanoate, ethyl decanoate and ethyl octanoate was monitored during storage at 4 ^{o}C and room temperature (RT), in the final beer by Chromatography. The expression levels of the ester synthetase genes under conditions that resulted in the highest increase in ester production were quantified by Real-Time PCR. For the lager beer, the best fermentation performance was achieved at RT (±22.5^{o}C), resulting in the utilization of the highest amount of nutrients and production of 4.86% (v/v) ethanol. This was accompanied by the highest production of acetate and ethyl esters, which were 40.86% and 87.21%, respectively, higher than that of the control. Spent yeast density ranged from 2.492 to 3.358 mg/ml for all parameters tested, with the highest yield produced when wort was supplemented with 0.120 g/l zinc sulphate. Fermentations at 14 °C yielded the highest foam head stability and spent yeast viability with a foam head rating of 2.67 and a spent yeast viability of 3.85×10^{7} cfu/ml. Ester compounds were relatively stable at 4 °C than at room temperature decreasing by only 7.93% after three months. Of all the volatile esters produced, ethyl decanoate was the least stable, with a 36.77% decrease in concentration at room temperature. For the ale beer, the best fermentation performance which resulted in the highest nutrient utilization was achieved when wort was supplemented with 0.75 g/l L-leucine resulting in the utilization of the

highest amount of nutrients (51.25% FAN and 69.11% reducing sugar utilization) and production of 5.12% (v/v) ethanol. At the optimum fermentation pH of 5, 38.27% reducing sugars and 35.28% FAN were utilized, resulting in 4.32% ethanol (v/v) production. Wort supplemented with 0.12 g/l zinc sulphate resulted in 5.01% ethanol (v/v) production and 54.32% reducing sugar utilization. Spent yeast density ranged from 1.985 to 2.848 mg/ml for all parameters tested with the highest yield produced when wort was supplemented with 0.120 g/l zinc sulphate. This was also accompanied by the highest yeast viability of 2.12×10^7 cfu/ml achieved on day 3 of fermentation. Supplementation with 0.75 g/l L-leucine yielded the highest foam head stability with a rating of 2.67. Overall, ester compounds were relatively more stable at 4 °C than at RT decreasing by only 6.93% after three months, compared to a decrease of up to 16.90% observed at RT at the same time. Of all the volatile esters produced, ethyl octanoate was the least stable, with a 32.47% decrease in concentration at RT, phenyl ethyl acetate was the most stable ester at RT, decreasing by 9.82% after three months. Wort supplemented with 0.75 g/l L-leucine resulted in an increase in isoamyl acetate and phenyl ethyl acetate production by 38.69% and 30.40%, respectively, with a corresponding high expression of alcohol acetyltransferases, *ATF2* (133.49-fold higher expression than the control). Elevation of fermentation temperature to RT resulted in the upregulation of *ATF2* (27.11-fold), and producing a higher concentration of isoamyl acetate. These findings indicate that ester synthesis during fermentation is linked to both substrate availability and the regulation of gene expression. Therefore, it would be possible to manipulate the expression of certain ester synthestase genes to create new yeast strains with desirable ester production characteristics. Results from this study also suggest that supplementing wort with essential nutrients required for yeast growth and optimizing the fermentation conditions could be effective in controlling aroma-active ester concentrations to a desired level in beer.

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CHAPTER 1: Introduction and Literature Review

1.1 Introduction

When considering brewing of beer in its most simplistic form, it undoubtedly represents mankind's oldest biotechnology. Whether the discovery of the desirable beverage can be ascribed to accidental contamination of grains or the natural curiosity of humans remains a mystery (Lodolo *et al*., 2008). Its success depends on combining a sound understanding of the science involved with an equally clear grasp of the practicalities of production. Beer consumption continues to rise in Africa, Latin America and Asia, driven by growth in population and incomes and improvements in beer quality and appearance. Despite economic pressures, total global beer consumption increased in 2010, growing at over 2% after a downturn in 2009, caused by the global economic recession. Over the past five years, the global beer category has maintained an average compound annual growth rate (CAGR) of 3.3%. In 2010, emerging markets grew at an average CAGR of 5.7% – the main growth coming from China, Africa and South America. Within the emerging markets, China recorded volume growth of 6% and, despite inflationary pressures, an increase in volumes of premium lager. Africa saw healthy growth of 8% with increased volume in both the premium and more affordable price segments, driven by Angola, Nigeria, Tanzania, Ghana, Uganda and the Democratic Republic Congo. Looking ahead to 2015, it is likely that growth will continue to be led by emerging markets. The 25 fastest-growing markets are forecast to deliver over 5% CAGR in beer volumes. China is expected to account for almost 40% of this growth with Vietnam, Brazil, Ukraine, Nigeria, India and Peru contributing significantly (SABMiller, 2011).

Beer is a complex mixture of constituents, brewed from raw materials including water, yeast, malt, and hops and contains a broad range of different chemical components that may react and interact at all stages of the brewing process. Fundamentally, beer is the product of the alcoholic fermentation by yeast of extracts of malted barley. Barley starch supplies most of the sugars from which the alcohol is derived in the majority of the world's beers. In the brewery, the malted grain must first be milled to produce relatively fine particles, which are for the most part starch. The particles are then intimately mixed with hot water in a process called mashing. The enzymes that break down the starch are amylases. They are developed during the malting process, but only start to act once the gelatinization of the starch has occurred in the mash tun

(vessel). After perhaps an hour of mashing, the liquid portion of the mash, known as wort, is recovered either by straining through the residual spent grains (lautering) or by filtering through plates. The wort is run to the kettle where it is boiled, usually for one hour. Boiling serves various functions, including sterilization of wort, precipitation of proteins (which would otherwise come out of solution in the finished beer and cause cloudiness), and the driving away of unpleasant grainy characters originating in the barley. Thereafter, brewers introduce a proportion of hops. Hops are responsible for the bitterness of beer. The hopped wort is cooled and pitched with yeast. There are many strains of brewing yeast (*Saccharomyces cerevisiae*) that are used that give beer its distinct flavour profile (Bamforth, 2000).

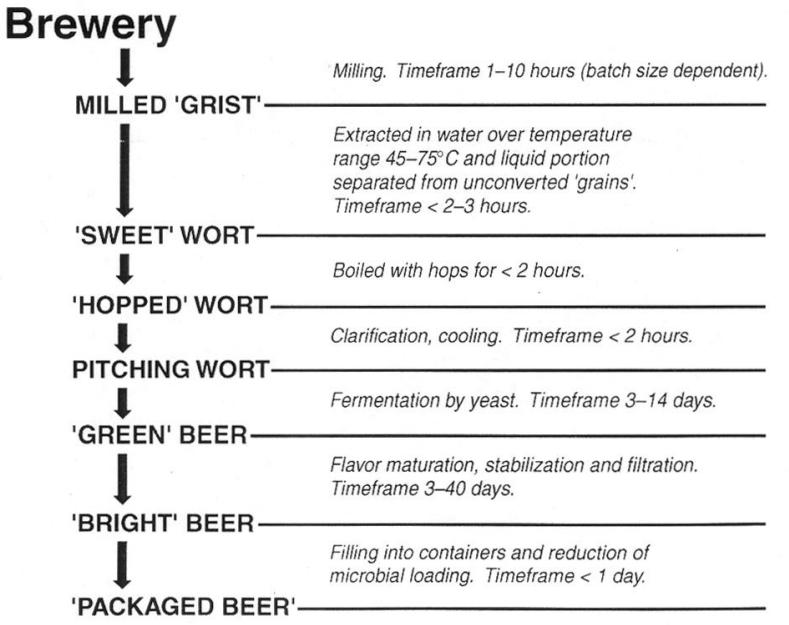


Figure 1.1: Key stages of brewing (adapted from Bamforth, 2000)

Flavour compounds in beer significantly influence the taste, and the rich variety of volatile, low concentration compounds in beer contributes to the sensory properties which affect the sale of beer; therefore, it is very important to study flavour characteristics of the beer (Guido *et al.*, 2004). Many classes of compounds have been shown to play an important role in the flavour characteristics. The volatile fraction can be composed of over 800 different compounds but only several tens of which can be flavour-active (directly involved in producing a flavour sensation when the product is consumed) and must be considered for differentiation purposes (Palamand and Aldenhoff, 1973). The main flavour active compounds can be derived from four

groups namely (i) ingredients such as barley and hops, (ii) by-products of yeast metabolism, (iii) contaminant microorganisms, and (iv) from the stability of flavour compounds during product storage (Kobayashi *et al.,* 2007).

Several reviews are available on the general composition of beer but only a limited amount of work has been reported concerning the flavour influence of some of the beer constituents (Kobayashi *et al.,* 2007; Brown and Hammond, 2003). The complex nature of beer aroma is still not comprehensively understood. The objective of this chapter is to present an overview of the major components that influence beer flavour; the various strategies used to manipulate these flavour compounds; and, the techniques available for their measurement.

1.2 Beer volatile compounds

Apart from ethanol and carbon dioxide, fermenting yeast cells also produce a broad range of secondary metabolites. While these substances are only produced in very low concentrations, they are responsible for the complex aromas of fermented beverages such as beer, wine and sake (Verstrepen *et al*., 2004). The following provides a brief overview of some of these compounds.

1.2.1 Higher alcohols

The major higher alcohols (also known as fusel alcohols) found in alcoholic beverages are the aliphatic alcohols, *n*-propanol, 2-methylpropan-1-ol, 3-methylbutan-1-ol and the aromatic alcohols β-phenylethanol and benzyl alcohol. These compounds may have both positive and negative impacts on aroma and flavour. Large quantities of these higher alcohols (>300 mg/l) in beer can lead to a strong, pungent smell and taste, whereas optimal levels impart desirable characters. Hexan-1-ol, for example, is usually a minor constituent, but its herbaceous and greasy aromas have been linked to negative effects in beverages (Rodrigues *et al.,* 2008). Isoamyl alcohol is the most quantitatively important compound for flavour in the higher-alcohol group. It influences drinkability because beer flavour becomes heavier if isoamyl alcohol concentration increases. Secondly, isobutyl alcohol has an undesirable effect on the quality of beer if its concentration surpasses 20% of the total amount of n-propanol, isobutyl alcohol, and isoamyl alcohol (Kobayashi *et al.,* 2006).

Biosynthesis of higher alcohols involves the decarboxylation of α-keto-acids to form aldehydes, followed by a reduction of the aldehydes to generate the corresponding alcohols. The

α-keto-acids are produced via two key pathways: the catabolic Erhlich pathway which involves degradation of amino acids to their corresponding alcohols and an anabolic pathway, involving *de novo* synthesis of branched-chain amino acids from glucose. The rate-limiting step in the catabolism of branched-chain amino acids is catalysed by mitochondrial and cytosolic branched-chain amino acid aminotransferases encoded by *BAT1* and *BAT2*, respectively (Gee and Ramirez, 1994). Overexpression of *BAT1* increases the concentration of isoamyl alcohol, isoamyl acetate and, to a lesser extent, isobutanol. The uptake of branched-chain amino acids by *S. cerevisiae* is mediated by the *BAP2*-encoded branched-chain amino acid permease. Constitutive expression of the *BAP2* gene results in increased production of isoamyl alcohol, but no increase in isobutanol or amyl alcohol production (Saerens *et al.,* 2008b). The higher alcohols are also involved in the biochemical pathways leading to the synthesis of esters which is another important flavour group (Gee and Ramirez, 1994).

1.2.2 Esters

Esters are plentiful volatile constituents of different foods and beverages such as fruits and fruit juices, olive oil, fermented dairy derivates, beer, wine or distilled alcoholic beverages. During alcoholic fermentation, a sizeable quantity of esters can be produced as a result of yeast metabolism (Campo *et al.,* 2007). Esters are one of the more volatile compounds in beer and hence, impact a great deal on beer aroma. In moderate quantities, they can add a pleasant, full-bodied character to beer aroma. When present in surplus, however, they give beer aroma an overly fruity quality, which is considered undesirable by most consumers (Gee and Ramirez, 1994). The presence of different esters can have a synergistic effect on the individual flavours, which means that esters can also affect beer flavour well below their individual threshold concentrations (Rodrigues *et al.,* 2008).

The synthesis of aroma-active esters by yeast is of great importance because they represent a large group of flavour active compounds, which confer to beer a fruity-flowery aroma (Branyik *et al*., 2008). Ester formation is closely linked to lipid metabolism and growth and is a product of fermentation. Two potential routes for ester formation have been recognized. These are the reaction between an alcohol (such as ethanol) or higher alcohols with a fatty acyl-CoA ester and by esterases working in a reverse direction. Different alcohol acetyltransferases (*ATF* genes) have been identified and the expression of these genes is required for ester

formation. Confirmation from gene disruption and expression analysis of members of the *ATF* gene family indicated that different ester synthases are involved in the synthesis of esters during alcoholic fermentation. Control mechanisms that underpin the oxygen-mediated regulation of *ATF1* gene transcription appear to be closely linked to those involved in the regulation of fatty acid metabolism (Lodolo *et al.,* 2008). There are two main groups of volatile esters in fermented beverages. The first group contains the acetate esters (in which the acid group is acetate and the alcohol group is ethanol or a complex alcohol derived from amino acid metabolism), such as ethyl acetate (solvent-like aroma), isoamyl acetate (banana aroma), and phenyl ethyl acetate (roses, honey). The second group is the ethyl esters (in which the alcohol group is ethanol and the acid group is a medium-chain fatty acid) and includes ethyl hexanoate (anise seed, apple-like aroma), ethyl octanoate (sour apple aroma), and ethyl decanoate (floral aroma) (Saerens *et al*., 2008a). Of these esters, ethyl acetate is typically present in the highest concentration (Kobayashi *et al*., 2008) and represents approximately one third of all esters in beer. The taste threshold concentration for ethyl acetate in beer is 30mg/l, but for lager type beers the recommended concentration is lower than 5 mg/l. The very intensive „fruity' aroma caused by isoamyl acetate is found at concentrations higher than 2 mg/l and 2-phenyl ethyl acetate at 3.8 mg/l. Ethyl hexanote has a low concentration threshold of 0.005 mg/l, ethyl octanoate at 0.5 mg/l and ethyl decanoate at 1.5 mg/l (Smogrovicova and Dömény, 1999). When these concentrations exceed their threshold, it gives the beer an undesirable flavour. Therefore it is important to keep the concentration of volatile esters in the final product below their taste threshold.

The role of ester production in yeast metabolism is unclear, but several hypotheses have been suggested. Some researchers have suggested that esters might be formed to remove toxic fatty acids from the yeast cell (Nordström, 1964), whereas another proposes that esters could simply be overspill products from the yeast's sugar metabolism during fermentation and might be of no advantage to the yeast cell (Peddie, 1990).

1.2.3 Carbonyl compounds

Carbonyls (aldehydes and ketones) have a functional group that consists of a carbon atom double bonded to an oxygen atom. Concentrations of carbonyl compounds in beer are relatively low. Even acetaldehyde, the predominant carbonyl compound in beer, is present at no more than 10 mg/l (Kobayashi *et al.,* 2008). Aldehydes are formed during wort preparation (from

processes, such as Maillard reactions and lipid oxidation) and as a function of the anabolic and catabolic pathways for higher alcohol formation during the fermentation process. Acetaldehyde from yeast metabolism as a step in the production of alcohol from glucose has a crisp green apple flavour. Acetaldehyde is the major aldehyde to consider due to its importance as an intermediate in the formation of ethanol and acetate. Acetaldehyde has a flavour threshold of 10–20 mg/l and its presence in beer above the threshold value results in „grassy' off-flavours (Meilgaard, 1975). However, many tasters can detect this compound at much lower levels. (Lodolo *et al.,* 2008).

1.2.4 Vicinal diketones

Several vicinal diketones (VDKs) are present in beer but the most noteworthy when considering beer flavour is diacetyl (2,3- butanedione) and 2,3-pentanedione (Lodolo *et al*., 2008). The concentrations of these two VDKs are of critical significance for beer flavour. Diacetyl has extensively been considered a serious off-flavour component in beer (Thompson *et al.,* 1970) and has a potent "butterscotch" aroma at concentrations above the flavour threshold around 0.1-0.15 ppm in lager beers. The currently accepted pathway is that diacetyl is formed from the chemical oxidative decarboxylation of surplus α-acetolactate leaked from the valine biosynthetic pathway to the extracellular environment. 2,3-pentanedione is formed similarly from α-acetohydroxybutyrate. This chemical conversion is considered the rate-limiting step of VDK formation. At the end of the main fermentation and maturation phase, diacetyl is re-assimilated and reduced by yeast to acetoin and 2,3-butanediol, compounds with relatively high flavour thresholds. It seems that numerous enzymatic systems are involved in the reduction of VDKs by brewing yeast (Brányik *et al.,* 2008).

1.3 Factors contributing to the production of flavour active compounds in beer

1.3.1 Yeast metabolism

Saccharomyces cerevisiae is extensively used as a biotechnological production organism, along with a eukaryotic model system. It is attractive to work with due to its nonpathogenicity, which has made it to be categorized as a GRAS (generally regarded as safe) organism and its long history of application in the production of consumable products (such as ethanol and bakers' yeast/breads). Currently, fermentative ethanol production by *S. cerevisiae* is still the

most economical method, with many attempts having been made to achieve higher ethanol concentrations using other organisms (Pham *et al*., 2006). The yeasts currently used in breweries are conventionally divided into two main classes, bottom-fermenting and top-fermenting. Beer is therefore divided into two very broad categories, lager and ale, based on which yeast is used. Lager yeast, known as *Saccharomyces pastorianus* or *Saccharomyces carlsbergensis*, is used at relatively cool temperatures (8 to 15 °C), and develops a cloudy mass (flocculates) on the bottom of the vessel. Lager beers produced by bottom-fermenting yeasts are the most common beer types throughout the world (more than 90%). To produce ale beers, strains of *S. cerevisiae* are commonly used in the temperature range of 16 to 25 °C (Ferreira *et al*., 2010).

The unique flavour profiles of beer can be attributed principally to the biochemical activities during fermentation within the yeast cell. Sugars in the wort are converted to ethanol and volatile compounds such as higher alcohols and esters, which are simultaneously formed as by-products of yeast metabolism. These volatile compounds are distinct from the aromatic compounds in malt and hops (Kobayashi *et al.,* 2006). These compounds make an important impact to the aroma and taste of beer (Polaina, 2002).

The flavour compounds are intermediates in pathways leading from the catabolism of wort components (sugars, nitrogenous compounds and sulphur compounds) to the synthesis of components required for yeast growth (amino acids, proteins, nucleic acids, lipids, etc.) (Figure 1.2). The yeast-derived flavour-active compounds can roughly be listed as ethanol, CO_2, carbonyls (aldehydes/ketones), higher/ fusel alcohols, esters, VDKs (diacetyl and pentanedione), fatty and organic acids and sulphur compounds. Ethanol and CO_2 are the primary by-products formed during fermentation as indicated in Figure 1.2 (Lodolo *et al.,* 2008). The by-products discussed in this chapter along with its organoleptic threshold and the flavour it contributes to beer is listed in Table 1.1.

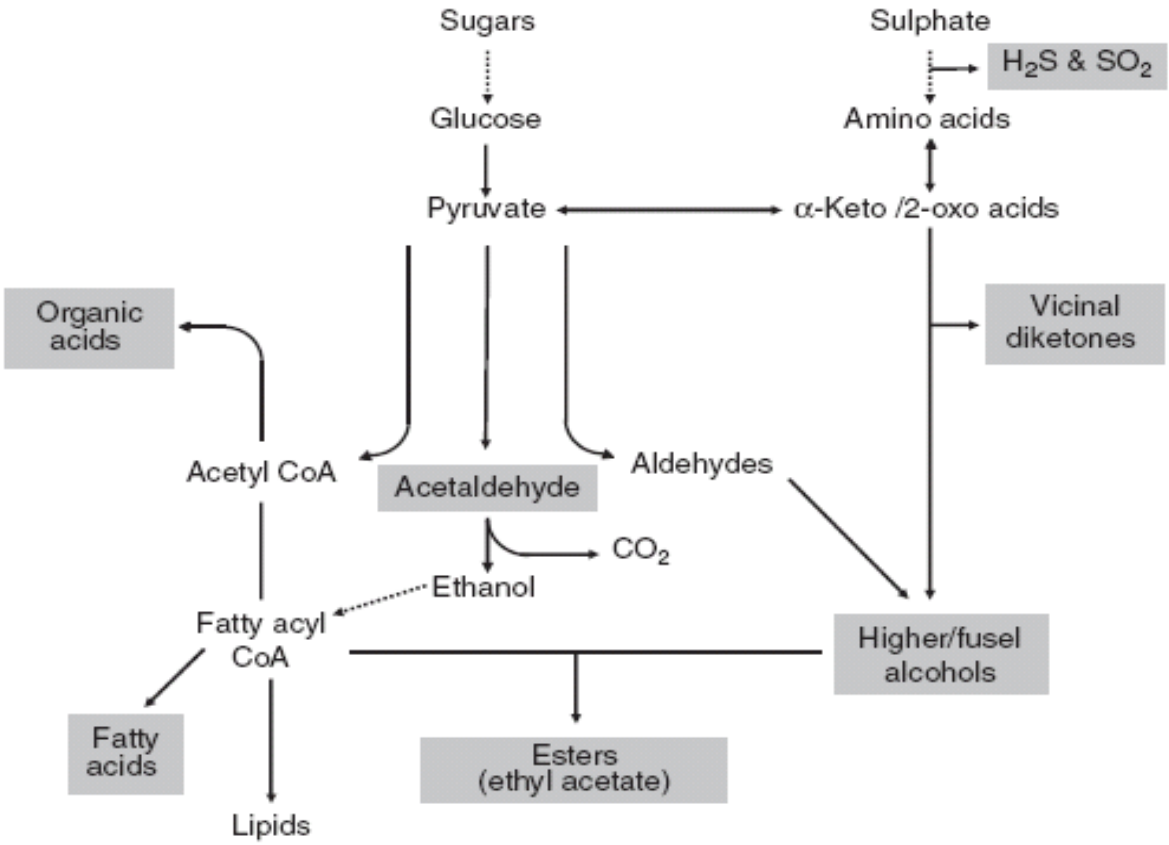


Figure. 1.2: Interrelationships between the main metabolic pathways contributing towards flavour-active compounds in beer (adapted from Hammond, 1993).

The two key nutrient classes that will influence brewing yeast performance are carbohydrates and nitrogenous compounds. Assimilation of these individual nutrients is reliant on the yeast response to the various components. Brewing strains can use numerous carbohydrates (glucose, sucrose, fructose, maltose, galactose, raffinose and maltotriose), with the major characteristic difference between ale and lager strains being the ability of lager yeasts to ferment melibiose (Bamforth, 2000). The generalized sugar uptake pattern initiates with sucrose, which is hydrolysed, resulting in an increase in glucose and fructose concentrations. This is followed by the uptake of the simplest sugars (the monosaccharides glucose and fructose), followed in increasing order of complexity by disaccharides (maltose) and trisaccharides (maltotriose) (Stewart, 2006).

The second major class of nutrients is nitrogenous compounds. A major function of the malt is to provide assimilable nitrogen sources to the yeast. The leading sources of nitrogen in wort are amino acids, ammonium ion and some di- and tripeptides. The greater part of wort-free amino nitrogen (FAN) in wort is utilized by yeast for the purpose of protein formation (structural and enzymic) which is needed for yeast growth (Pierce, 1987) and other functions such as osmoregulation (Hohmann, 2002). The level and composition of wort FAN has a significant influence on higher alcohol, ester, VDK and H_2S formation due to the function of amino acid metabolism in the formation of these flavour compounds (Pierce, 1987; O'Connor-Cox and Ingledew, 1989). Conditions that stimulate fast yeast growth (high temperature and high dissolved oxygen (DO) concentrations) will result in a high FAN utilization, which in turn leads to flavour imbalances (Lodolo *et al*., 2008).

Table 1.1: List of compounds associated with beer flavours (Kobayashi *et al.,* 2008)

Compounds	**Flavour in beer**	**Organoleptic threshold (ppm)**
Higher alcohols		
Propan-1-ol (n-propanol)	Alcohol	800
2-Methyl propanol (isobutyl alcohol	Alcohol	200
2-Methyl butanol (active amyl alcohol)	Alcohol, banana, medicinal, solvent	65
3-Methyl butanol (isoamyl alcohol)	Alcohol	70
2-Phenly ethanol	Roses, sweetish, perfumed	125
Esters		
Ethyl acetate	Solvent, fruity, sweetish	30
Isoamyl acetate	Banana, apple, solvent, estery	1.2
2-Phenylethyl acetate	Roses, honey, apple, sweetish	3.8
Ethyl caproate	Sour apple	0.21
Ethyl capeylate	Sour apple	0.9
Carbonyl compounds		
Acetaldehyde	Green leaves, fruity	25
2,3-Butanedione (diacetyl)	Butter-scotch	0.15

1.3.2 Raw materials

1.3.2.1 Barley Malt

Barley is the most vital raw material for the production of beer. It is a widely grown cereal crop, used for human and animal feed and for beer brewing. This is due to its high enzymatic content that converts starch into fermentable sugars. It is a cereal that contains a husk that protects the embryo during the handling of the grain which is essential during the wort filtration (Silva *et al*., 2008). Malting commences with steeping of barley in water at 14–18 °C for up to 48 h, until it reaches a moisture content of 42–46%. This is usually achieved in a 3-stage process. Raising the moisture content allows the grain to germinate, a process that usually takes 3–5 days at 16–20 °C. In germination, the enzymes break down the cell walls and some of the protein in the starchy endosperm. Amylases, produced in germination, are important for the mashing process in the brewery. Lowering the moisture content by kilning arrests germination, and regimes with progressively increasing temperatures from 50 to perhaps 110 °C are used to allow drying to <5% moisture whilst preserving heat-sensitive enzymes. The more intense the kilning process, the darker the malt and the more roasted and burnt are its flavour characteristics (Bamforth, 2000).

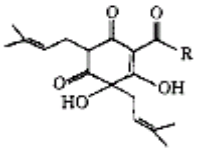
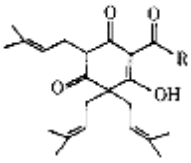
Malt volatiles can be divided into four groups: those that are derived from oxidation of lipid precursors, those formed in the Maillard or browning reactions during wort boiling, various aliphatic sulphur compounds, and phenols. Barley lipids are oxidized by specific lipoxygenases during the germination process to intermediate hydroperoxides which are responsible for the break down during subsequent processing stages (malt kilning through to beer storage) (Bamforth, 2000) to flavour-active aldehydes. If reductases are active, the corresponding alcohols will be produced as a result. The aldehydes are accountable for the grassy, beany taste of green malt and green malt wort, but they are present at much lower levels in kilned malts where some inactivation of the lipoxygenase has occurred. Besides hexanal and 2,4-decadienal these are nearly absent from more highly coloured malt such as crystal malt, caramalt or roasted malt. The yeast has the ability to remove these aldehydes completely during fermentation by reduction to their saturated alcohol counterparts. It is well known, however, that the green, grassy, pea-like flavours do not completely vanish and dominate the flavour of green malt beer, which contains elevated levels of l-hexanol (from reduction of hexanal and 2-hexenal) (Moir, 1992).

Green malt wort contains a series of alkenols probably formed by the action of a thermo-labile reductase, which can decrease the aldehyde portion of alkenols and leave the double bond intact. The yeast is unable to reduce these flavour-active alcohols further and they remain in the finished beer. When reducing sugars (such as glucose or maltose) are heated with little amounts of nitrogen compounds (amines, amino-acids or simply ammonium salts) reductones and dyhydroreductones are produced by a series of rearrangements and dehydrations. These reductones are subsequently polymerised to yellow, brown and black melanoidin pigments. These are the important sugar caramelization reactions, which transpire when a broad range of foodstuffs is boiled, roasted, toasted or fried. Fortunately, from a sensory point of view, there is much more to Maillard reactions than this. Many volatile heterocyclic compounds are formed by degradation of the reductones with or without incorporation of nitrogen or sulphur from amines or hydrogen sulphide. These provide a rich source of flavour whose character depends on the composition and concentration of the starting sugars, nitrogen compounds etc., and on conditions of heating. This is why, for example, crystal malt with a higher level of reducing sugars present during roasting tastes quite different to roasted malt (Moir, 1992).

1.3.2.2 Hop and its degradation products

Generally, beer is „„hopped'' by the addition of dried hop cones (female flowers of the species *Humulus lupulus* L.) into the wort kettle during the boiling step of beer making. The addition of hops has been found to enhance the quality of the beer as well as introducing the characteristic bitter taste, which is a desired flavour preferred by consumers (Royle *et al.,* 2001). Hops contain many diverse groups of organic compounds. Of specific interest are the resins containing mainly hop acids, hop oil and polyphenol. These three classes are important as biochemical markers to differentiate hop varieties. The hop acids, part of the soft resin fraction, consist of related series: α-acids (humulone, cohumulone and adhumulone); and β-acids (lupulone, colupulone and adlupulone) (Royle *et al.,* 2001).

Table 1.2: Structures and Properties of Major Hop Resins (Palamand *et al.,* 1973)

Compound	Name	R Group
 α acids	Hurnulone	$CH_2CH(CH_3)_2$
	Cohumulone	$CH(CH_3)_2$
	Adhumulone	$CH(CH_3)CH_2CH_3$
 β acids	Lupulone	$CH_2CH(CH_3)_2$
	Colupulone	$CH(CH_3)_2$
	Adlupulone	$CH(CH_3)CH_2CH_3$

These compounds (Table 1.2) occur as pale-yellowish solids in the pure state, are weak acids, exhibit very poor solubility in water and have almost no bitter taste (Cortacero-Ramırez *et al*., 2003). The α-acids are flavourless, but, upon continued boiling in the wort, they are isomerized to the bitter-tasting iso-α-acids or isohumulones (beer bitter acids) (Royle *et al.,* 2001). Since, the efficiency (utilization) of isomerization here is of the order of 30%, a processing industry has developed producing iso-α-acids in almost quantitative yields from the hop α-acids (Cortacero-Ramırez *et al*., 2003).

Iso-α-acids are hop-derived compounds, present in low concentrations in beers (≈100 mg/l), and are primary flavour constituents, next to ethanol and carbon dioxide. The threshold value for the bitter taste of the iso-α-acids in water has been estimated at 6 mg/l. The concentration of iso-α-acids in beer is in the range 10-100 mg/l. The solubility of the iso-α-acids in beer is much higher than that of the α-acids. In addition to imparting bitter taste to beer, the iso-α-acids display other attractive features: it has tensioactive properties, thus stabilizing the beer foam; and, they inhibit the growth of Gram-positive bacteria, thus protecting beer against these microorganisms (Royle *et al.,* 2001).

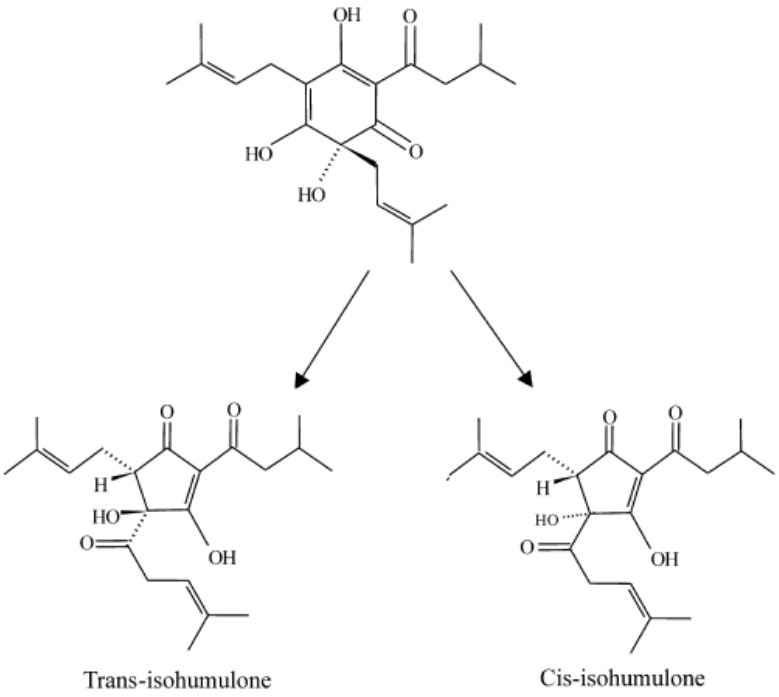


Figure 1.3: Isomerization of α acids to iso- α acids (Cortacero-Ramırez *et al*., 2003)

Iso-α-acids is the main source of bitter flavour in beer and is concentrated in beer foam. Numerous researchers have found that the isocohumulones were concentrated to a lesser extent than their less polar isohumulone and isoadhumulone counterparts, and one report has indicated a seven-fold concentration of trans-isoadhumulone in beer foam relative to the remaining liquid beer (Royle *et al.,* 2001). This suggests that it is the hydrophobicity of the hop acids that influences their partitioning in beer foam (Royle *et al.,* 2001).

1.4 Stability of flavour compounds in beer

Beer shelf-life is generally determined by its microbiological, colloidal, foam, colour and flavour stabilities. Previously, the appearance of hazes and the growth of beer spoilage micro-organisms were considered the primary cause of shelf life deterioration (Vanderhaegen *et al*., 2006). However, with progress in the field of brewing chemistry and technology, these difficulties are now largely under control. Most of the interest has shifted to factors affecting the changes in beer aroma and taste, as beer flavour is regarded as the most important quality parameter of the product (Vanderhaegen *et al*., 2006). The flavour of bottled beer changes during storage. Beer aging is considered to be a major quality problem because many of the aging

flavours are unpleasant. Furthermore, the type of flavour evolution during storage is difficult to control, making it hard for brewers to guarantee a consistent product quality or to meet some consumers' expectations regarding flavour (Vanderhaegen *et al.,* 2007). Through storage, flavour appears to deteriorate significantly with time at a rate depending on the composition of beer (pH, oxygen, antioxidants, precursor concentrations, etc.) and storage conditions (packaging, temperature, light, etc.) (Callemien *et al.,* 2006)

The literature on beer staling exposes the inadequacies in dealing with the actual sensory changes during storage of beer. Dalgliesh (1977) described the changes in the most detail. However, the Dalgliesh plot (Figure 1.4) is a generalization and shows broad view of the sensory evolution during beer storage and is therefore not applicable to every beer. A steady decrease in bitterness is observed during aging. This is a result of an increasing sweet taste, hence masking the bitterness. In contrast to an initial acceleration of sweet aroma development, the formation of caramel, burnt sugar and toffee-like aromas (also called leathery) corresponds with the sweet taste upsurge. Furthermore, a rapid formation of what is described as ribes flavour is observed. The term ribes denotes to the characteristic odour of blackcurrant leaves (*Ribes nigrum*). Thereafter, the potency of the ribes flavour decreases. According to Dalgliesh (1977), cardboard flavour forms after the ribes aroma. In contrast, according to Meilgaard (1972), cardboard flavour constantly increases to reach a maximum, followed by a decrease. Besides these general findings, other described changes in flavour are harsh after-bitter and harsh notes in taste and wine- and whiskey-like notes in strongly aged beer. Positive flavour attributes of beer, such as fruity/estery and floral aroma have a tendency to decrease in intensity. For the overall impression, the decrease of positive flavours may be just as important as development of stale flavours (Vanderhaegen *et al.,* 2007).

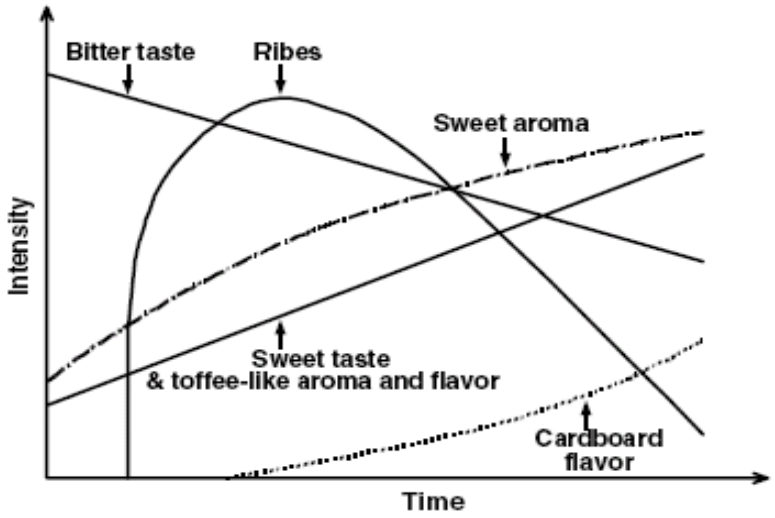


Figure 1.4: Sensory changes during beer aging according to Dalgliesh (1977).

Contact of beer with oxygen results in a rapid deterioration of the flavour and the type of flavour changes depends on the oxygen content of bottled beer. There is a close correlation between the ribes odour and headspace air, and this flavour can be prevented by limiting excessive contact with air (Clapperton, 1976). Furthermore, it is found that beer staling still occurs at oxygen levels as low as possible (Bamforth, 1999), which suggests that beer staling is partly a non-oxidative process.

Apart from oxygen concentration, storage temperature affects the aging characteristics of beer, by affecting the many chemical reactions involved. The reaction rate increase for a certain temperature increase depends on the activation energy of a particular reaction. This activation energy varies with the reaction type, which implies that the rates of different reactions do not increase with increasing temperature in a similar manner. Consequently, beer storage at different temperatures does not produce the same level increase of the staling compounds. Few sensory studies confirm this prediction. According to Furusho *et al*. (1999), cardboard flavour shows different time courses during lager beer storage at 20 and 30 ºC. In the early phase of beer aging, this results in a sensory pattern with relatively more cardboard character when beer is stored at 30 ºC compared to 20 ºC. Lager beer aged at 25 ºC tends to develop a predominantly caramel character whereas, at 30 or 37 ºC, more cardboard notes are dominant. From these examples, it follows that the Dalgliesh plot (Figure 1.4) is a general overview of the sensory changes during

storage. Beer flavour changes are intricate and mostly depend on the beer type, the oxygen concentration and the storage temperature (Vanderhaegen *et al.,* 2007).

1.5 Contaminant microorganisms in beer

Beer does not spoil readily due to its exceptional microbiological stability and it has been recognized for hundreds of years as a safe beverage. The reason as to why beer is an unfavorable medium for many microorganisms is due to the presence of ethanol (0.5–10% w/w), hop bitter compounds (≈17–55 ppm of iso-α-acids), high carbon dioxide content (approximately 0.5% w/w), low pH (3.8–4.7), reduced oxygen content (< 0.1 ppm) and the presence only trace amounts of nutritive substances such as glucose, maltose and maltotriose. These carbon sources are utilized by brewing yeast during fermentation. As a consequence, pathogens such as *Salmonella typhimurium* and *Staphylococcus aureus* are not capable of surviving in beer (Sakamoto *et al.,* 2003). Though, in spite of these unfavorable features, few microorganisms are still able to grow in beer. These, beer spoilage microorganisms, can cause an increase in turbidity and unpleasant sensory changes of beer.

A number of microorganisms have been identified to be potential beer spoilage microorganisms, including a number of Gram-positive and Gram-negative bacteria, and certain wild yeasts. Gram-positive beer spoilage bacteria include lactic acid bacteria belonging to the genera *Lactobacillus* and *Pediococcus*. These microorganisms are recognized as the most hazardous bacteria for breweries and account for approximately 70% of the microbial beer-spoilage incidents. Among the lactobacilli the most important spoilage organisms according to the brewing literature are *Lactobacillus brevis, L. lindneri, L. curautus, L. casei, L. buchneri, L. coryneformiss* and *L. phantarum* (Priest, 1996). Furthermore, the following potential beer spoilage species have been reported: *L. brevisimilis, L. fermentum* and *L. fructivoransi.*. Among the *pediococci* only *Pediococcus damnosus, P. inopjnatus* and to some extent *P. dextrinicus* are of importance for spoilage of beer. However, growth of *P. inopinatus* and *P. dextrinicus* is only possible above pH 4.2 and at low ethanol and hop bitter concentrations (Lawrence, 1988). Only some strains of the above species are capable of growth in beer. *P. pentosuceus* is found on malt and can grow during the early stages of wort production provides the temperature is below 50 °C and hops have not been added, but they have never been reported to cause any defect in the beer produced (Simpson and Taguchi, 1995). The spoilage caused by lactic acid bacteria appears to be

dependent on the composition of the beer produced and to be most hazardous during conditioning of beer and in packaged products. In particular *L. casei* and the *Pediococcus* produce extensive amounts of diacetyl. Honey-like flavours and extended fermentation time have been linked with infections caused by *pediococcus* spp., while *L. brevis* has been shown to cause super-attenuation due to its ability to ferment dextrins and starch (Lawrence, 1988). A fruity atypical aroma has been described even when present at low numbers. Historically, *Acetobacter aceti* and *A. pasteurianus* were both paid a great deal of attention since they are able to convert ethanol into acetic acid thereby changing the flavour of the beer significantly, giving rise to vinegary off-flavours (Lawrence, 1988). However, being aerobes they are not considered a major problem in modern breweries.

The second group of beer spoilage bacteria is Gram-negative bacteria of the genera *Pectinatus* and *Megasphaera*. The importance of these strictly anaerobic bacteria in beer spoilage has increased since the improved technology in modern breweries has resulted in substantial reduction of oxygen content in the final products. Beer spoilage organisms belonging to the genera *Pectinatus* have been reported to grow in beer at pH above 4.3-4.6 and at ethanol concentrations below 5 % (w/v). The spoilage caused by these organisms includes the production of propionic, acetic, and succinic acids, methyl mercaptan, dimethyl sulphide and hydrogen sulphide as well as turbidity (Lawrence, 1988).

Wild yeasts would appear to be less of a serious spoilage problem than bacteria but are considered a serious bother to brewers because of the difficulty in discriminating them from brewing yeasts (Sakamoto *et al.,* 2003). The diversity of wild yeasts in terms of beer spoilage means that no general description can be given; however, wild yeasts are commonly divided into *Saccharomyces* and non-*Saccharomyces* wild yeasts. Often the most severe infections will be caused by *Saccharomyces* spp. which, once isolated, can often be distinguished from lager yeasts by cell morphology and the formation of spores (Jespersen *et al.,* 1996). Among the *Saccharomyces* wild yeasts, most isolates belong to *Saccharomyces cerevisiae* with majority of strains previously described as *S. daststicus, S. pastorianus, S. ellipsoideus* and S. *willianus*. Infections with these yeasts typically cause phenolic off-flavours and super-attenuation of the final beer. The production of phenolic off-favours is due to the ability of these wild yeasts to decarboxylate different phenolic acids such as ferulic and trans-cinnamic acids while the super-attenuation is due to the production and secretion of glycoamylases with starch debranching

activity which enables the wild yeasts to use dextrins not normally fermented by the culture yeast (Jespersen *et al.,* 1996).

1.6 Strategies for controlling the production of BVCs in beer

1.6.1 Alteration of operational and fermentation parameters

1.6.1.1 High gravity brewing (wort specific gravity)

While high-gravity brewing generates most of today's lager beers, it is well known that the fermentation of worts of high specific gravity regularly leads to unbalanced flavour profiles (Verstreppen *et al.,* 2003a). The motivation behind high gravity brewing is to increase the capacity of the brewhouse and the fermentation. High gravity technology decreases the costs of energy, labour and equipment per volume of beer produced, and increases the capacity of the brewery without new investments by utilizing raw materials more efficiently. The quality and stability of beer flavour have been valuable to brewers.

The benefits of high gravity technology are accomplished only if the fermentation time remains moderate and the attenuation and flavour of the beer and the viability of yeast are adequate. These demands have limited the gravity of worts usually used in industry to 16-18 ºP. As the wort gravity increases the factors inhibiting yeast growth, viability and overall fermentation performance include osmotic pressure, metabolites such as ethanol, carbon dioxide, fatty acids and esters and the deficiency of yeast nutrients, amino nitrogen and oxygen (or oleic acid and ergosterol). These problems can be alleviated by using a high pitching rate or higher fermentation temperatures than normal gravity brewing systems (Suihko *et al.,* 1993).

Palmer and Rennie (1974) [cited by Verstrepen *et al*., 2003] detected a four-fold increase in ethyl acetate and isoamyl acetate production when the specific gravity of the wort was increased from 10.5 °P to 20 °P. Likewise, Anderson and Kirsop (1974) [cited by Verstrepen *et al*., 2003] found that the acetate ester concentration increased four- to eight-fold when the specific gravity of the pitching wort doubled. Apart from the total sugar content of the medium, the amounts of different assimilable sugars in wort similarly have an influence on ester production. Generally, worts containing higher levels of glucose and fructose produce more esters than worts with high maltose contents (Verstrepen *et al*., 2003a).

1.6.1.2 Pitching rate

The use of high cell concentrations has been known to enhance fermentation performance. Hence, the use of higher pitching rates can provide a solution to accelerate the fermentation of wort to beer considerably without severe modifications in flavour, on condition of selecting the suitable yeast strain. High pitching rates leads to lower relative yeast growth. This implies that the yeast population at the end of fermentation is relatively older than the yeast population of normal pitched wort. This can have significant economic and technological consequences for the physiology of yeast during subsequent fermentations with the same yeast population. Therefore, it would be intriguing to analyse the impact of pitching rate on yeast physiology in more detail (Verbelen *et al.,* 2008). Several reports on the effects of pitching levels on beer fermentation and flavour compounds are available, but contradictory results have been reported. Anderson and Kirsop (1975) indicated that an increased pitching rate decreased the levels of ethyl acetate and isoamyl acetate. Slaughter and McKernan (1988) showed that inoculum size affected yeast properties and production of flavour compounds, but seemed to have little effect on ethanol production. It was reported that it was difficult to draw general correlations between all the properties studied and the inoculum size. Suihko *et al.* (1993) stated that as the pitching rate increased, the maximum amount of yeast in the fermenting wort increased and the development of ethyl acetate and isoamyl acetate decreased, but the effect of pitching rate on the formation of diacetyl and higher alcohols was not clear. Edelen *et al.* (1996) showed that increasing the pitching rate had a significant effect on beer fermentations in terms of shortening fermentation time, attaining yeast peak counts, lowering ester levels and increasing levels of some higher alcohols. Erten *et al.* (2007) found that production of 2-methyl-1-propanol increased with increasing pitching rate, but the formation of 2- and 3-methyl-1-butanol decreased. The pitching rate did not modify ester formation with the exception of isoamyl acetate, where the level declined with an increased pitching rate. Lower pitching rates led to higher levels of diacetyl and 2,3-pentanedione. The effect of pitching rate on flavour compounds clearly merits further investigation.

Pitched yeast is often re-used in the industry to cut cost therefore cropping, storage and washing of spent/flocculated yeast is important. Acid washing of cropped yeast is a common practice used in major breweries and is used to eliminate bacterial contaminants with bactericidal substances such as phosphoric acid. (Cunningham and Stewart, 1998). The typical process

requires a reduction in pH to a value of pH 2.2–2.5 for a few hours at a temperature below 4 °C. Though, the drawbacks of this process are that it may not eliminate all bacteria and it may negatively influence the yeast condition. Reports suggest that acid washing can decrease cell viability (Van Bergen and Sheppard, 2004) and changes in cell surface charge and hydrophobicity (Wilcocks and Smart, 1995). Incorrect use of this application (acid „hot spots', elevated temperatures and extended exposure periods) will lead to increased yeast damage impacting on fermentation performance (Lodolo *et al*., 2008).

1.6.1.3 Oxygen content

In ale and lager fermentations, little oxygen is required to initiate metabolism, but otherwise the alcoholic fermentation is anaerobic. Molecular oxygen has many functions in yeast physiology (Figure 1.5) and various genes are differentially expressed in response to different oxygen environments to normalize cellular metabolism. Furthermore differences in gene expression under aerobic and anaerobic growth conditions, cells respond to decreases in oxygen tension (Lodolo *et al.,* 2008). Oxygen is an essential nutritional element for the synthesis of ergosterol and unsaturated fatty acids (UFA) (Rattray *et al*., 1975). The sterols and UFA are incorporated into the membrane bilayer structure (Prasad and Rose, 1986) of growing cells. Cell membranes support cellular function because the uptake of nutrients occurs across this natural barrier. Yeast that is not provided with a suitable oxygen supply will possess inferior membranes with a sub optimum transport capability and a decreased ability to withstand osmotic stresses and high exterior ethanol levels (Piper *et al*., 1984) (Figure 1.5).

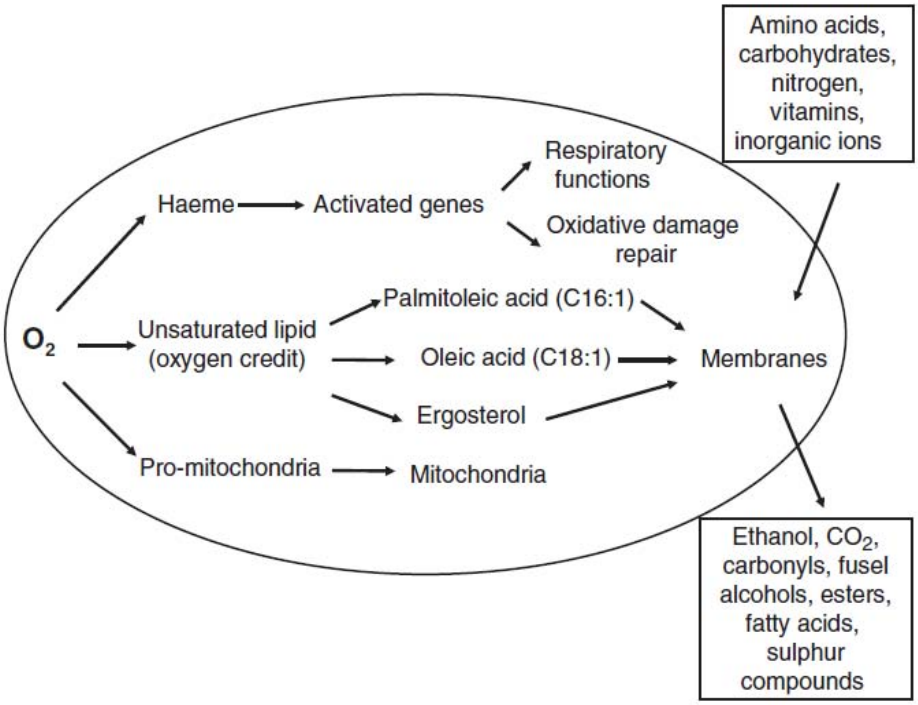


Figure 1.5: Graphical summary of the numerous anabolic roles of oxygen in yeast metabolism (Lodolo *et al.,* 2008)

Oxidation processes are deemed to be a key cause of stale flavour development in beer (Depraetere *et al*., 2008). It is often reported that absorption of oxygen in the mash, during filtration, during boiling, in wort and beer, results in oxidation, which can damage the flavour. The general belief is that wort aeration has a negative influence on wort quality, which leads to a more rapid beer staling, though literature describing aeration related wort oxidation processes is very scarce (Depraetere *et al*., 2008). Depraetere *et al.,* (2008) reported that wort oxygenation does not play an important role for determining flavour stability as wort oxygenation is not continuous.

1.6.1.4 Carbon dioxide

The importance of carbon dioxide (CO_2) for the growth and metabolism of microorganisms has been documented for several decades (Kruger *et al.,* 1992). The inhibitory and regulatory outcomes of CO_2, on yeast metabolism and fermentation have been broadly studied and a number of review articles have been published (Kruger *et al.,* 1992). Much of this

work centered on the use of a deliberately applied top pressure of CO_2, to regulate fermentation. More current studies have observed at the effects of CO_2 under milder conditions in the absence of any deliberately applied top pressure. Some of the effects of CO_2 supersaturution that have been described include: inhibition of cell growth, loss of cell viability, reduced fermentation rate, increased cell volume, approximate doubling of DNA content, increased RNA content, decreased protein content, increased amino acid content and changes in the lipid content and degree of unsaturation of the cell membrane fatty acids (Kruger *et al.,* 1992).

Knatchbull and Slaughter (1987) [cited by Kruger (1992)] summarized the effects of low CO_2 pressures on the formation of flavour-active volatiles by yeast. Results showed that CO_2 pressures of 0.5 and 1.0 atm (100 kPa) lead to reduced fusel alcohol and ester concentrations whereas acetaldehyde levels were increased. Nevertheless, the overall formation of fusel alcohols was less affected than that of the esters. The reduction in ester and fusel alcohol formation is partially caused by the inhibition of growth of metabolically active biomass (Renger *et al.,* 1992).

1.6.2 Alteration of microbial and physiological factors

1.6.2.1 Genetic manipulation of yeast

Yeasts that are used for production of ethanol, wine, beer and bread belong, almost without exception, to the genus *Saccharomyces,* and are genetically closely related (Hansen *et al.,* 1996). Although some research has been done on the genetic make-up of other strains, the most thorough studies on industrial strains have been made on lager brewing yeast (Hansen *et al.,* 1996). *Saccharomyces cerevisiae* is one of the best genetically characterized single cell eukaryotes as its genome is fully sequenced and has been analyzed exhaustively. There are principally two kinds of yeast used in brewing that correspond to the ale and lager types of beer. Genetic engineering can be used to alter the characteristics of yeast and barley in ways that enhance their performance in brewing. Different experimental approaches are directed to the modification of the brewer's yeast, to produce beer with better properties or new characteristics. In most cases, technical advances allow the construction of new strains of yeast with the desired properties. Currently however, the public concerns about the use of genetically modified food pose a barrier to the use of these strains (Polaina *et al.,* 2002).

Genetic manipulation can be used to eliminate problems during fermentation such as preventing the production of undesired flavour compounds. For example, during the lager beer process (‚alcoholic fermentation') a small amount of the first intermediate in valine biosynthesis, α-acetolactate, leaks out of the cells. This compound is gradually converted to diacetyl in a non-enzymatic reaction, and imparts an undesirable, butter- like flavour to the beer. The key purpose of the time-consuming secondary fermentation (lagering) in lager brewing is the elimination of diacetyl and α-acetolactate. The homologue from the isoleucine biosynthetic pathway, 2,3-pentanedione, plays a similar, although less distinct function. During the secondary fermentation (lagering), the yeast takes up the diacetyl and reduces it to acetoin and further to 2,3-butanediol which have higher flavour thresholds. Numerous methods have been taken to reduce the concentration of α-acetolactate in the yeast cytoplasm, and thus leakage to the beer (Hansen *et al.,* 1996). This will provide a substantial benefit for the brewing industry. The first approach requires manipulation of the isoleucine-valine biosynthetic pathway, either by blocking the biosynthesis of the diacetyl flavour or α-acetolactate into valine before it is converted into diacetyl. Cabane *et al.* (1974) were first to propose that the deleterious mutation of the brew's yeast *ILV2* gene in brewers yeast would resolve the diacetyl issue. This or any alternative action on the valine pathway needs the manipulation of specific genes encoding enzymes of the pathway. These genes have been cloned from *S. cerevisiae* and characterized (Poliana *et al.,* 1984). *S. carlsbergensis*-specific alleles of the *ILV* genes from the brewer's strain have also been cloned (Peterson *et al.,* 1986). Due to the genetic complexity of the brewing strain (a hybrid with about four copies of each gene, two from each parent), the elimination of the *ILV2* function requires the very laborious task of eliminating each of the four copies of the gene present in the yeast. This result has not been described so far. An alternative can be to boost the activity of the enzymes that direct the following steps in the conversion of α-acetolactate into valine: the reductoisomerase, encoded by *ILV5* and possibly the dehydrase, encoded by *ILV3* (Villanueva *et a*l., 1990). To accomplish the desired effect, it could be satisfactory to manipulate only one of the four copies of the *ILV* genes present in the brewer's yeast. A clever procedure to inhibit the *ILV2* function, by using an antisense RNA of the gene, has been described (Xaio *et al.,* 1988). Additional approaches make use of an enzyme, acetolactate decarboxylase, which catalyzes the direct conversion of acetolactate into acetoin, bypassing the production of diacetyl. Different microorganisms produce this enzyme. Its usage for the accelerated maturation of beer was

recommended years ago, and presently is commercially accessible for this use. A clear alternative is to express a gene encoding a-acetolactate decarboxylase in the brewing yeast. This has been carried out by different groups of researchers (Polaina *et al.,* 2002).

The yeast metabolism during beer fermentation leads to the formation of higher alcohol, esters and other compounds, which are vital to the aroma and taste of beer. A first group of compounds important to beer flavour are isoamyl and isobutyl alcohol and their acetate esters. These compounds originate from the metabolism of valine and leucine (Dickinson *et al.,* 1992). Two genes, *ATF1* and *LEU4*, encoding enzymes involved in the formation of these compounds, have been effectively manipulated to increase their biosynthesis. *ATF1* encodes alcohol acetyl transferase and its over-expression has been shown to increase the production of isoamyl acetate (Fujii *et al.,* 1994). *LEU4* encodes α-isopropylmalate synthase, an enzyme that regulates a key step in the formation of isoamyl alcohol from leucine. This enzyme is inhibited by leucine (Santayanarayama *et al.,* 1968) but mutant strains resistant to a toxic analog of leucine are insensitive to leucine inhibition have been obtained from a lager strain and shown to produce increased amounts of isoamyl alcohol and its ester (Polaina *et al.,* 2002).

1.6.2.2 Reactor design

As a result of mergers, acquisitions and large-scale production, it has become economically desirable to build larger fermentors. Nevertheless, depending on the shape of the fermentor, critical difficulties are encountered when volumes reach about 10,000-12,000 hectoliter. Larger fermentors lead to poor yeast growth, poor diacetyl reduction and poor ester production. A good example is the „Apollo' fermentors, in which isoamyl acetate levels decrease from 4 ppm at 1 m depth to 0.3 ppm at 18 m depth. The impact of fermentor design on flavour production is principally attributed to increase of carbon dioxide as a result of higher hydrostatic pressure in tall fermentors. Excessive dissolved carbon dioxide usually leads to an inhibition of yeast growth and metabolism, presumably because of the inhibition of essential decarboxylation reactions. As decarboxylation reactions are also necessary for the formation of both fuse1 alcohols and acetyl-CoA, it is alleged that the effect of carbon dioxide on ester production is due to the inhibition of substrate formation. The inhibitory effect of carbon dioxide on ester production is found in most strains, but it has been shown that the specific response differs from strain to strain (Verstreppen *et al.,* 2003a).

1.6.2.3 Fermentation with immobilized yeast

Continuous beer fermentation presents a varied range of benefits, mostly of an economic nature over the traditional batch process. Rapid processing, low capital and production cost, and high volumetric productivity are among the most repeated arguments in favour of continuous fermentation. However, the improved productivity cannot be achieved at the expense of an unbalanced flavour profile of the final product and hence, process optimization is indispensable for any technological innovation (Branyik *et al.,* 2004).

During traditional batch beer fermentation, brewing yeast adapt their relatively versatile metabolism to a changing environment. Altered metabolic fluxes are associated with distinct phases recognizable on the growth curve. The metabolic changes related with entry into individual growth phases are applied at the level of gene expression and enzyme activity. The capability of yeast to sense the changing external environment starts the induction or repression of specific genes corresponding to the actual growth phase, while the modulation of metabolic pathways is mediated through stimulatory or inhibitory effects of intracellular metabolites. Events such as dissolved oxygen and substrate depletion prompt the selective and sequential assimilation of individual compounds, e.g. fermentable sugars and wort amino acids, as well as the formation of metabolic by-products in a coordinated fashion. Therefore the beer flavour results from a mixture of aerobic and anaerobic metabolic products produced during growth phases of different intensity (Branyik *et al.,* 2008). Therefore the flavour profile produced is distinctively different to that of beer fermented traditionally. Higher diacetyl content and lower concentration of esters in beer have been reported (Kronlof *et al.,* 1992). An adequate oxygen supply at the beginning of fermentation is necessary for sufficient biomass growth, though its excess may result in growth related (amino acid metabolism) overproduction of vicinal diketones, decrease of ester synthesizing activity level of cells as well as an increased level of acetaldehyde (Branyik *et al.,* 2004).

1.7 Techniques for measuring flavour compounds in beer

Beer is a complex mixture of constituents containing a broad range of different chemical components in varying concentration. With the increasing export of beer, as a result of market globalization, shelf life problems have become a tremendously important issue for some breweries (Pinho *et al*., 2006). A variety of flavour compounds may arise depending on beer type

and the storage conditions. In contrast to some wines, beer aging is usually considered negative for flavour quality. Currently, the main quality problem of beer is the change of its chemical composition during storage, which alters the sensory properties (Pinho et al., 2006). A better understanding of the key aroma and flavour compounds in beer is of utmost importance for modern brewing technology, helping the selection of raw materials and yeast strains, as well as for routine quality control (da Silva *et al*., 2008). Thus, reliable, and sensible analytical methodologies are required for the extraction and analysis of a great number of beer volatile compounds (Pinho *et al*., 2006).

1.7.1 Traditional Methods: High-Pressure Liquid Chromatography (HPLC) and Capillary Electrophoresis

High-pressure liquid chromatography (HPLC) is a technique commonly used to measure BVC's. However, analysis by HPLC becomes difficult and time consuming if very complex mixtures need to be separated, due to their sensitivity to trace metals in HPLC columns and commercial stationary phases. Furthermore, reproducibility problems are sometimes encountered (Royle *et al.,* 2001). In recent years, Capillary Electrophoresis (CE) has progressed into an attractive alternative to HPLC because has higher efficiencies, faster separation times, ease of operation and requires lower sample volume. The attractive features of CE include its small sample requirement and almost zero solvent consumption; these, and its speed, enable high-throughput chemical analysis of a wide variety of substances. It is widely recognized that CE is a very adaptable technique, partly because of the range of separation modes available (Cortacero-Ramırez *et al*., 2003). The most frequently used modes of CE have been capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), isotacophoresis (ITP), capillary electrochromatography (CEC), capillary gel electrophoresis (CGE) and isoelectric focusing (IEF) (Royle *et al.,* 2001). Limitations of this technique include: instability and irreproducibility of migration times and peak areas, moderate sensitivity, manipulation of separation selectivity is difficult, detection options are limited, routine applications are limited (Haddad *et al*., 1999).

1.7.2 Chromatographic separation techniques

One of the major factors in the quality assessment of a beer is its flavour, determined by a complex mixture of volatile constituents varying in chemical structures and concentration levels. Common methods to study beer volatiles include chromatographic techniques such as: Gas Chromatography and sensory analyses using panel tests. Gas chromatography with mass spectrometry detection (GC-MS) offers a powerful tool to identify and quantify volatile beer compounds (Sikorska *et al.,* 2007). Direct injection that is used for GC detection is not suitable for the quantitative analysis of beer samples because they contain large amounts of nonvolatile compounds that may damage the column. Although the headspace (HS) sampling technique has an advantage over direct injection in which only the volatile compounds in the sample can be injected, its sensitivity is low. Therefore, several extraction methods, which can be performed before injection, have been examined for the measurement of volatile compound concentrations in beer. Recently, two main types of extraction method have been investigated: solid-phase microextraction (SPME) and single-drop microextration (SDME). Both extraction methods may be used in combination with the Head Space (HS) method for beer samples (Kobayashi *et al.,* 2008).

Solid-phase microextraction (SPME) is a new technique that has been applied to the beverage industry. Since its introduction, publications of applications of this promising technique have described the analysis of volatiles in whiskey, hops, wine and spirits. Solid-phase microextraction has also been applied in beer research for specific analysis of sulphur compounds, alcohols and esters, aldehydes with on-fibre derivatisation and in 2006 a general method developed and for the analysis of the volatile fraction from whole beer (de Schutter *et al*., 2008). In SPME, the analyte in the sample is adsorbed to an immobilized polycoat fiber bound to a fine needle and removed by heating in the inlet of the GC or GC/MS device (Kobayashi *et al.,* 2008). This technique eliminates most shortcomings of conventional extraction techniques. It does not require solvents, is experimentally simple and fast and the sampling can be carried out directly under field conditions or on-line. Also, SPME can be coupled with HPLC or CE, but in this case a solvent desorption step is needed (Tankeviciute *et al*., 2001). The ease of the utilization of this technique relies on the possibility of using different thicknesses of solid-phase extraction fiber coatings instead of conducting a series of sample

dilutions. The downside of such an approach is the small number of commercially available fibers of different thicknesses (Plutowska et al., 2008).

In SDME, a microdroplet is held in a large flowing aqueous phase or above an aqueous sample solution, and the analytes in the sample are extracted into the droplet. SDME is simple and is inexpensive when compared with the SPME method. To date, in beer, only alcohols have been quantified (Kobayashi *et al.,* 2008). These methods show clear advantages compared to traditional techniques: (i) eliminates the use of (toxic) organic solvents; (ii) allows the quantification of a large number of molecules with low limits of detection and good linearity over a considerable dynamic range; (iii) integrates sampling, extraction and concentration into a single step; (iv) requires no or little manipulation/preparation of sample; (v) substantially shortness the time of analysis; and moreover (vi) are simple and faster techniques, and (vii) covers a wide range of sampling techniques, including field, *in situ* and air sampling (Rodrigues *et al.,* 2008). Generally accepted disadvantages of SDME are its sensitivity towards organic solvents (the ratio between the chromatographic retention factors of analytes and interferences must be high in order to get good separations) and the limited range of commercially available stationary phases (Campo *et al.,* 2007; Rodrigues *et al.,* 2008). Since the first SPME fibres became commercially available, it has been more frequently used and fields of application have been continuously growing. This includes a wide range of food analysis, to determine the volatile composition of wines, beers, whiskeys and several kinds of fruits, clinical chemistry, environmental chemistry and pharmaceutical analysis (Rodrigues *et al.,* 2008).

1.7.3. Gas chromatography-olfactometry

GC-olfactometry (GC-O) is a valuable analytical procedure for the detection of aroma-active compounds among various volatiles extracted from food samples (Grosch, 1993). The methodology of GC-O can be split into dilution, intensity, and detection frequency methods (Kim *et al*., 2003). Gas chromatography with olfactometric detection is based on the sensory evaluation of the elute from the chromatographic column intended at discovering the active odour compounds. Qualitative and quantitative evaluation of the odour is carried out for each analyte exiting the chromatographic column. This allows establishing whether a given compound is sensory active at a given concentration (i.e. whether it appears in the sample at a level higher than the threshold of sensory detection) and what its smell is, as well as the determination of the

time of sensory activity and the intensity of the odour. Determination of the analyte's odour is possible due to the presence of a special attachment, an olfactometric port, connected in parallel to conventional detectors, such as flame-ionization detector (FID) or mass spectrometer (MS) (Plutowska *et al.,* 2008)

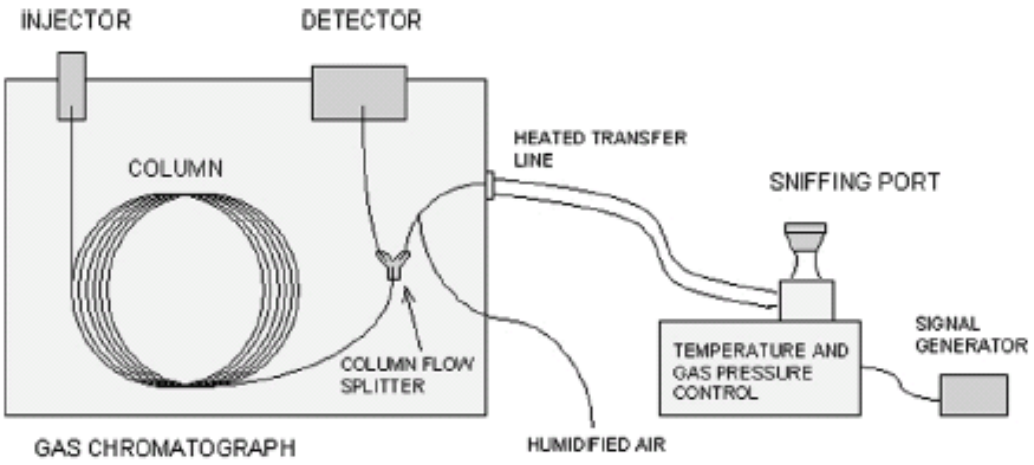


Figure 1.6: Scheme of the gas chromatograph equipped with the olfactometric detector (Plutowska *et al.,* 2008).

Regardless of the fact that odour detectors have been in use for over 40 years, literature indicates that in recent years they have been used more frequently, finding applications especially in the analysis of food and beverages (including alcoholic beverages). Further investigations are still being conducted in order to improve GC–O technique, i.e. to achieve a higher sensitivity and better reliability and repeatability of the results. Another noticeable trend in the development of GC–O is aimed improving reporducibility and reliability of the obtained results by unifying, simplifying and shortening of applied procedure (Plutowska *et al.,* 2008). Recent research by GC-O has given first evidence that some aged stimulated wines contain other novel and naturally rare ethyl esters which may have some impact on their aroma (Campo *et al*., 2007).

1.7.4 Aroma extraction dilution analysis

Several quantitative methods exist for the evaluation of the intensity of odours and their relative influence on the odour of the sample (Plutowska *et al.,* 2008). In recent years, new techniques, such as aroma extraction dilution analysis (AEDA), have been developed to evaluate

the relevance of detected volatiles to odour perception in foods. Using this method, several staling compounds have been identified in beer. In AEDA, stepwise dilutions of an aroma extract are perceived to provide flavour dilution (FD) factors, which are proportional to the relative aroma potency of each compound (Kim *et al*., 2003). In this technique, the diluted flavour extract of beer is assessed by GC–O and by a small number of judges. The extraction method is very important, as it is essential to ensure that extracts with an odour representative of the original product are obtained. The flavour dilution of an odourant corresponds to the maximum dilution at which that odourant can be perceived by at least one of the judges. Consequently, the FD factors give an estimation of the importance of volatiles for the perceived flavour of a beer sample. The higher the dilution factor, the more important the odour impact (Guyot-Declerck *et al.,* 2005). The method should be regarded as a first step in the screening and not to obtain conclusive results about the relevance of flavour compounds (Vanderhaegen *et al.,* 2006).

The dilution methods have also some drawbacks. The total analysis time is long, especially in the case of large evaluator panels; therefore the number of evaluators taking part in the analysis is usually limited. This, in turn, increases the probability of obtaining low-precision and subjective results. In addition, the results depend on the sensory detection threshold of the analytes rather than on the realistic intensity of the analyte odour in a given sample (Etievant *et al*., 1999). Dilution to threshold methods is also criticized for the underlying false assumption that the odour intensity increases in parallel with the concentration for all odour components in a sample (Petka *et al*., 2005).

1.8 Scope of this study

Most of the previous studies on esters have focused on the production of acetate esters using ale brewing strains, because of their relatively high abundance with very little attention paid to ethyl esters and use of other yeast strains in the fermentation process. Despite the extensive information published on flavour chemistry, odour thresholds, and aroma descriptions, the flavour of complex products such as wine and beer cannot be predicted. In this study, the influence of important fermentation and nutritional parameters on the production of both acetate and ethyl esters by an ale and a lager brewing strain was investigated. The expression levels of the biosynthesis genes responsible for the production of esters during fermentation under the different conditions were also investigated to allow for the prediction of the effect of

fermentation parameters on flavour beer profiles. This could help brewers to identify yeast strains that could produce the desired amounts of esters and higher alcohols in accordance with specific consumer preferences. This study also investigated the effect of storage temperature and time on the stability of the flavour-active ester compounds in both ale and lager.

1.8.1 Hypotheses

It was hypothesized that the fermentation parameters and wort nutritional supplements will strongly influence the concentration and composition of volatile esters in beer and hence the flavour and aroma profiles. It was further hypothesized that the profiles of the volatile compounds in beer will be dependent on the expression level of ester biosynthetase genes under the different fermentation and nutritional conditions.

1.8.2 Objectives

1.8.2.1 To investigate the influence of certain fermentation parameters and wort nutritional supplements on the ester profile and overall quality of the beer produced by an ale and a lager brewing yeast strain.

1.8.2.2 To correlate the expression levels of yeast alcohol acetyltransferase genes *ATF1* and *ATF2*, Acyl-coenzymeA:ethanol *O*-acyltransferase gene *EEB1* and ethanol hexanoyl transferase gene *EHT1* under the optimum fermentation conditions and wort nutritional supplements to the ester profiles in the produced beer.

1.8.2.3 To investigate the influence of storage time and temperature on the stability of acetate and ethyl esters present in beer.

1.8.3 Aims

1.8.3.1 To generate wort from pale and crystal malted barley

1.8.3.2 To pitch the wort with ale brewing strain and lager brewing strains and produce beer

1.8.3.3 To determine the effect of fermentation temperature and pH on acetate and ethyl ester formation.

1.8.3.4 To determine the effect of amino acids and yeast growth factors on the production of acetate and ethyl esters.

1.8.3.5 To determine the expression levels of *ATF1, ATF2, EEB1* and *EHT1* genes using quantitative PCR and correlate with the ester profiles in beer.

1.8.3.6 To determine the influence of storage temperature and duration on the ester flavours and aroma of beer.

CHAPTER 2: Effect of fermentation parameters and wort nutritional supplements on the production of acetate and ethyl esters by a lager brewing yeast strain

2.1 Introduction

During fermentation, the yeast *Saccharomyces cerevisiae* produces a broad range of aroma-active esters that are vital for the desirable complex flavour of beer. The sensory threshold levels in beer are low, ranging from 0.2 ppm for isoamyl acetate to 15-20 ppm for ethyl acetate esters (Peddie, 1990). Although esters are only present in trace amounts in beer, they are extremely important as minor changes in their concentration may have dramatic effects on beer flavour (Saerens *et al.,* 2008a). Many variables are known to affect ester production, including the yeast strain used, the composition of the fermentation medium (wort), and the fermentation conditions.

Wort is a highly complex medium consisting of fermentable sugars (fructose, sucrose, glucose, maltose and maltotriose), dextrins, nitrogenous materials, vitamins, metal ions, mineral salts, trace elements, and many other constituents. In wort, the main nitrogen sources for yeast metabolism are individual amino acids, small peptides, and ammonium ions formed from the proteolysis of barley malt proteins during malting and mashing, collectively known and measured as Free Amino Nitrogen (FAN) (Lekkas *et al.*, 2007). FAN stimulates the active growth of yeast and increases the final cell mass, thereby enhancing fermentation rates (Dragone *et al*., 2004). Adequate levels of FAN in wort ensure efficient yeast cell growth and appropriate fermentation performance. Provision of adequate assimilable nitrogen in the fermentation medium is critical for rapid and full sugar utilization. Nitrogen limitation in wort results in protracted and/ or incomplete (sluggish or stuck) fermentations, while nitrogen concentrations exceeding the recommended level lead to more rapid fermentations and increased yeast mass (O'Connor-Cox *et al*., 1991).

In addition to FAN, metal ions play an important role in brewing and yeast performance in particular (Rees *et al*., 1998). These metal ions are required in the micromolar or the nanomolar range as trace elements necessary for growth (Jones and Greenfield, 1984) and to ensure efficient and complete fermentation (Rees *et al*., 1998). A variety of metal ions are present in wort, the concentration of which depends on the raw materials used and the method employed to produce the wort (Rees *et al*., 1998). Many different ions can be present at

suboptimal levels in brewer's wort due to several factors, including the introduction of stainless steel vessels and poor barley crops (Rees *et al*., 1998). Supplementation of wort with metal ions has been reported to be effective in counteracting these disadvantages (Nabais *et al*., 1988; Hu *et al*., 2003). Magnesium and calcium are two well-studied metal ions that exert significant effects on cell viability and ethanol production (Dombek and Ingram, 1986; Nabais *et al*., 1988), while the effects of other metal ions on yeasts are less understood. Zinc is required by *S. cerevisiae* to maintain its normal growth and metabolism (Eide, 2006). It has been reported that suitable zinc supplementation in the culture medium was beneficial for ethanol production (Tosun and Ergun, 2007). Almost 3% of the *S. cerevisiae* proteome function requires zinc (Eide, 1998), since it is an essential cofactor for many enzymes. Thus far, a total of one hundred and five *S. cerevisiae* proteins that use zinc as a cofactor have been identified. Therefore, supplementing wort with zinc shows promise in alleviating stuck fermentations and enhancing fermentation performance.

Apart from the chemical composition of the medium, there are other process characteristics that affect fermentation performance and hence ester production, namely, aeration of the medium, hydrostatic pressure of the fermentation tank, and fermentation temperature and pH (Saerens *et al*., 2008b). Temperature influences fermentation by increasing the yeast growth and speeding up enzyme action. Moreover, cell sensitivity to the toxic effect of alcohol increases with temperature due to increased membrane fluidity (Reddy *et al*., 2010). Higher temperatures increase the rate of yeast metabolism but the quantitative influence of a temperature change will be different for each biochemical reaction, changing the balance of flavour compounds (Šmogrovičovà *et al.,* 1999). An optimum pH is essential for yeast growth and ethanol production since most of the yeasts propagate well between pHs 4.5 and 6.5, and nearly all yeast species are unable to grow outside this pH range (Reddy *et al*., 2010).

Previous studies on aroma-active esters have focused on acetate esters, because of the relatively high levels that can be produced when using ale brewing strains. This study focuses on the effect of certain nutritional supplements, i.e., L-leucine and zinc sulphate on acetate and ethyl ester production as well as fermentation performance and overall beer quality. In addition, the effect of temperature and pH on fermentation performance, formation of esters and beer quality was also investigated. It is anticipated that findings of this investigation will enable brewers to manipulate ester concentrations in beer to a desired level.

2.2 Materials and Methods

2.2.1 Yeast strain and cultivation conditions

Yeast strains were from the microbiology culture collection (School of Biochemistry, Genetics, and Microbiology, Pietermaritzburg Campus, UKZN). Yeast strains were maintained on malt agar slants and were sub-cultured monthly. All experiments were carried out using a lager strain grown in malt extract broth for 24 h at 30 ºC with shaking at 120 rpm. Two millielitre pre-culture was inoculated into 200 ml malt extract broth for 6 h at 30 ºC with shaking at 120 rpm until it reached an OD_{600} of 1.120. Samples were centrifugated at 4000 rpm for 15 min at 4 °C and the pellet was resuspended in 200 ml wort. 20 ml inoculum was used to pitch 2 L wort at a pitching rate of 20×10^6 cfu/ml.

2.2.2 Beer production

2.2.2.1 Wort preparation

Wort was prepared in a custom designed microbrewery set-up. Malt wort was prepared by crushing 3.080 kg pale malt and added to 9.2 L of water. Mashing was carried out at the following temperatures: 63.5 ºC for 60 min to allow for β-amylase activity, 71 ºC for 30 min to allow for α-amylase activity and 74 ºC for 10 min to inactivate all enzymes. Mash was heated in a beaker that was placed in a water bath. Mash was consistently stirred manually and temperature monitored every 2-3 min. Mash was then centrifuged to separate spent grain and wort. The residual mash was washed with approximately 4 L of water. Mash was then transferred to beakers and washed with 9 L of warm water to remove residual sugars. The wort was then brought to a boil; 5 g of Southern hops was added and allowed to boil for 1 h, followed by the addition of 2.5 g Saaz hops and allowed to boil for a further 10 min. Wort was used immediately after preparation. Several batches of wort were made to test the different parameters.

2.2.2.2 Wort fermentation

Fermentations experiments were set up to determine the effect of temperature, pH, zinc sulphate and L-leucine on fermentation performance and ester production using mini-fermenters (3.5 L) to facilitate the fermentation process on a small scale. Fermentation was performed in duplicate and duplicated readings were taken for each sample collected. Fermentation vessels

containing 2 L of wort which were fermented under various supplementations and fermentation parameters as follows: Zinc sulphate (0.03, 0.06, and 0.12 g/l); L-leucine (0.25, 0.50 and 0.75 g/l), temperature (14, 18 and room temperature [22.5 °C]); and pH (3, 5 and 7). The control fermentation was not supplemented with zinc sulphate or L-leucine and was carried out at 14 °C for the lager strain at pH 5. Fermentations were monitored by an air lock mechanism to ensure the fermentations are not stuck. During fermentation, samples were taken and analysed as described below. Once fermentation was complete fermenter vessels were incubated at 4 °C for 5 days to allow yeast to settle.

2.2.2.3 Fermentation analysis

Samples were collected daily from the fermentation vessel; this was performed by opening the tap at the bottom of the fermentation vessel and removing 5 ml of wort to carry out analysis. Thereafter, the tap was immediately closed and incubated to allow fermentation to continue. Samples were analysed immediately to determine total yeast cell density, free amino nitrogen, reducing sugar and ethanol concentration.

2.2.2.3.1 Reducing Sugar utilization

Three millilitres of the DNS reagent was added to 3 ml wort that was diluted 10 times, in a test tube and the tube contents heated in boiling water for 5 min. While the contents of the test tube was still warm, 1 ml of 40% Rochelle salt solution was added. This was then cooled and the intensity of the dark red colour that had developed was read at 510 nm. Standards were run using varying concentrations of glucose to generate a standard curve from which the amount of reducing sugars present in the sample was estimated (Sadasivam and Manickum, 1996).

2.2.2.3.2 Free amino nitrogen utilization

Free alpha amino nitrogen (FAN) levels in the wort were determined by the standard ninhydrin method using glycine as the reference amino acid. 1 ml of wort was diluted with 9 ml of distilled water and 2 ml transferred into test tubes. Ninhydrin colour reagent (1 ml) was added before heating the tubes in boiling water for 16 min. Samples were allowed to cool and absorbance recorded at 570 nm against a blank containing water in place of the sample (Sadasivam and Manickum, 1996).

2.2.2.3.3 Viable yeast cell

A 1 ml sample was serially diluted and 0.1 ml of the appropriate dilution was spread plated onto Malt Extract agar plate. The plates were incubated at 30 ºC for 48 h, and the final number of colonies on the plates for the dilution containing 30 to 300 colonies were counted and expressed as colony forming units per millilitre (cfu/ml) of the sample.

2.2.2.3 Bottling and conditioning

Settled yeast was removed from each fermenter and beer was transferred into sterile 750 ml sample bottles. The bottles containing the beer were allowed to stand for 30 min before capping. Eight millilitres of a brown sugar (1 g/ml) was added to each bottle to allow for carbonation. The bottles were capped and incubated at 14 °C for five days to allow for bottle conditioning, and thereafter stored at 4 °C until required for further analysis.

2.2.3 Measurement of foam head stability

Foam head stability was assessed according to the modified mini-foam shake tests developed by Van Nierop *et al.* (2004). Twenty milliliters of beer was dispensed into 50 ml glass measuring cylinders, in triplicate and all of the cylinders were sealed with parafilm. Each set of cylinders were shaken at the same time, vigorously up and down 10 times, after which the cylinders were set down on the counter and the parafilm pieced, and a timer set for 15 min. After 15 min the foam was evaluated visually and the cylinders were arranged from best to worst. Ratings of 1 through 5 were given, where 5 is the greatest stability and 1 is the worst.

2.2.4 Analysis of beer colour

Beer colour was measured spectrophotometrically at a wavelength of 430 nm based on the method of Seaton and Cantrell (1993) using distilled water as a blank. Experiments were performed in triplicate and a commercial beer was included in the analysis as a positive control.

2.2.5 Measurement of spent yeast density

Spent yeast density was measured by the method of Soley *et al.* (2005). Ten millilitre samples were centrifuged (6000 rpm for 10 min at 4 ºC). The pellet was then resuspended in a NaCl solution (0.9%, w/v), filtered through a previously dried and weighted Whatman grade

GF/A (Ø 47 mm) glass microfiber filter, and dried at 105 ºC to a constant mass. Thereafter, weight of the filter was subtracted from the weight of the filter containing the dried cellular material to obtain the mass of spent yeast produced. In order to reduce the experimental error, measurements were performed in triplicate.

2.2.6 Analysis of beer volatile esters

The composition and concentrations of esters in the samples were measured using Headspace gas chromatography (HS-GC) coupled with flame ionisation detection (GC-FID). The volatiles from 100 ml of each sample were assessed for acetate esters and ethyl esters in the beer. Beer samples were collected in 250 ml serum bottles (Wheaton) and were immediately closed. Samples were heated for 25 min at 70 °C in a water bath before injecting 1 ml using a gas tight syringe of the headspace into the GC. The oven temperature was held at 50 °C for 5 min, then increased to 200 °C at a rate of 5 °C per min and finally held at 200 °C for 3 min. The FID temperature was kept constant at 250 °C and nitrogen was used as the carrier gas. Standards were run using varying concentrations of ethyl acetate, isoamyl acetate, phenyl ethyl acetate, ethyl decanaoate, ethyl hexanoate and ethyl octanoate to generate standard curves from which the concentration of esters present in the sample was estimated. Standards were heated for 25 min at 70 °C in a water bath before injecting 1 ml using a gas tight syringe of the headspace into the GC.

2.3 Results (Data for results presented in Chapter is shown in Appendix B)

2.3.1 Fermentation performance under different nutritional and fermentation conditions

2.3.1.1 Effect of zinc sulphate supplementation

The profile of sugar utilization and ethanol production during the fermentation period in the absence and presence of varying concentrations of zinc sulphate ($ZnSO_4$) is shown in Figure 2.1a, while the FAN content and yeast density at the same period is shown in Figure 2.1b. Time 0 concentration of reducing sugars and FAN in the wort was 2478 µg/ml and 2386 mg/ml, respectively. The fermentation performance was significantly improved with increasing concentration of $ZnSO_4$ added to the wort. Wort supplemented with 0.120 g/l of $ZnSO_4$ resulted in the highest utilization of reducing sugars and FAN (48.44% and 57.80%, respectively), after the six day fermentation period. Furthermore, addition of $ZnSO_4$ (0.120g/l) resulted in the highest production of 4.86% (v/v) ethanol which was 19.70% higher than the control. Wort supplemented with of 0.06 and 0.120 g/l $ZnSO_4$ increased the yeast cell density up to day 3 of fermentation achieving a maximum cell population of 4.54×10^{11} and 8.97×10^{11} cfu/ml, respectively, thereafter yeast cell density gradually decreased. Wort that contained 0.03 g/l and no $ZnSO_4$ increased the yeast cell population up to day 4 of fermentation reaching a cell density of 4.95×10^{11} and 3.21×10^{11} cfu/ml, respectively, thereafter slowly decreasing (Figure 2.1 b).

2.3.1.2 Effect of L-Leucine supplementation

The profile of sugar utilization and ethanol production during the fermentation period in the absence and presence of varying concentrations of L-leucine is shown in Figure 2.2a, while the FAN content and yeast density during the same period is shown in Figure 2.2b. Initial concentration of reducing sugars in the wort was 2497 µg/ml. In the control experiment the yeast utilized 31.80% of the available reducing sugars. Increasing concentration of L-leucine in the wort resulted in an increase in reducing sugar utilization. Wort supplemented with 0.75 g/l L-leucine resulted in 57.60% reducing sugar utilization which was 81.13% higher than the unsupplemented control. The addition of L-leucine to wort resulted in an increased amount of FAN concentration in the medium.

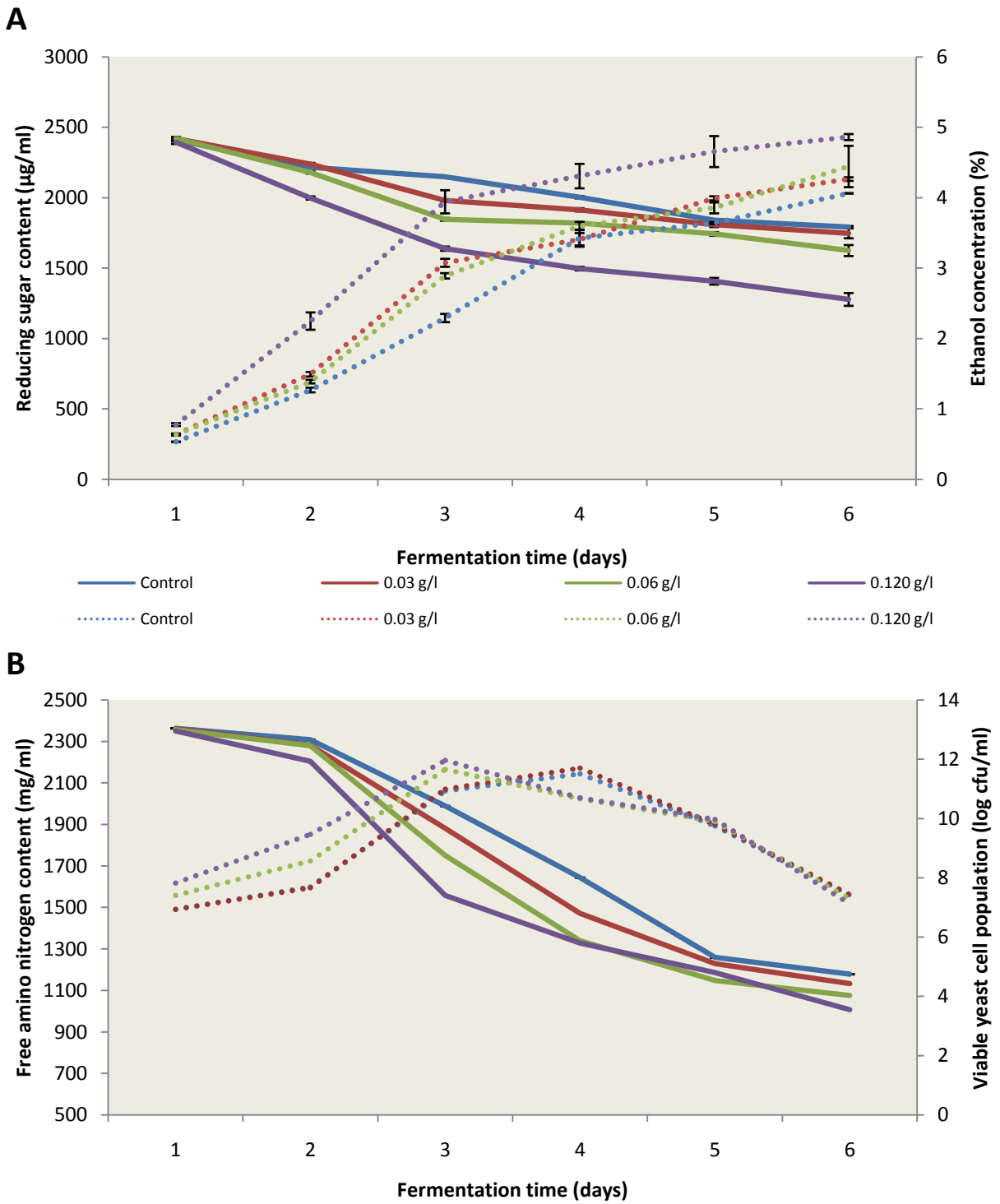


Figure 2.1: Profiles of (a) average reducing sugar and ethanol content and (b) average free amino nitrogen concentration and yeast cell density in the wort during fermentation at varying concentrations of zinc sulphate. Time 0 vales for reducing sugars and FAN are 2478 µg/ml and 2386 mg/ml, respectively. Solid lines represent reducing sugar and free amino nitrogen content and dotted lines represent ethanol concentration and yeast cell density.

The control experiment resulted in 46.86% of FAN utilization by the yeast, with increasing concentration of L-leucine in the medium resulting in more FAN being consumed by yeast. In the control experiment, 3.92% (v/v) ethanol was produced whereas wort containing 0.75 g/l L-leucine produced 4.32% (v/v) ethanol which was 17.65% higher than that in the unsupplemented control. Wort supplemented with 0.50 and 0.75 g/l L-leucine resulted in increased yeast cell density up to day 3 of fermentation achieving a maximum cell population of 4.54×10^{11} and 3.97×10^{11} cfu/ml, respectively, thereafter yeast cell density gradually decreased. Wort that contained 0.25 g/l and no L-leucine supplement resulted in increased yeast cell population up to day 4 of fermentation reaching a peak density of 4.95×10^{11} and 3.23×10^{10} cfu/ml, respectively, thereafter gradually decreasing.

2.3.1.3 Effect of fermentation temperature

The profile of sugar utilization and ethanol production during the wort fermentation at varying temperatures is shown in Figure 2.3a, while the FAN content and yeast density at the same period is shown in Figure 2.3b. Initial concentration of reducing sugars and FAN in the wort was 2253 µg/ml and 2271 mg/ml, respectively. Increased fermentation temperature resulted in greater utilization of both the reducing sugar and FAN. Fermentation at 14 °C resulted in 31.67% reducing sugar utilization; while 40.78% and 50.38% reducing sugar utilization was observed at 18 °C and room temperature (RT), respectively. This was 28.77% and 59.08% higher than the control experiment. Fermentation at 14 °C resulted in 48.60% FAN utilization and increasing the temperature to 18 °C and room temperature (22.5 °C) resulted in 56.70% and 62.23% utilization, respectively, which was 16.67% and 28.05% higher than the control (Figure 2.3 b). Furthermore, the highest fermentation temperature resulted in the highest ethanol production (4.85% v/v) which was 23.10% more than fermentation at 14 °C. Fermentation at 14 °C gradually increased the yeast cell density until day 3 reaching a peak of 3.2×10^{11} cfu/ml and thereafter decreased. Fermentation at 18 °C and room temperature resulted in an increase in yeast cell density until day 4 of fermentation having a maximum of 2.54×10^{11} and 4.52×10^{11} cfu/ml; thereafter a decrease in yeast cell density was observed.

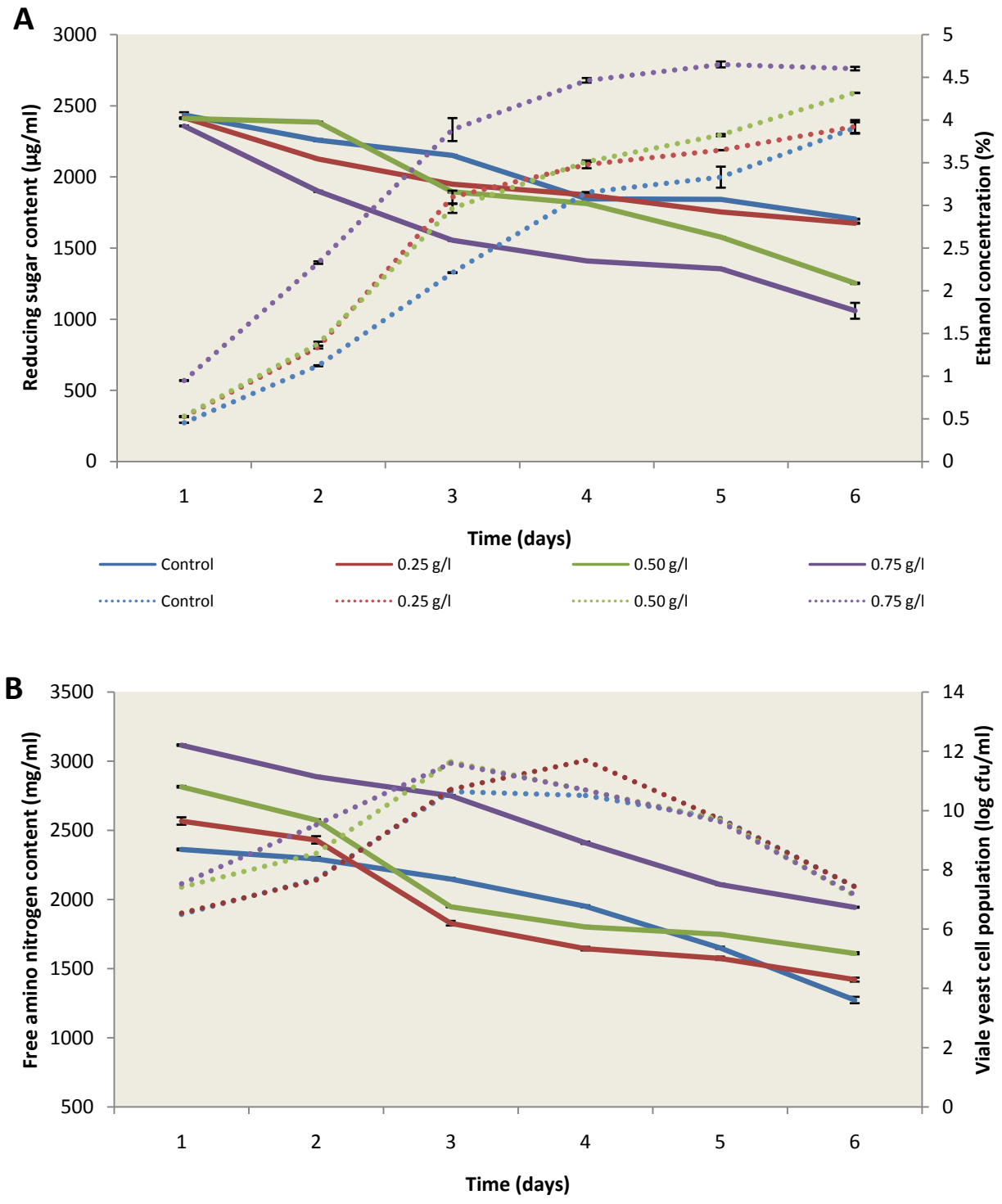


Figure 2.2: Profiles of (a) average reducing sugar and ethanol content and (b) average free amino nitrogen concentration and yeast cell density in the wort during fermentation period at varying concentrations of L-leucine. Time 0 vales for reducing sugars are 2497 µg/ml. Solid lines represent reducing sugar and free amino nitrogen content and dotted lines represent ethanol concentration and yeast cell density

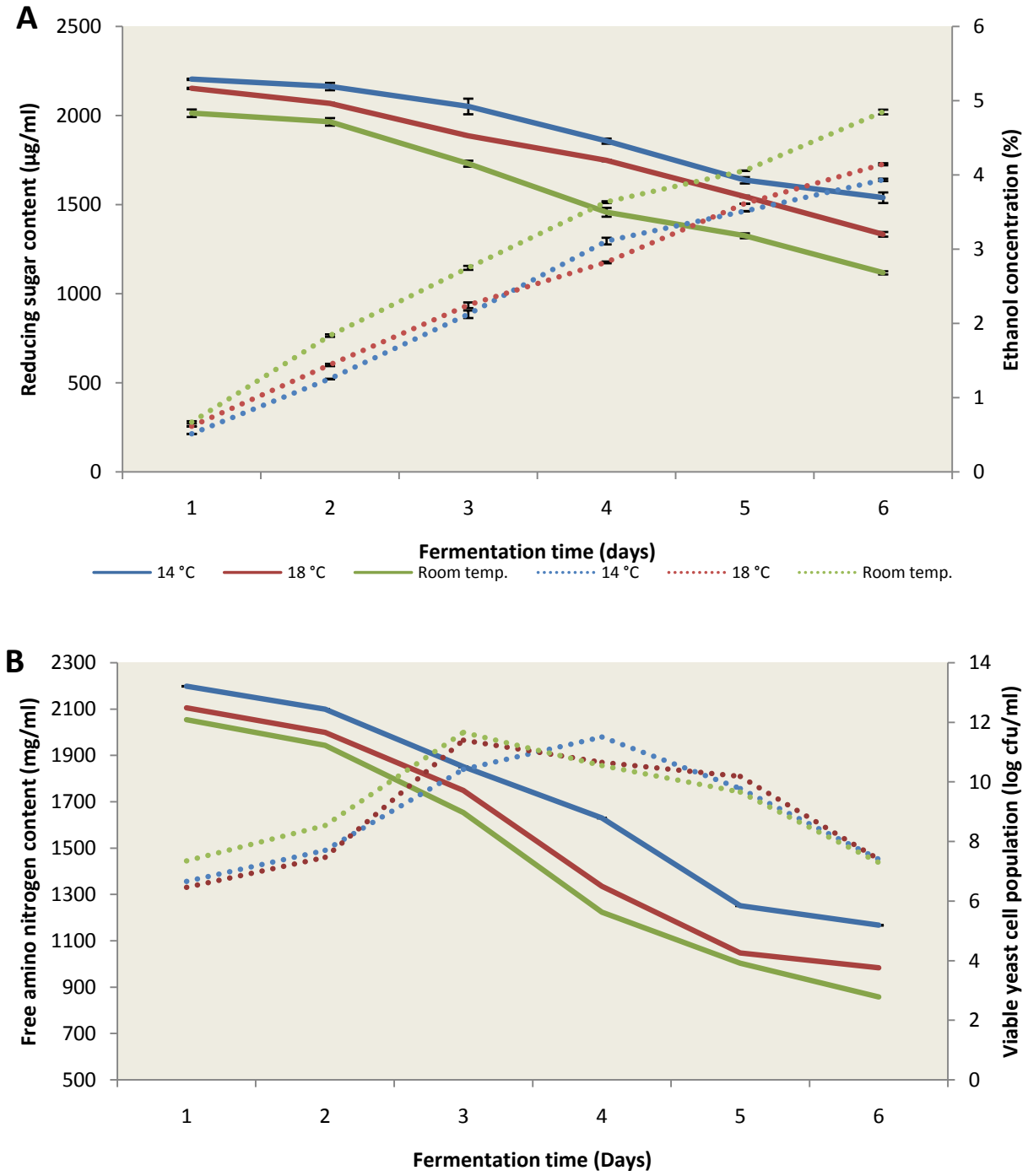


Figure 2.3: Profiles of (a) reducing sugar and ethanol content and (b) free amino nitrogen concentration and yeast cell density in the wort during fermentation at varying fermentation temperatures. Time 0 vales for reducing sugars and FAN are 2253 µg/ml and 2271 mg/ml, respectively. Solid lines represent reducing sugar and free amino nitrogen content and dotted lines represent ethanol concentration and yeast cell density.

2.3.1.4 Effect of fermentation pH

Effect of fermentation pH ranging from 3 to 7 was investigated. The profile of sugar utilization and ethanol production during fermentation at different pHs is shown in Figure 2.4a, while the FAN content and yeast cell density at the same period is shown in Figure 2.4b. pH 5 was found to support the highest utilization of nutrients. Initial concentration of reducing sugars and FAN in the wort was 2348 μg/ml and 2340 mg/ml, respectively. Fermentations at pH 3, 5 and 7 resulted in 21.01%, 30.86% and 24.04% reducing sugar utilization, respectively. At pH 5, 50.95% of FAN was utilized whereas fermentation at pH 3 and 7 resulted in 20.21% and 33.99% FAN utilization, respectively. Furthermore, at pH 5 the highest amount of ethanol was produced (4.022% [v/v]) which was 85.89% and 77.89% higher alcohol produced than at pH 3 and 7, respectively. Yeast cell population increased during fermentation at pH 5 and 7 up to day 4, reaching a maximum of 3.95×10^{11} cfu/ml and 4.54×10^{9} cfu/ml, respectively thereafter decreased. At pH 3, yeast cell density increased until day 3 of fermentation attaining a maximum of 8.41×10^{9} cfu/ml and gradually decreased thereafter.

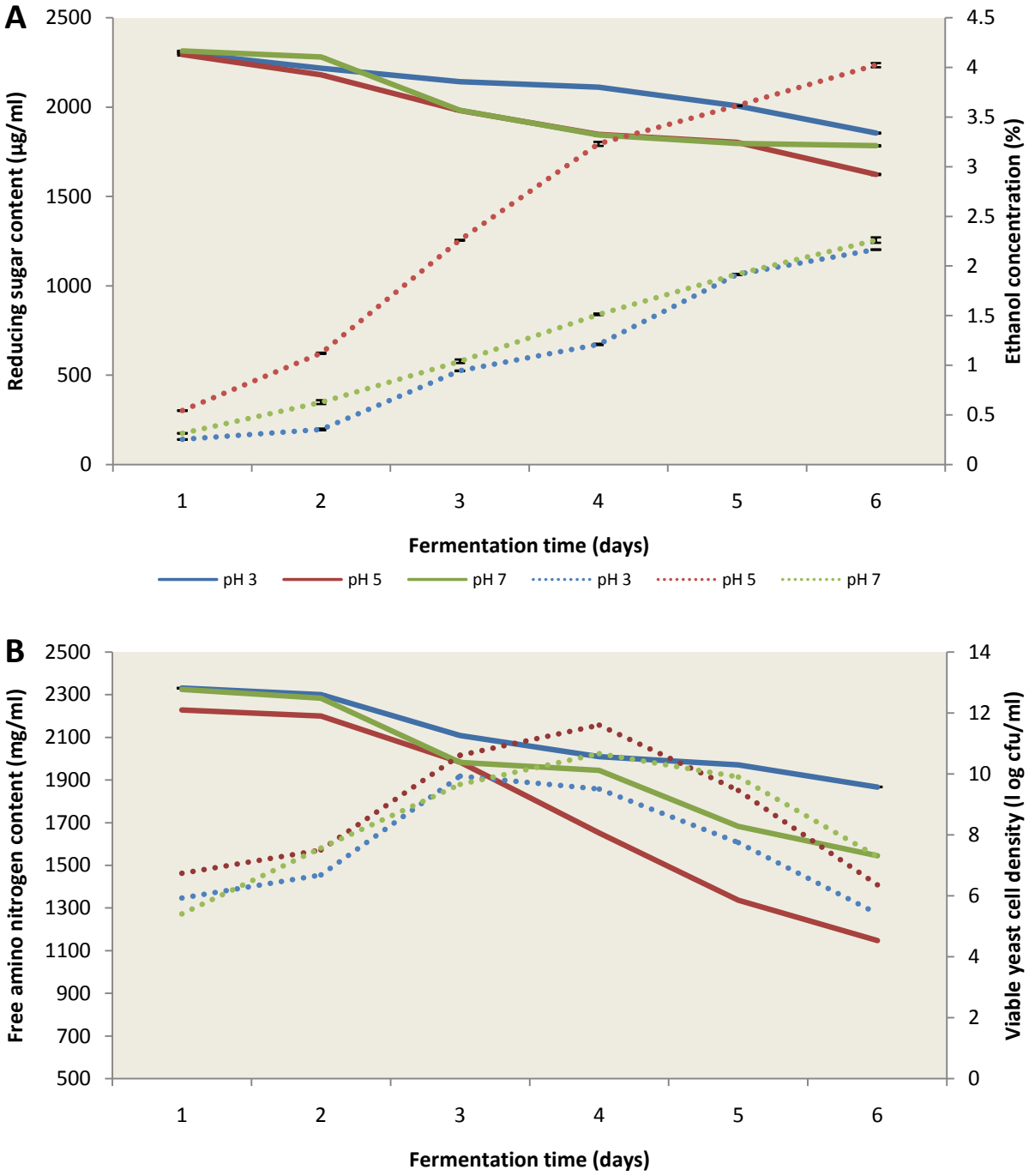


Figure 2.4: Profiles of (a) reducing sugar and ethanol content and (b) free amino nitrogen concentration and yeast cell density in the wort during fermentation at varying fermentation temperature. Time 0 vales for reducing sugars and FAN are 2348 µg/ml and 2340 mg/ml, respectively. Solid lines represent reducing sugar and free amino nitrogen content and dotted lines represent ethanol concentration and yeast cell density.

2.3.2 Post fermentation analysis

2.3.2.1 Spent yeast density

Spent yeast density determined under the various nutritional and fermentation conditions ranged from 2.182 to 3.358 mg/ml. Supplementation of wort with $ZnSO_4$ and L-leucine, resulted in an increase in spent yeast density, except for when 0.5 g/l of L-leucine was added (Table 2.5). Fermentation in the presence of 0.12 g/l $ZnSO_4$ produced the highest spent yeast density of 3.358 mg/ml which was 34.75% more compared to that obtained during fermentation without any $ZnSO_4$. Addition of 0.75 g/l L-leucine into wort resulted in 16.17% increase in spent yeast density. Similarly, increase in fermentation temperature from 14 °C to RT resulted in 25.8% increase in spent yeast density compared to fermentation at 14 °C. However, increase in wort acidity and alkalinity resulted in a decrease in spent yeast density. Highest spent yeast density was observed at pH 5. There was a 12.69% and 8.88% reduction in spent yeast density at pH 3 and pH 7, respectively compared to that at pH 5.

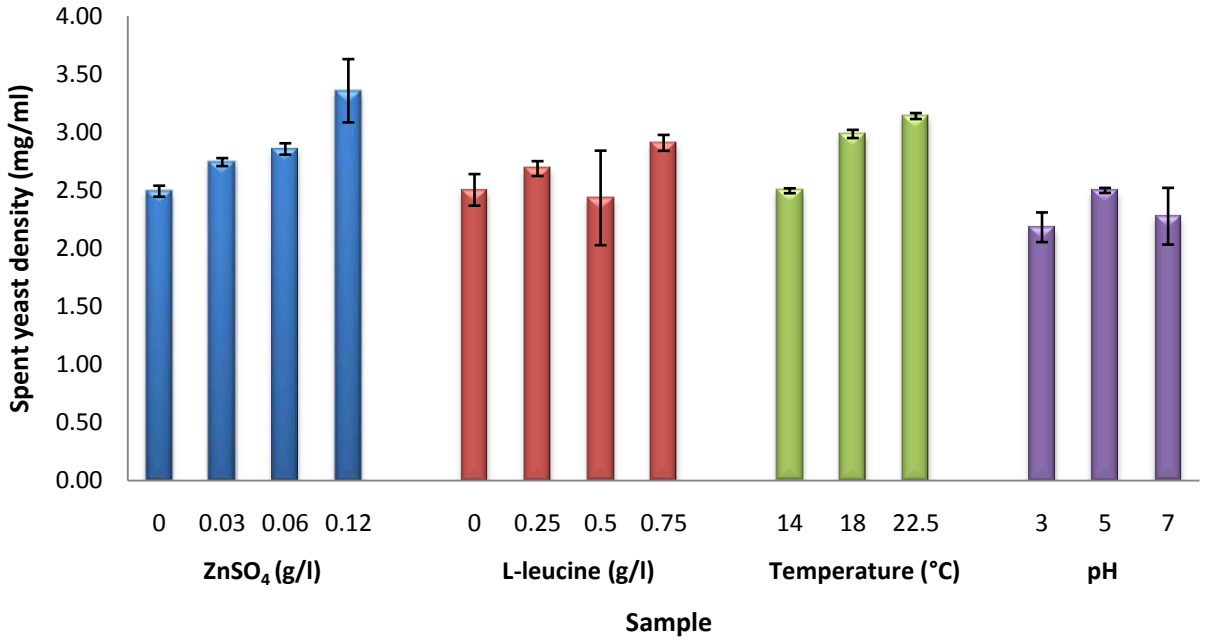


Figure 2.5: Spent yeast density produced under the various nutritional and fermentation conditions

2.3.2.2 Beer colour and foam head stability

Beer colour was measured after bottle conditioning to determine the colour intensities in the beers produced under the different conditions. The differences in colour intensity between the different experimental beers were similar. A commercial beer which served as a control had the deepest colour intensity as its absorbance at 430 nm was 0.86 (Table 2.1). The experimental beer produced at room temperature resulted in the deepest colour intensity producing an absorbance of 0.690 at 430 nm.

The commercial beer had the best foam head stability compared to all the experimental beers. The best foam head stability was produced in the experimental beers with 0.75 g/l L-leucine, retaining as high as 53.4% foam head stability compared to the commercial beer, while those prepared at pH 7 and those supplemented with 0.12 g/l $ZnSO_4$ had the least foam head stability rating (Table 2.1). An increase in $ZnSO_4$ in wort from 0.03 g/l to 0.12 g/l resulted in a decrease in foam head stability by 16.5% while increasing L-leucine concentration in the wort resulted in an increase in foam head stability. Wort supplemented with 0.75 g/l L-leucine had 33.5% better foam head stability than the unsupplemented control. Alteration of fermentation temperature had no effect on foam head stability since all experimental beers had a rating of 2. However, an increase in pH of the fermentation medium resulted in a decrease in foam head stability.

Table 2.1: Colour profiles and foam head stability of beer produced under the various nutritional and fermentation conditions

Sample	Beer colour (430nm)	Foam head stability rating
Zinc sulphate (g/l)		
0.00	0.611 ± 0.00	2.00 ± 0.00
0.03	0.664 ± 0.02	2.00 ± 0.00
0.06	0.645 ± 0.02	2.34 ± 0.58
0.12	0.665 ± 0.04	1.67 ± 0.58
L-leucine (g/l)		
0.00	0.620 ± 0.00	2.00 ± 0.00
0.25	0.633 ± 0.02	2.34 ± 0.58
0.50	0.641 ± 0.01	2.34 ± 0.58
0.75	0.632 ± 0.01	2.67 ± 0.58
Temperature (°C)		
14	0.682 ± 0.00	2.00 ± 0.00
18	0.675 ± 0.00	2.00 ± 0.00
Room temperature (22.5)	0.690 ± 0.02	2.00 ± 0.00
pH		
3	0.653 ± 0.03	2.00 ± 0.00
5	0.632 ± 0.02	2.34 ± 0.58
7	0.663 ± 0.01	1.67 ± 0.58
Commercial beer	0.861 ± 0.02	5.00 ± 0.00

Values are averages of triplicate results ± standard deviation

2.3.2.3 Detection of beer volatile ester compounds

Volatile ester compounds produced during the fermentations were quantified by Gas Chromatographic analysis of the head space samples and results shown in Figures 2.6 to 2.9, while ester threshold levels and ester concentrations measured in a commercial beer are shown in Table 2.2. Addition of 0.12 g/l $ZnSO_4$ into the fermentation medium resulted in a 9.51% increase in total acetate esters and 80.06% increase in total ethyl esters (Figure 2.6) compared to the unsupplemented sample. Ethyl acetate constituted roughly 67% of the total esters. There was an increase in all ester concentrations with the highest increase of 143.51% in isoamyl acetate concentration when wort was supplemented with 0.12 g/l $ZnSO_4$. This was followed by ethyl decanoate, ethyl hexanoate, phenyl ethyl acetate and ethyl octanoate increasing by 95.59%,

56.14%, 23.14% and 6.90%, respectively, compared to the control. There is a good correlation between total acetate ester concentration and $ZnSO_4$ concentration (R^2= 0.985). Of these acetate esters, phenyl ethyl acetate and isoamyl acetate showed a good correlation with $ZnSO_4$ concentration (R^2= 0.959 and R^2= 0.980, respectively). All acetate esters and ethyl decanoate exceeded their threshold level under all $ZnSO_4$ concentrations.

Addition of L-leucine (0.750 g/l) into the fermentation medium resulted in a 21.24% increase in total acetate ester concentration and 31.29% increase in total ethyl ester concentration compared to the control which was not supplemented with L-leucine (Figure 2.7). The highest increase was observed for isoamyl acetate which increased by 481.63% compared to the unsupplemented control. This was followed by ethyl hexanoate, phenyl ethyl acetate, ethyl decanoate and ethyl octanoate, increasing by 59.22%, 53.72%, 32.44% and 15.31%, respectively. There was a good correlation between increasing L-leucine concentration and phenyl ethyl acetate and ethyl octanoate production (R^2= 0.983 and R^2= 0.973, respectively). All acetate esters and ethyl decanoate exceeded their threshold levels under all L-leucine concentrations.

Increasing the fermentation temperature from 14 °C to room temperature (22.5 °C) (Figure 2.8) resulted in an increase in total acetate ester concentration by 50.68%, and total ethyl ester concentration by 87.19%. The highest increase of 312.19% was observed for isoamyl acetate. This was followed by ethyl decanoate, phenyl ethyl acetate, ethyl hexanoate, ethyl acetate and ethyl octanoate increasing by 105.37%, 50.59%, 45.26%, 40.19% and 14.54%, respectively, compared to the control. There was a good correlation between total acetate esters (R^2= 0.997), specifically ethyl acetate (R^2= 0.991) and isoamyl acetate (R^2= 0.983) and the fermentation temperature. Of the ethyl esters there was a good correlation between fermentation temperature and ethyl octanoate (R^2= 0.966) and ethyl hexanoate (R^2= 0.975). All acetate esters and ethyl decanoate exceeded their threshold level under all fermentation temperature.

Table 2.2: Ester concentration and flavour threshold levels in a commercial beer

Ester compound	Threshold concentration (mg/l)	Commercial beer concentration (mg/l)
Ethyl acetate	30	28.32 ± 0.65
Isoamyl acetate	1.2	1.56 ± 0.41
Phenyl ethyl acetate	3.8	3.81 ± 0.77
Ethyl decanoate	1.5	1.99 ± 0.32
Ethyl hexanoate	0.21	0.176 ± 0.04
Ethyl octanoate	0.9	0.541 ± 0.16

Fermentation pH that produced the highest ester production was 7 resulting in a 4.02% increase in total acetate ester and 16.14% total ethyl ester production when compared to the control (pH 5) (Figure 2.9). There was a 29.23% increase in isoamyl ester production at pH 7, followed by phenyl ethyl acetate, ethyl decanoate, ethyl hexanoate and ethyl octanoate increasing by 23.62%, 19.41%, 9.59% and 1.11%, respectively. However, there was a slight decrease in ethyl acetate concentration by 0.17% at pH 7 compared to the control (pH 5) and 3.40% decrease in total ester concentration at pH 3, with isoamyl acetate decreasing by 38.52%. There was a good correlation between fermentation pH and total acetate and ethyl ester concentration (R^2= 0.993 and R^2= 0.961, respectively) All acetate esters and ethyl decanoate exceeded their threshold level under all fermentation pH.

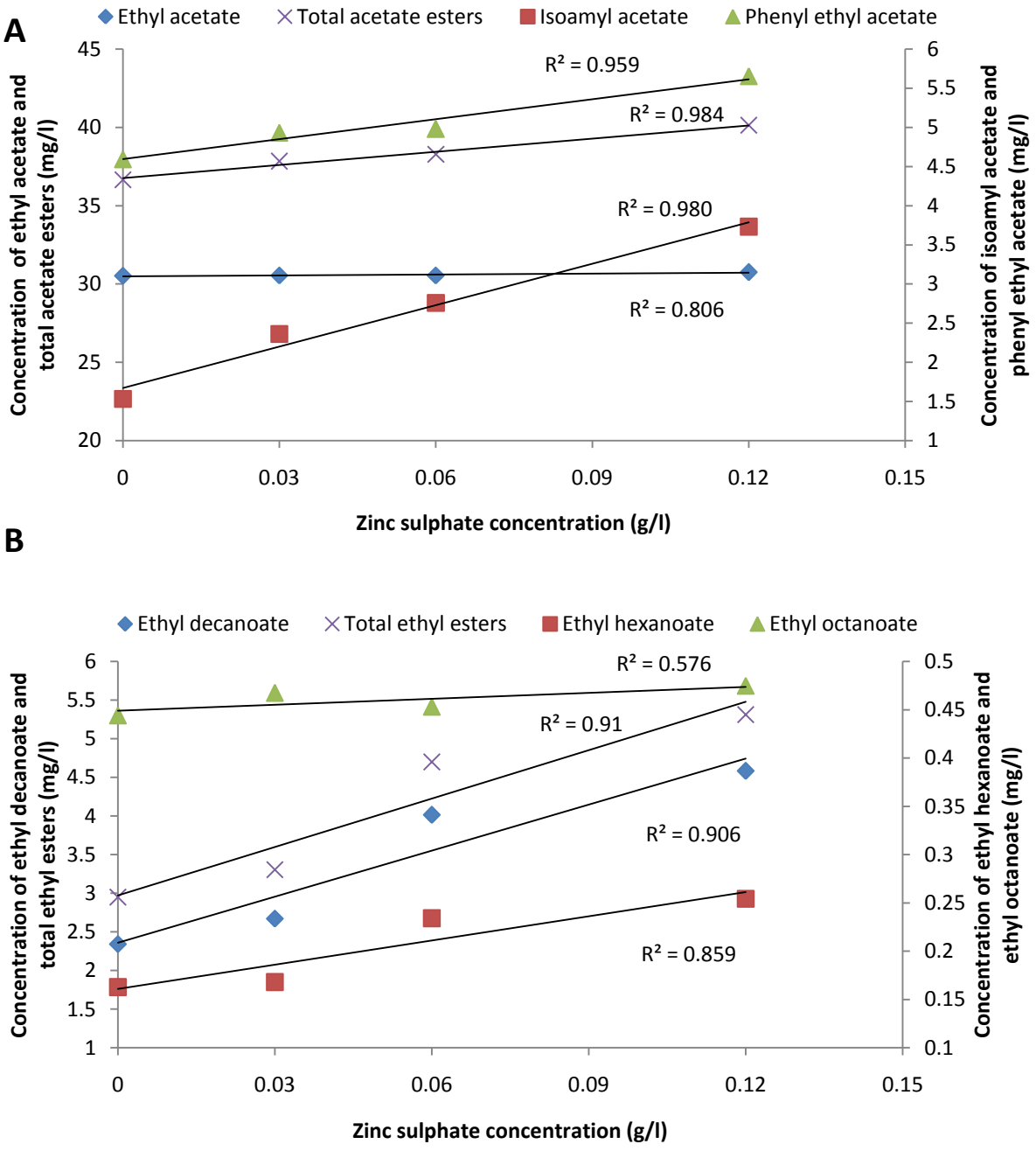


Figure 2.6: Profiles of acetate esters (a) and ethyl esters (b) produced in lager beer at varying concentrations of zinc sulphate supplements

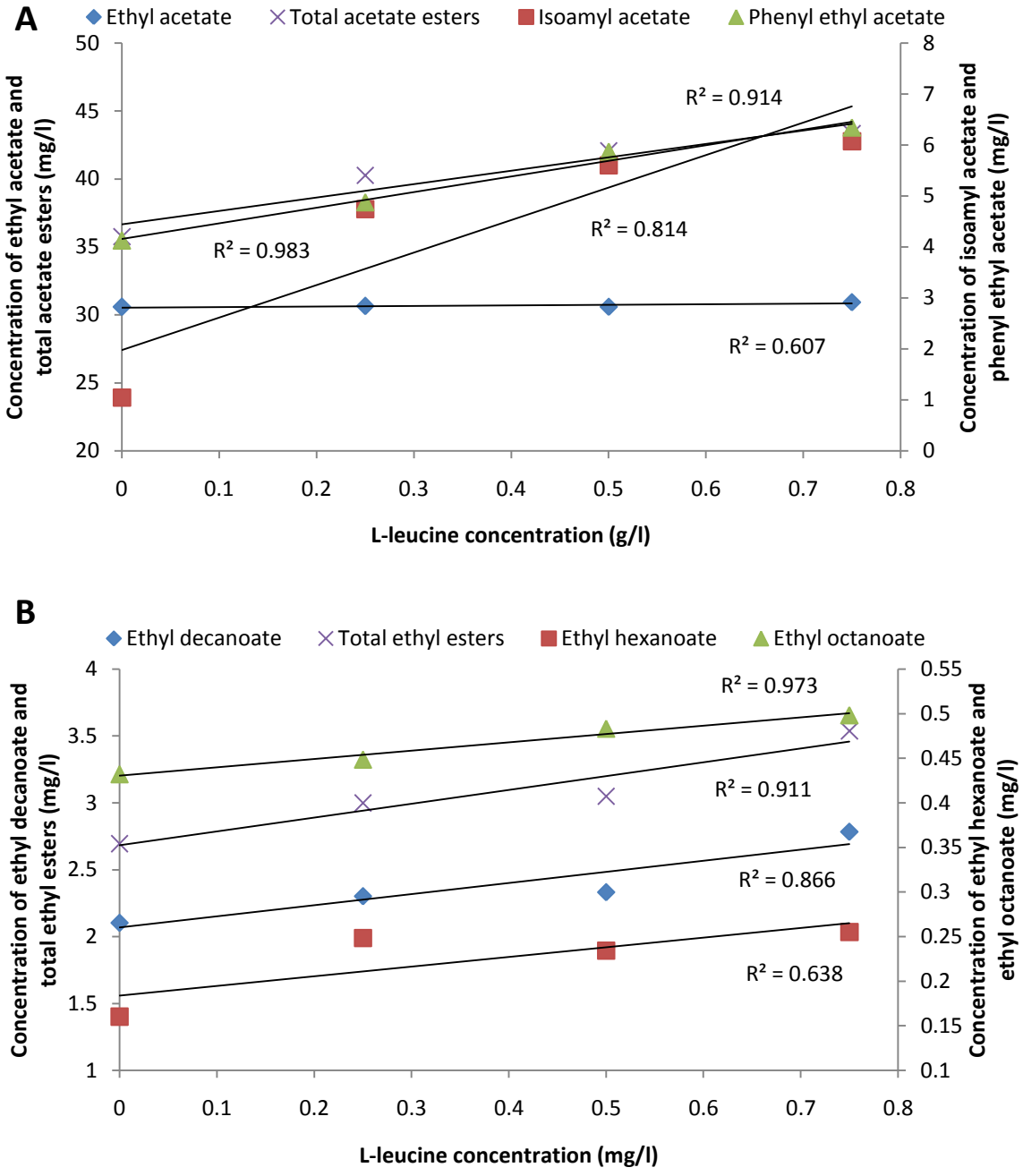


Figure 2.7: Profiles of acetate esters (a) and ethyl esters (b) produced in lager beer at varying concentrations of L-leucine supplements

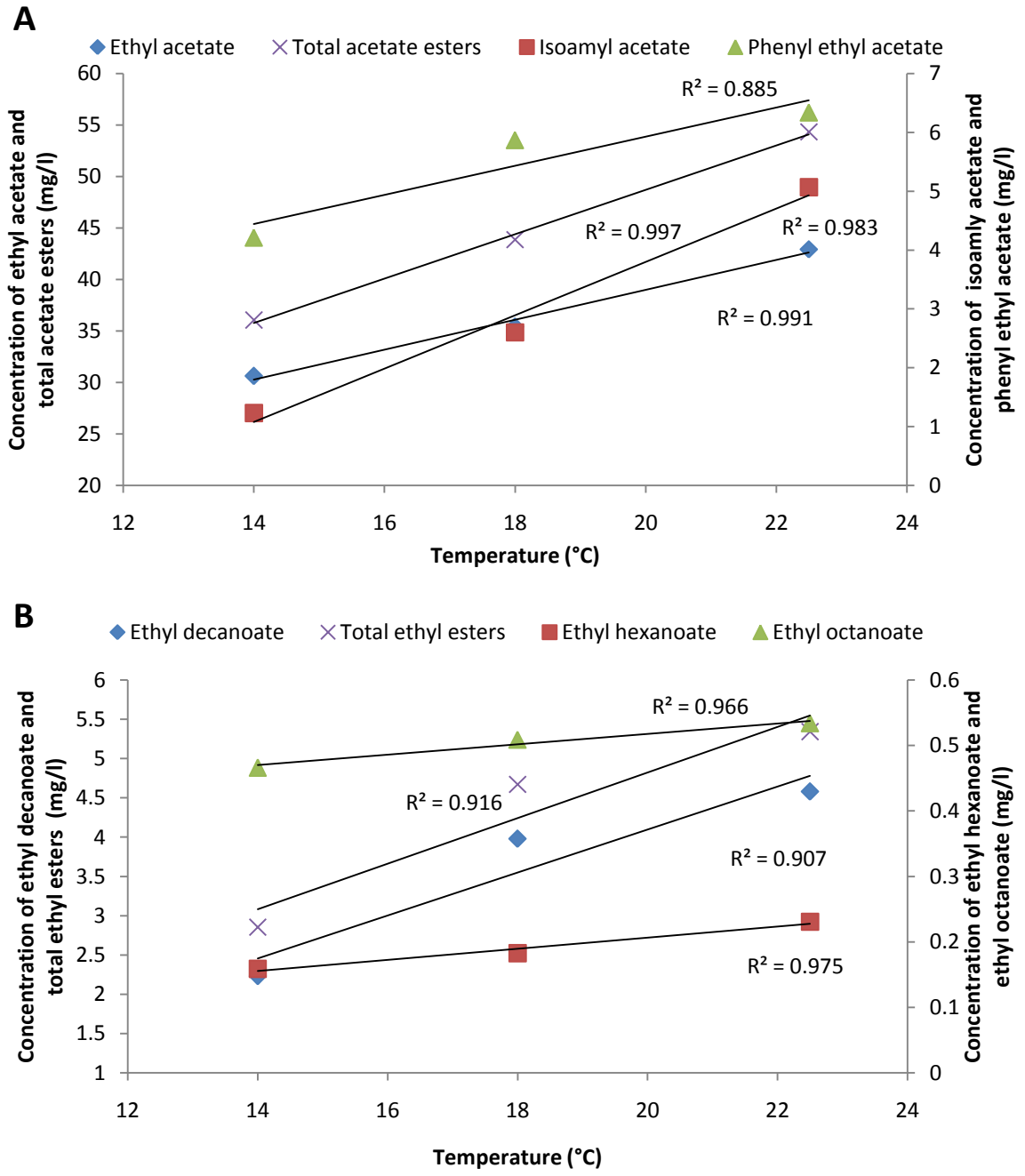


Figure 2.8: Profiles of acetate esters (a) and ethyl esters (b) produced in lager beer at varying fermentation temperatures

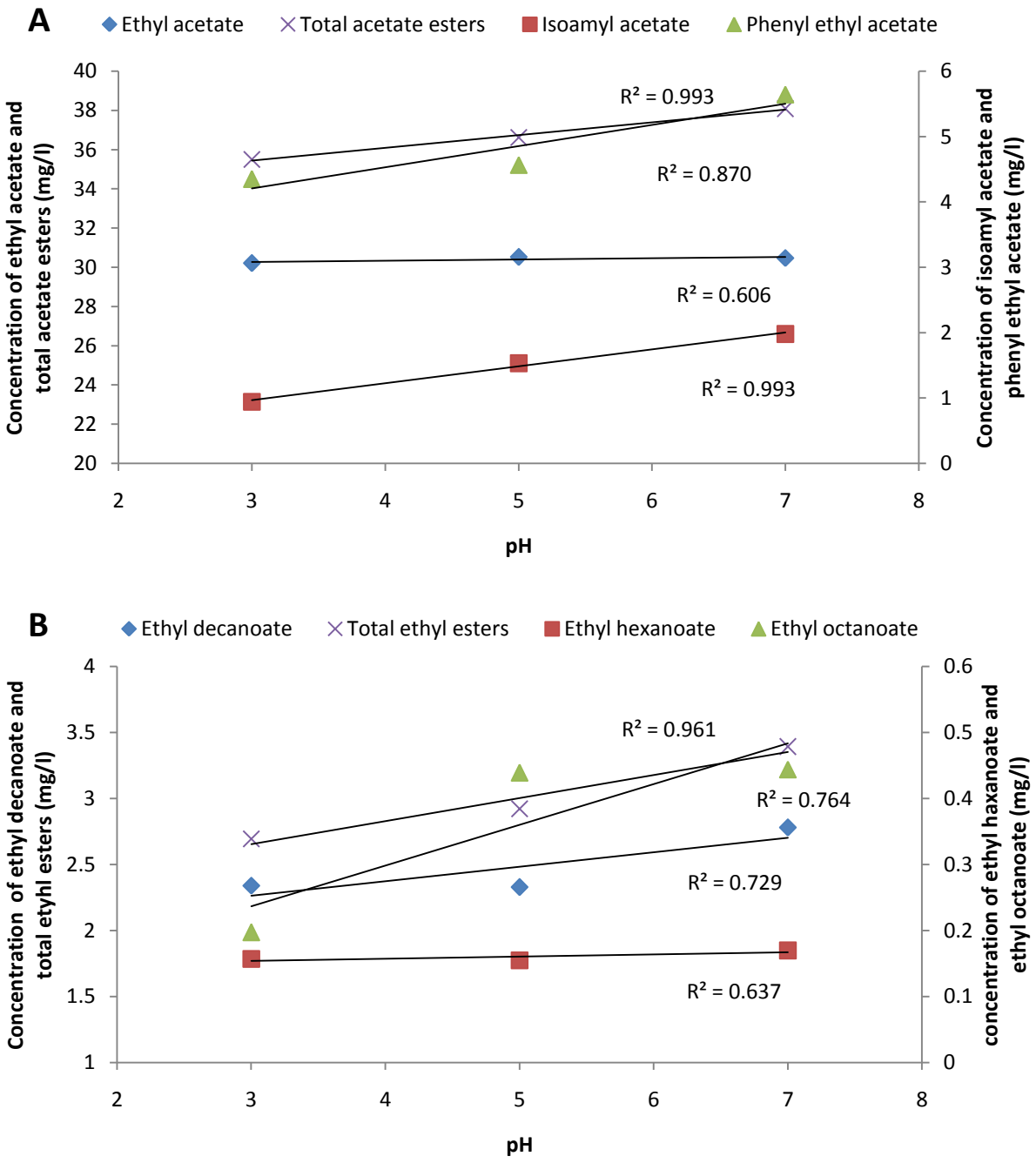


Figure 2.9: Profiles of acetate esters (a) and ethyl esters (b) produced in lager beer at varying fermentation pH

2.3.2.4 Stability of volatile ester compounds in lager beer over time

Stability of aroma-active esters was monitored in lager beer stored at 4°C and room temperature over a three month period. Generally, beer produced in this study was more stable at 4 °C compared to room temperature after three months (Table 2.3). At 4 °C there was a decrease in total ester concentration by 7.92% with ethyl decanoate being the least stable compound decreasing by 14.04%. Phenyl ethyl acetate was the most stable ester compound deceasing by only 3.79% after three months. Acetate esters were more stable than ethyl esters decreasing by 7.48% compared to 13.19% observed for ethyl esters. By week 8, ethyl acetate concentration decrease below the threshold level. After 12 weeks of storage at 4 °C, phenyl ethyl acetate and ethyl decanoate concentrations remained above the threshold level while isoamyl acetate, ethyl octanoate and ethyl hexanoate remained below the flavour threshold. At room temperature, there was a 13.32% decrease in total ester concentration. Ethyl decanoate was the least stable compound decreasing by 36.53%, while isoamyl acetate was the most stable compound at room temperature decreasing by 10.65% after three months. Acetate esters were more stable at room temperature than ethyl esters as it decreased by 11.65% compared to ethyl esters that decreased by 33.08%. Ethyl acetate and phenyl ethyl acetate decreased below the threshold level on week 6 and 12, respectively. Ethyl decanoate remained above the threshold level after 12 weeks of storage at room temperature. Isoamyl acetate, ethyl octanoate and ethyl hexanoate remained below the threshold level after 12 weeks.

Table 2.3: Stability of aroma-active esters in lager beer during storage at 4 °C and room temperature (±22.5 °C)

Storage temperature	Storage time (weeks)	Acetate esters (mg/l)			Ethyl esters (mg/l)		
		Ethyl acetate	Isoamyl acetate	Phenyl ethyl acetate	Ethyl decanoate	Ethyl octanoate	Ethyl hexanoate
4 °C	0	30.69 ± 0.11	1.06 ± 0.01	4.17 ± 0.02	2.42 ± 0.03	0.455 ± 0.01	0.163 ± 0.01
	2	30.66 ± 0.03	1.06 ± 0.00	4.14 ± 0.02	2.39 ± 0.04	0.451 ± 0.01	0.163 ± 0.00
	4	30.66 ± 0.03	1.05 ± 0.01	4.12 ± 0.02	2.37 ± 0.00	0.453 ± 0.01	0.162 ± 0.00
	6	30.51 ± 0.03	1.06 ± 0.00	4.11 ± 0.01	2.32 ± 0.15	0.447 ± 0.01	0.157 ± 0.00
	8	29.31 ± 0.43	1.04 ± 0.01	4.07 ± 0.01	2.11 ± 0.01	0.422 ± 0.00	0.151 ± 0.00
	10	28.54 ± 0.08	1.02 ± 0.01	4.04 ± 0.00	2.11 ± 0.01	0.415 ± 0.00	0.148 ± 0.00
	12	28.21 ± 0.42	1.01 ± 0.01	4.01 ± 0.01	2.08 ± 0.02	0.410 ± 0.00	0.147 ± 0.00
Room temperature (± 22.5 °C)	0	30.70 ± 0.01	1.06 ± 0.00	4.17 ± 0.01	2.42 ± 0.04	0.457 ± 0.00	0.166 ± 0.00
	2	30.27 ± 0.02	1.06 ± 0.00	4.13 ± 0.01	2.33 ± 0.03	0.441 ± 0.01	0.157 ± 0.00
	4	30.16 ± 0.05	1.05 ± 0.00	4.08 ± 0.02	2.31 ± 0.03	0.429 ± 0.01	0.156 ± 0.00
	6	29.95 ± 0.07	1.05 ± 0.00	4.02 ± 0.01	2.30 ± 0.02	0.413 ± 0.01	0.153 ± 0.00
	8	28.39 ± 0.10	1.03 ± 0.00	4.00 ± 0.01	2.12 ± 0.01	0.421 ± 0.01	0.134 ± 0.00
	10	28.21 ± 0.01	1.02 ± 0.01	3.90 ± 0.05	2.05 ± 0.01	0.406 ± 0.00	0.127 ± 0.00
	12	27.10 ± 0.01	0.95 ± 0.04	3.70 ± 0.04	1.53 ± 0.51	0.389 ± 0.01	0.111 ± 0.01

2.4 Discussion

Fermentation conditions and nutritional supplements are important in beer brewing due to its influence on fermentation performance and in final product characteristics. In this study, the effect of important fermentation parameters and nutritional supplements on the fermentation performance and production of acetate and ethyl esters by a lager brewer's yeast strain was investigated. It was found that all fermentation and nutritional parameters tested increased the rate of fermentation and the synthesis of aroma-active esters, thus affecting the flavour of the lager beer produced.

Control fermentations were performed at 14 °C at pH 5 and were not supplemented with any nutrients. Experimental fermentations where there was either a supplementation or an alteration of fermentation condition were compared to the control fermentation. This will provide information on how single parameters affected the fermentation performance, beer characteristics and ester profiles. Yeast activity during fermentation is important in achieving consistent fermentations that result in beers of acceptable quality (Verbelen *et al*., 2009); therefore it is necessary to monitor fermentation performance. Addition of $ZnSO_4$ to the fermentation medium resulted in a higher utilization of nutrients during initial growth phase and a higher production of ethanol. Stimulatory effects of elevated zinc levels observed in this study on several fermentation parameters could be explained by the essential role zinc plays in yeast metabolism including: the stabilization of proteins and membrane systems, acting as a catalytic centre of essential enzymes (e.g. alcohol dehydrogenase, aldolase and acetaldehyde dehydrogenase), enhancing riboflavin synthesis, activating acid and alkaline phosphatases as well as stimulating the uptake of maltose and maltotriose (Jones and Gadd, 1990). The observed increase in ethanol production as a result of $ZnSO_4$ supplementation in wort could be due to the fact that zinc is an essential cofactor for multiple alcohol dehydrogenases that convert acetaldehyde to ethanol during glucose fermentation, (viz, *ADH1, ADH3* and *ADH4*) (de Smidt *et al*., 2008). Therefore, addition of $ZnSO_4$ to the fermentation medium can be used for enhancing fermentation performance as it increased the rate of fermentation.

Amino acids are utilized by yeast for protein formation (structural and enzymic) required for growth (Lodolo *et al*., 2008) and other functions such as osmoregulation (Hohmann, 2002). It is therefore not surprising that the addition of the amino acid, L-leucine enhanced the production of ethanol as well as increased the utilization of nutrients. Adequate levels of FAN in wort

ensure efficient yeast cell growth and, hence, an appropriate fermentation performance (O'Connor-Cox and Ingledew, 1989). A good index for potential yeast growth and efficiency is FAN utilization (Lekkas *et al*., 2007). Thus, the addition of FAN allowed for the enhanced growth of yeast by utilizing more nutrients and reaching a higher cell density. Similarly, increase in fermentation temperature stimulated yeast growth, leading to a higher utilization of nutrients. Fermentation is usually conducted at pH 5 as this is the preferred pH for yeast growth and metabolism. This is clearly demonstrated in this study as there was the highest utilization of nutrients at pH 5. High cell densities were achieved under all experimental fermentations and the control experiment. This may have occurred due to oxygen being introduced into the fermentation vessels when sampling was done daily. This will create an aerobic environment that will results in more yeast growth.

In the brewing industry, surplus yeast is recovered by natural sedimentation at the end of the fermentation and conditioning. Spent yeast is very high in protein and B vitamins, and may be given to livestock as a feeding supplement (Goldammer, 2008). Increasing concentration of $ZnSO_4$ in wort resulted in a decrease in spent yeast viability. Negative effects of increased $ZnSO_4$ concentration on viability may be due to several inter-related factors including: activation of degradative enzymes, suppression of secondary metabolism by which detoxification takes place, disruption of membrane structure leading to the leakage of K^+ and UV absorbing materials, induction of (self) autolysis due to the stimulatory effect on proteolytic activity blocking functional groups of important biomolecules, e.g. enzymes, inhibition of transport systems for essential ions and nutrients, displacement and substitution of essential metal ions, and denaturation of enzymes (Rees *et al.,* 1998). Increase in spent yeast density was a result of a high biomass yield obtained during the fermentation. A similar trend was observed when increasing the fermentation temperature where there was an increase in spent yeast density and a decrease in yeast viability. These results correlate with previous reports that yeast viability decreases as the temperature increases (Ough, 1966; Nagodawithana *et al*., 1974; Casey *et al*., 1984). This decrease has been attributed to a greater accumulation of intracellular ethanol at higher temperatures, which could cause cell toxicity (Nagodawithana *et al*., 1974) and would alter the structure of the membrane, thus decreasing its functionality (Lucero *et al.,* 2000). Ethanol inhibits cell growth, cell viability and fermentation rate (Pratt *et al*., 2003). Effects of ethanol stress on yeast viability were in agreement with the findings of Jiménez and Benítez

(1987) who showed that increase in ethanol concentration in the growth medium resulted in a corresponding decrease in yeast viability. Similarly, the increase in L-leucine supplementation resulted in an increase in spent yeast viability. This could be due to excess nutrients that remained in the wort, thus preventing the yeast from entering death phase (Blieck *et al*., 2007).

Colour development in beer has been mostly attributed to the malt extract used in the respective beers instead of the fermentation parameters (Kopsahelis *et al*., 2007). Generally, the malt extract used has been reported to have the greatest effect on beer colour as the degree of colour intensity of the malt extract depends on the degree of kilning or roasting of the malted barley (Seaton and Cantrell, 1993; Kopsahelis *et al*., 2007). Colour differences may also be due to the use of different malts of different proportions on malt. Thus, it is possible that the control beer may have been produced from a malt extract which was differentially roasted compared to the malt extract used in this study, hence the deepest colour intensity. Also, the quality of beer foam is one of the most important criteria for the brewing industry. Foaming potential of beer is determined by the brewing process and the raw materials used. Furthermore, it depends on a number of compounds which affect foam formation and stability (Bamforth, 1985). Components in beer that influence foam stability include proteins, bittering substances from hops (e.g. iso-α-acids), metal-ions, polysaccharides and melanoidins (Evans *et al*., 1999; Lusk *et al*., 1998; Simpson *et al.,* 1993). Proteins associated with beer foam formation and stability are protein Z, lipid transfer protein 1 (LTP1), hordein and glutelin fragments (Asano and Hashimoto, 1980). Of those, protein Z (M_r ~ 40 kDa) and the 9.6 kDa LTP1, both originating from barley, are the predominant proteins in beer (Evans *et al*., 1999). It has been previously suggested that protein Z at concentrations ranging from 50–200 mg/L is the major foaming component in beer due to its physico-chemical properties. Later work has substantiated that protein Z promotes foam stability by interacting with other proteins and foam-positive compounds (Sørensen *et al*., 1993). Since amino acids are a building block for proteins, increase in L-leucine concentration allows for more proteins to interact with Protein Z, therefore resulting in an increase in foam head stability.

Esters are responsible for the fruity character of fermented beverages, and constitute an important group of aromatic compounds in beer. Addition of $ZnSO_4$ (0.120 g/l) into the fermentation medium resulted in a 9.51% increase in acetate esters and 80.06% increase in ethyl esters. Two potential routes for ester formation have been recognized; the reaction between an alcohol (such as ethanol) or higher alcohols with a fatty acyl-CoA ester (Nordström, 1963) and

by esterases working in a reverse direction (Suomalainen, 1981). Hodgson and Moir (1990) (cited by Verstrepen *et al*., 2003a) showed that ester production is enhanced when zinc is added to the medium, due to stimulation of higher alcohol formation, which can subsequently be converted to esters. Since ethanol is the most abundant alcohol and acetic acid is one of the most abundant acids present in the fermentation, ethyl acetate is normally the most abundant ester (Lambrechts and Pretorius, 2000). A good correlation between acetate esters and $ZnSO_4$ concentration was observed in this study (R^2= 0.985), therefore, addition of $ZnSO_4$ can be used to control acetate ester concentrations in beer, specially isoamyl acetate and phenyl ethyl acetate.

The concentration and composition of wort FAN have an impact on the production of higher alcohol and esters, due to the role of amino acid metabolism in the formation of these flavour compounds (Pierce, 1987; O'Connor-Cox and Ingledew, 1989). Nitrogen compounds of the fermentation medium can influence the production of esters as amino acids and ammonium determine the pool of intracellular nitrogen which regulates the metabolic pathways for ester formation (Henschke and Jiranek, 1993 cited by Torrea *et al*., 2003). Moreover, some amino acids are precursors for the formation of these volatile compounds (Boulton *et al*., 1996 cited by Torrea *et al*., 2003). Addition of L-leucine (0.750 g/l) into the fermentation medium resulted in a 21.24% increase in acetate ester concentration and 31.29% increase in ethyl ester concentration. Acetate esters are formed from the reaction of acetyl-CoA with higher alcohols, which arise directly from neutral amino acids via Ehrlich reaction. Ethyl esters come from the reaction of acyl-CoA compounds with ethanol. These acyl-CoA compounds are normally generated through the metabolism of fatty acids, although they can also be synthesized through the carbon skeletons of certain amino acids (Boulton *et al*., 1996 cited by Torrea *et al*., 2003). Peddie (1990) suggested that, in all-malt wort, when the C:N ratio is low (high nitrogen content) oxygen becomes the main growth-limiting factor. While growth ceases because of oxygen depletion, metabolites including higher alcohols and acetyl-CoA are still being formed, but cannot be used, resulting in the formation of esters as overspill products. On the other hand, when the C:N ratio is high (low nitrogen content), nitrogen may be a growth-limiting factor. When yeast growth ceases due to nitrogen depletion, the formation of metabolites such as acetyl-CoA may be reduced, resulting in a decreased overspill and thus lower ester production. Another possible reason for the influence of nitrogen compounds on ester formation is the link between nitrogen metabolism and the production of higher alcohols. It has been shown that addition of valine,

leucine and isoleucine strongly increased the production of the corresponding higher alcohols; isobutanol, isoamyl alcohol and amyl alcohol (Kodama *et al.,* 2001; Quilter *et al*., 2003; Sablayrolles and Ball, 1995). Higher levels of higher alcohols may in turn lead to enhanced ester production, as shown by Calderbank and Hammond (1994). This raises the possibility of steering the production of specific esters by manipulating the formation of the corresponding higher alcohols through the concentration of the corresponding amino acids in the pitching wort. Engan (1970) and Trinh *et al*. (2010) showed that the level of isoamyl acetate can indeed be changed by the addition of certain amino acids, such as leucine and, to a lesser extent, isoleucine. Results from this study revealed that the addition of L-leucine into the fermentation medium can be used to control the formation of phenyl ethyl acetate and ethyl octanoate.

Increasing the fermentation temperature from 14 °C to room temperature (22.5 °C) yielded an increase in acetate ester concentration and ethyl ester concentration by 50.68% and 87.19%, respectively. Engan and Aubert (1977) cited by Verstrepen *et al*., 2003a have shown that up to 75% more esters are produced at 12 °C than at 10 °C. Similarly, Titica *et al*. (2000) found a 40-50% increase in ester formation when the fermentation temperature was raised from 10 °C to 16 °C. Furthermore, it is well known that the formation of higher alcohols is also temperature dependent; this implies that changes in temperature may cause changes in the availability of higher alcohols that are necessary for ester formation (Calderbank and Hammond, 1994). An increase in temperature increase the fluidity of the membrane (Reddy *et al.,* 2010), and this may allow more ester to diffuse into the medium. Elevated fermentation temperature is therefore a good method for controlling ethyl acetate, isoamyl acetate, ethyl hexanoate and ethyl octanoate. However, aroma-active esters are volatile, and a certain amount will inevitably evaporate from the fermenting medium. This will be more intense at higher temperatures. Loss by evaporation affects to a higher degree compounds with a lower boiling temperature, such as higher alcohols and ethyl acetate. Compounds with a higher boiling temperature, such as the ethyl esters and 2-phenyl ethyl acetate, will be less affected. The differences observed cannot be (solely) due to the effect of temperature but to some extent the result of a direct effect on specific yeast biosynthetic pathways. However, the optimum fermentation pH for ester production was pH 7 resulting in a 4.02% increase in acetate ester and 16.14% increase in ethyl ester production when compared to the control (pH 5). Horton *et al*. (2003) studied the effect of pH on the

enzyme alcohol acetyltransferase (AATase) in brewer's yeast and found that the enzyme was most active in the pH range of 7 to 8.

In lager beers the intensity of ester aroma character is regarded as an important component of sensory quality. The concentration of the various ester compounds gradually decreased over the three month storage period possibly due to ester hydrolysis, with beers stored at room temperature resulting in a much faster decrease in ester concentration than those stored at 4 °C. Chemical hydrolysis is an acid-catalysed process, but the activity of the enzyme with esterase activity, sometimes detected in beer, can affect the ester profile. Neven *et al*. (1997) showed that some esterases are released by yeast into beer as a result of cell autolysis during fermentation and maturation. Such esterase activity is strain dependent and top-fermenting yeasts are more active than bottom fermenting yeasts. The optimal esterase activity in beer is between 15 and 20 °C, therefore, there was a higher decrease in ester concentration at room temperature.

Control of flavour ester levels in alcoholic beverages is often problematic and insufficient flavour ester synthesis or aberrant flavour ester profiles are quite common in beer fermentations. Results from this study suggest that supplementing wort with essential nutrients and altering fermentation conditions could be an effective way of controlling ester formation in beer. Thus, beneficial adaptation of the flavour profile may also be possible. Moreover, since these conditions increase the rate of fermentation, allowing yeast to utilize nutrients faster and producing ethanol faster, the fermentation time can be reduced by two to three days, resulting in lower operational costs. However, organoleptic properties need to also be taken into account.

CHAPTER 3: Aroma-active ester profile of ale beer produced under different fermentation and nutritional conditions

3.1 Introduction

Yeast has been used to produce alcoholic beverages, including beer, since ancient times. The task of choosing yeasts to produce desirable tastes and flavours for beer is very important and significant. The preferable flavours of beer depend on a balance of volatile constituents such as acids, alcohols, aldehydes, ketones, and esters (Patel and Shibamoto, 2003). There are many strains of brewing yeast (*Saccharomyces cerevisiae*) that are used to produce beer (Bamforth, 2000). Two types of brewing yeast were originally classified based on flocculation behaviour: top fermenting (ale yeast) and bottom fermenting (lager yeast) (Jentsch, 2007). Their behaviour is so distinct that the two main classes of beer types (ales and lagers) are based on the two yeast types. Ale yeast (top yeast) exhibit flotation and have the ability to trap CO_2 bubbles to form a yeast „head' at the top of fermentation vessels whereas with lager strains (bottom yeasts), the cells clump together, resulting in flocs that sediment from the medium to settle at the bottom of the fermentation vessel. This phenomenon, flocculation, is strain-dependent (Speers *et al*., 1992; Verstrepen *et al*., 2003a) and evidence exists that a number of FLO genes play a role in flocculation (Teunissen and Steensma, 1995).

Ale yeast is genetically more diverse and prefers higher fermentation temperatures (18–24 °C) whereas lager yeast is more conserved and prefers at lower temperatures (8–14 °C) (Lodolo *et al*., 2008). Both types need some oxygen to initiate their metabolism; however the alcoholic fermentation is anaerobic (Bamforth, 2000). Brewing strains can utilize various carbohydrates (glucose, sucrose, fructose, maltose, galactose, raffinose and maltotriose), with the major distinguishing difference between ale and lager strains being the capability of lager yeasts to ferment melibiose (Lodolo *et al*., 2008). The phenotypic characteristics used to distinguish these yeast types include colony morphology, microscopic appearance, fermentation characteristics (flocculation and flavour profiles), growth at 37 °C (ale yeasts) and utilization of melibiose (lager strains). Besides the differences in phenotypic behaviour, the yeasts can also be distinguished using electrophoretic karyotyping of their chromosomes (Casey, 1996).

One of the most important factors affecting ester production during fermentation is the yeast strain type. Not only the average ester production, but also the relative proportions of each

individual ester produced, differs dramatically from strain to strain. Furthermore, the influence of fermentation parameters, such as oxygen and temperature, is highly dependent on the strain background (Anderson and Kirsop, 1974; Peddie, 1990). Ramos-Jeunehomme *et al.* (1991) suggested that the differences in ester production between distinct ale and lager yeast strains are due to differences in alcohol acetyltransferase activity. Ideally, the production strain therefore should be selected from a pool of strains in order to find the strain that performs best in the particular circumstances of a certain production plant. However, in most practical cases, the yeast strain is not considered as a "variable", so that the use of different yeast strains is not seen as a practical method to control ester synthesis, except when "new" beers are developed. Apart from the difference in ester production between distinct yeast strain types, Watari *et al*. (2000) have shown that ester production by a specific strain may also be variable due to genetic drift during successive rounds of fermentation or cultivation on agar slants. Therefore, a complete periodic examination of the production strains and yeast types, including genetic fingerprinting and standard fermentation trials should be implemented.

Wort composition and fermentation conditions are known to affect fermentation performance and flavour profiles (Saerens *et al*., 2008a). Lager and ale brewing strains respond differently to the various nutritional and fermentation conditions (Peddie, 1990) creating unique flavour profiles in the final product. Generally, ale yeast strains produce a higher concentration of flavour-active esters in beer (Lodolo *et al*., 2008). Therefore, this chapter aims at identifying the effects of various nutritional and fermentation conditions on fermentation performance and ester profiles in beer produced by an ale yeast strain.

3.2 Materials and Methods

3.2.1 Yeast strain and cultivation conditions

Yeast strains were from the microbiology culture collection (UKZN - Pietermaritzburg). Yeast strains were maintained on malt agar slants and were sub-cultured monthly. All experiments were carried out using a ale strain grown in malt extract broth for 24 h at 30 ºC with shaking at 120 rpm. All experiments were carried out using an ale yeast strain (*Saccharomyces cerevisiae*). Yeast was grown in malt extract broth for 24 h at 30 ºC with shaking at 120 rpm. Two millielitre pre-culture was inoculated into 200 ml malt extract broth for 6 h at 30 ºC with shaking at 120 rpm until it reached an OD_{600} of 0.45. Samples were centrifugated at 4000 rpm for 15 min at 4 °C and the pellet was resuspended in 200 ml wort. 20 ml inoculum was used to pitch 2 L wort at a pitching rate of 6×10^6 cfu/ml.

3.2.2 Beer production

3.2.2.1 Wort preparation

As described in chapter two.

3.2.2.2 Wort fermentation

Fermentations were set up to determine the effect of fermentation temperature, pH, addition of zinc sulphate ($ZnSO_4$) and L-leucine, on fermentation performance and ester production using mini-fermenters (3.5L) to facilitate the fermentation process on a small scale. The control fermentation was not supplemented with zinc sulphate or L-leucine and was carried out at 18 °C. All fermentations were carried out in duplicated in fermentation vessels containing 2 L of wort which were fermented under various supplementations and fermentation parameters as follows: Zinc sulphate (0.03, 0.06, and 0.12 g/l); L-leucine (0.25, 0.50 and 0.75 g/l), temperature (14, 18 and room temperature [22.5 °C]); and pH (3, 5 and 7). Control set up was performed at 18 °C and at pH 5. Fermentations were monitored by an air lock mechanism to ensure the fermentations are not stuck. During fermentation, samples were taken and analyzed as described below. Once fermentation was complete fermenter vessels were incubated at 4 °C for 5 days to allow yeast to settle.

3.2.2.3 Fermentation analysis, bottling, conditioning, measurement of beer foam head stability, beer colour, spent yeast density, and beer volatile esters

As described in chapter two.

3.3 Results (Data for results presented in Chapter is shown in Appendix B)

3.3.1 Fermentation performance under different nutritional and fermentation conditions

3.3.1.1 Effect of zinc sulphate supplementation

The profile of sugar utilization and ethanol production during the fermentation period in the absence and presence of varying concentrations of $ZnSO_4$ is shown in Figure 3.1a, while the FAN content and yeast density at the same period is shown in Figure 3.1b. Time 0 concentration of reducing sugars and FAN in the wort was 2569 µg/ml and 2488 mg/ml, respectively. Fermentation performance improved with the addition of $ZnSO_4$ into wort. Wort supplemented with 0.120 g/l of $ZnSO_4$ resulted in the highest utilization of reducing sugars and FAN (54.32% and 49.82%, respectively), after the six day fermentation period. Furthermore, the addition of $ZnSO_4$ (0.120 g/l) resulted in the highest production of 5.01% (v/v) ethanol which was 24.94% higher than the control. Wort supplemented with 0.06 and 0.120 g/l $ZnSO_4$ increased the yeast cell density up to day 3 of fermentation achieving a maximum cell population of 2.54×10^{11} and 5.42×10^{11} cfu/ml, respectively, thereafter yeast cell density gradually decreased. Wort that contained 0.03 g/l and no $ZnSO_4$ increased the yeast cell population up to day 4 of fermentation reaching a cell density of 3.12×10^{11} and 2.69×10^{11} cfu/ml, respectively, thereafter slowly decreasing (Figure 3.1 b).

3.3.1.2 Effect of L-Leucine supplementation

The profile of sugar utilization and ethanol production during the fermentation period in the absence and presence of varying concentrations of L-leucine is shown in Figure 3.2a, while the FAN content and yeast density during the same period is shown in Figure 3.2b. Initial concentration of reducing sugars in the wort was 2514 µg/ml. In the control experiment the yeast utilized 35.73% of the available reducing sugars. Increasing concentration of L-leucine in wort resulted in an increase in reducing sugar utilization.

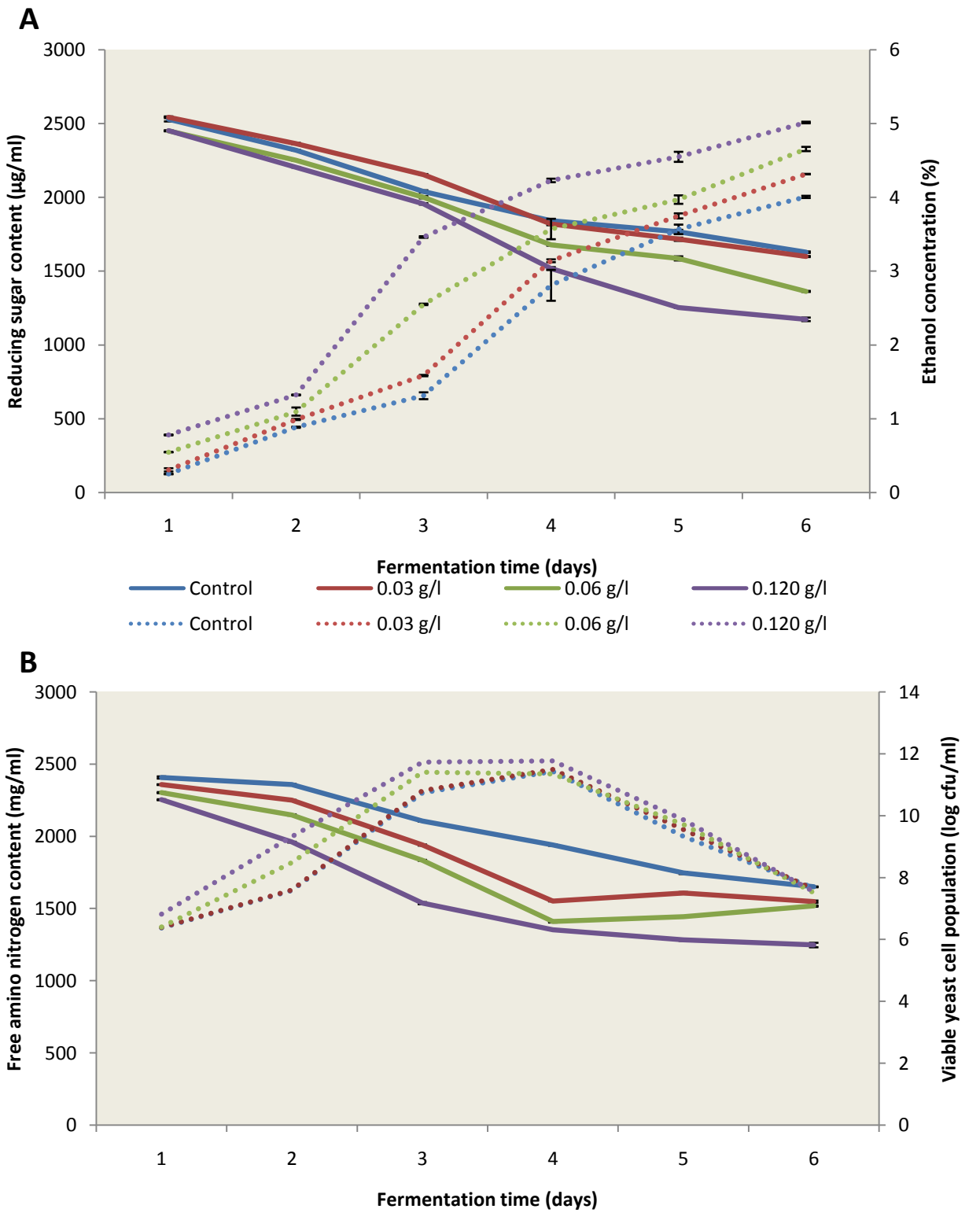


Figure 3.1: Profiles of (a) Reducing sugar and ethanol content and (b) Free amino nitrogen concentration and yeast cell density in the wort during fermentation period of varying concentrations of zinc sulphate. Time 0 vales for reducing sugars and FAN are 2569 µg/ml and 2488 mg/ml, respectively. Solid lines represent reducing sugar and free amino nitrogen content and dotted lines represent ethanol concentration and yeast cell density.

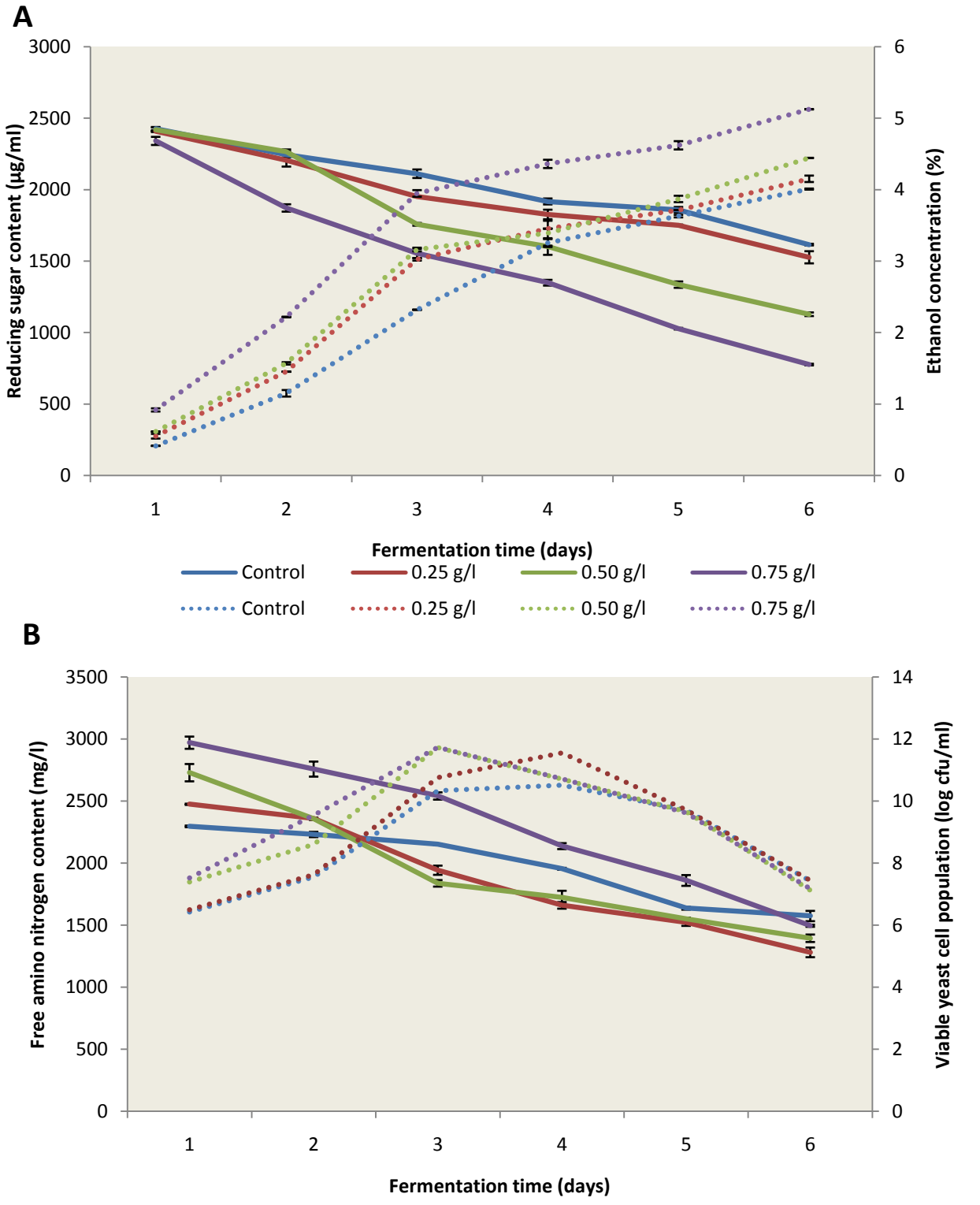


Figure 3.2: Profiles of (a) Reducing sugar and ethanol content and (b) Free amino nitrogen concentration and yeast cell density in the wort during fermentation period at varying concentrations of L-leucine. Time 0 vales for reducing sugars and FAN are 2514 µg/ml. Solid lines represent reducing sugar and free amino nitrogen content and dotted lines represent ethanol concentration and yeast cell density

Wort supplemented with 0.75 g/l L-leucine resulting in 69.11% reducing sugar utilization which was 93.42% higher than the unsupplemented control. The addition of L-leucine to wort resulted in an increased amount of FAN concentration in the medium. The control experiment resulting in 32.10% of FAN utilization by yeast, with increasing concentration of L-leucine in the medium resulted in more FAN being consumed by yeast. In the control experiment, 4.01% (v/v) ethanol was produced whereas wort containing 0.75 g/l L-leucine produced 5.12% (v/v) ethanol which was 27.68% higher than that in the unsupplemented control. Wort supplemented of 0.50 and 0.75 g/l L-leucine increased yeast cell density up to day 3 of fermentation achieving a maximum cell population of 5.21×10^{11} and 5.34×10^{11} cfu/ml, respectively, thereafter yeast cell density gradually decreased. Wort that contained 0.25 g/l and no L-leucine supplement resulted in increased yeast cell population up to day 4 of fermentation reaching a peak density of 3.56×10^{11} and 3.25×10^{10} cfu/ml, respectively, thereafter gradually decreasing.

3.3.1.3 Effect of fermentation temperature

The profile of sugar utilization and ethanol production during the wort fermentation at varying temperatures is shown in Figure 3.3a, while the FAN content and yeast density at the same period is shown in Figure 3.3b. Initial concentration of reducing sugars and FAN in the wort was 2348 µg/ml and 2361 mg/ml, respectively. Increased fermentation temperature resulted in greater utilization of both the reducing sugar and FAN. Fermentation at 14 °C resulted in 33.58% reducing sugar utilization; while 37.10% and 51.77% reducing sugar was utilized at 18 °C and room temperature, respectively. Fermentation at 18 °C and room temperature (22.5 °C) resulted in 33.85% and 52.40% FAN utilization, respectively, (Figure 3.3 b). Furthermore, the highest fermentation temperature resulted in the highest ethanol production (4.74% v/v) which was 17.03% more than fermentation at 18 °C. Fermentation at 14 °C gradually increased the yeast cell density until day 4 reaching a peak of 2.55×10^{11} cfu/ml and thereafter decreased. Fermentation at 18 °C and room temperature resulted in an increase in yeast cell density until day 3 of fermentation attaining a maximum of 2.64×10^{11} and 3.95×10^{11} cfu/ml, thereafter, a decrease in yeast cell density was observed.

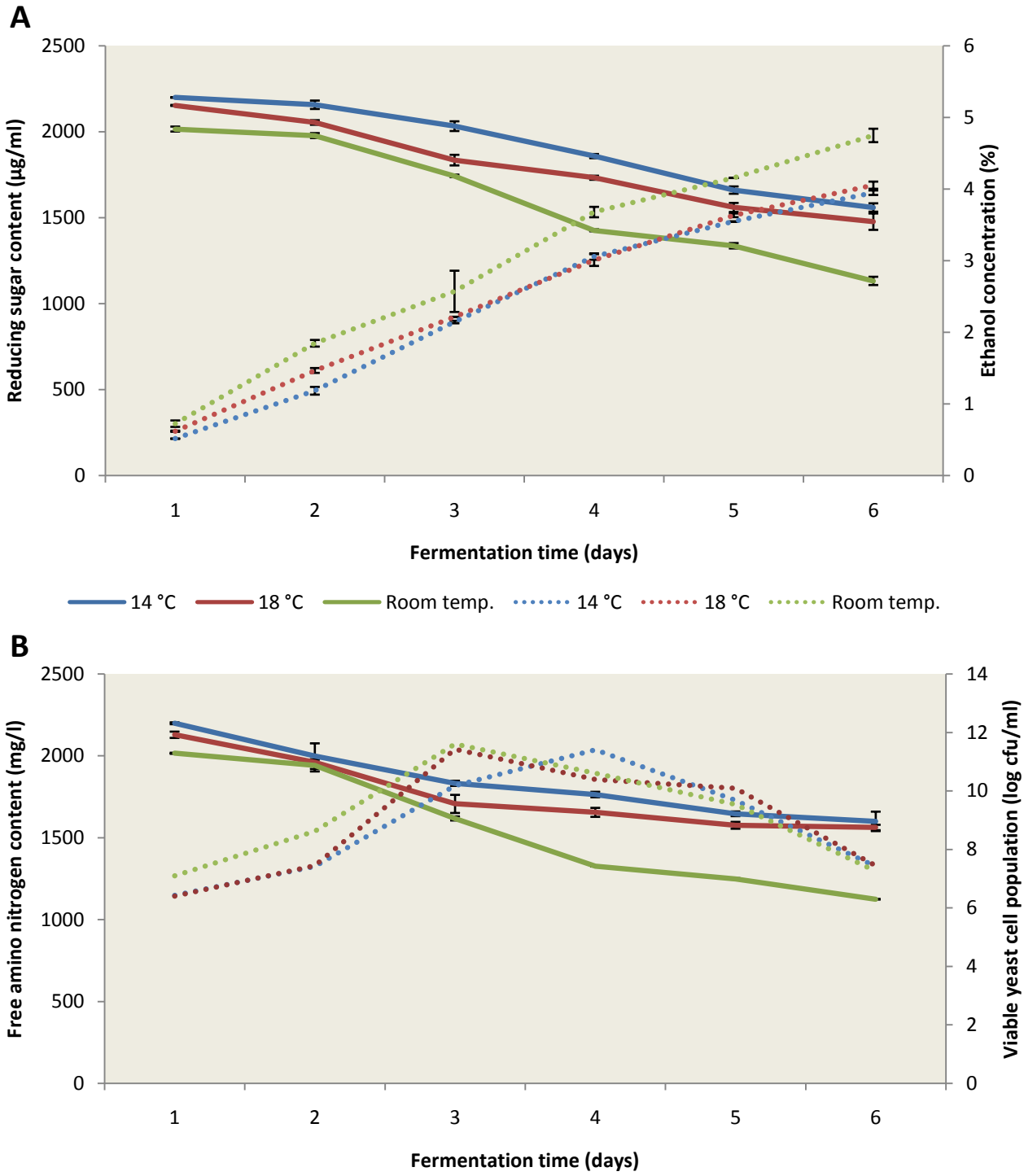


Figure 3.3: Profiles of (a) Reducing sugar and ethanol content and (b) Free amino nitrogen concentration and yeast cell density in the wort during fermentation at varying fermentation temperatures. Time 0 vales for reducing sugars and FAN are 2348 µg/ml and 2361 mg/ml, respectively Solid lines represent reducing sugar and free amino nitrogen content and dotted lines represent ethanol concentration and yeast cell density.

3.3.1.4 Effect of fermentation pH

Effect of fermentation pH ranging from 3 to 7 was investigated. The profile of sugar utilization and ethanol production during fermentation at different pHs is shown in Figure 3.4a, while the FAN content and yeast cell density at the same period is shown in Figure 3.4b. pH 5 was found to be optimum as the highest utilization of nutrients was observed. Initial concentration of reducing sugars and FAN in the wort was 2366 µg/ml and 2253 mg/ml, respectively. Fermentations at pH 3, 5 and 7 resulted in 21.09%, 38.27% and 26.31% reducing sugar utilization, respectively. At pH 5, 35.28% of FAN was utilized whereas fermentation at pH 3 and 7 resulted in 22.19% and 30.74% FAN utilization, respectively. Furthermore, at pH 5 the highest amount of ethanol was produced (4.07% [v/v]) which was 77.73% and 81.70% higher alcohol produced than at pH 3 and 7, respectively. Yeast cell density increased during fermentation at pH 5 and 7 up to day 4 reaching a maximum of 3.48×10^{11} cfu/ml and 2.54×10^{10} cfu/ml, respectively thereafter decreased. At pH 3, yeast cell density increased until day 3 of fermentation attaining a maximum of 2.22×10^{9} cfu/ml and gradually decreased thereafter.

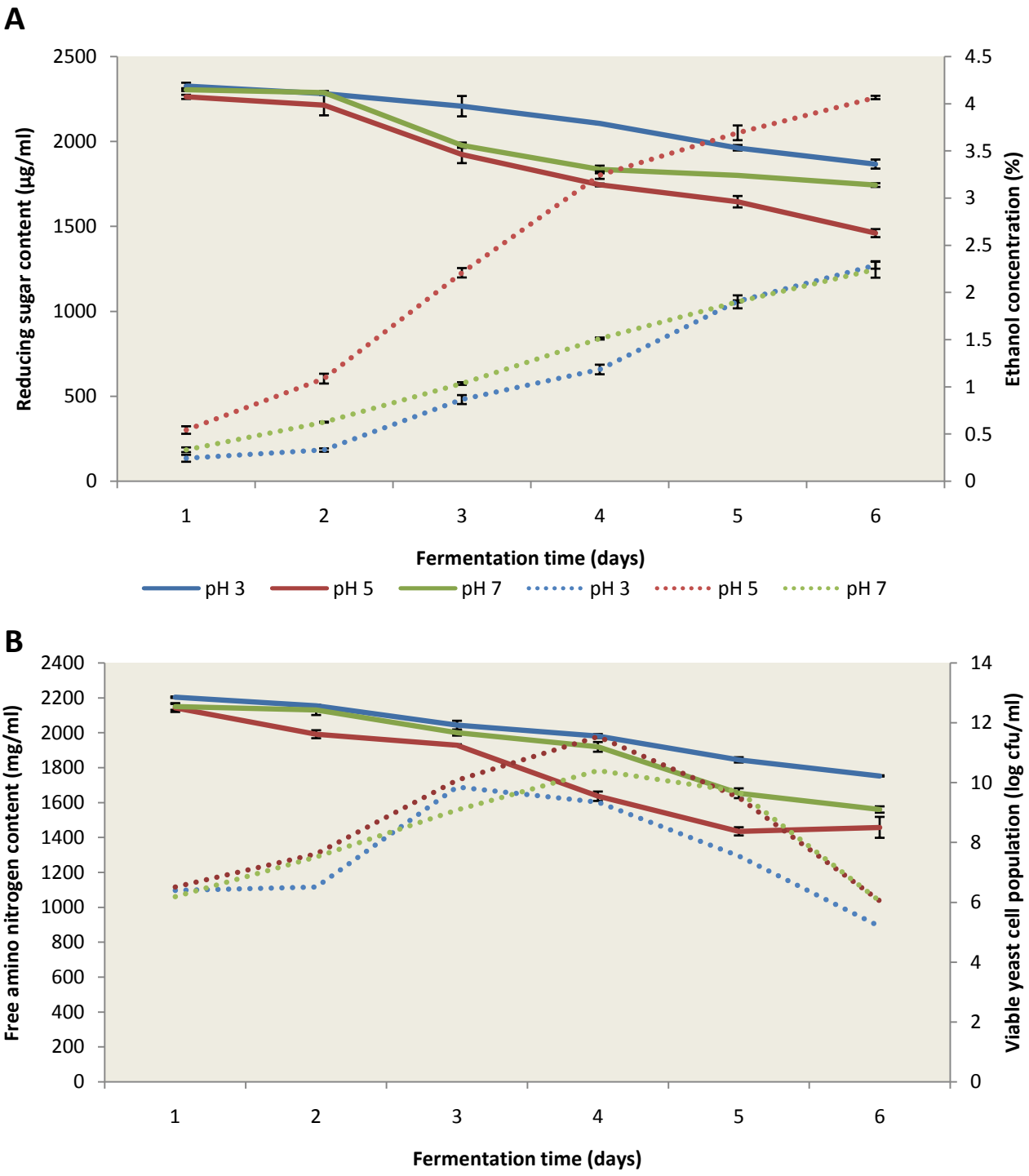


Figure 3.4: Profiles of (a) Reducing sugar and ethanol content and (b) Free amino nitrogen concentration and yeast cell density in the wort during fermentation at varying fermentation temperature. Time 0 vales for reducing sugars and FAN are 2366 µg/ml and 2253 mg/ml, respectively. Solid lines represent reducing sugar and free amino nitrogen content and dotted lines represent ethanol concentration and yeast cell density.

3.3.2 Post fermentation analysis

3.3.2.1 Spent yeast density

Spent yeast density determined under the various nutritional and fermentation conditions ranged from 1.985 to 2.848 mg/ml. Supplementation of wort with $ZnSO_4$ and L-leucine, resulted in an increase in spent yeast density (Figure 3.5). Fermentation in the presence of 0.12 g/l zinc sulphate produced the highest spent yeast density of 2.848 mg/ml which was a 32.52% more compared to that obtained during the fermentation without any $ZnSO_4$. Addition of 0.75 g/l L-leucine into wort resulted in 17.01% increase in spent yeast density compared to the unsupplemented sample. Similarly, increase in fermentation temperature from 18 °C to RT resulted in 10.31% increase in spent yeast density. However, increase in wort acidity and alkalinity resulted in a decrease in spent yeast density. The highest spent yeast density was observed at pH 5. There was an 8.82% and 7.73% reduction in spent yeast density at pH 3 and pH 7, respectively compared to that at pH 5.

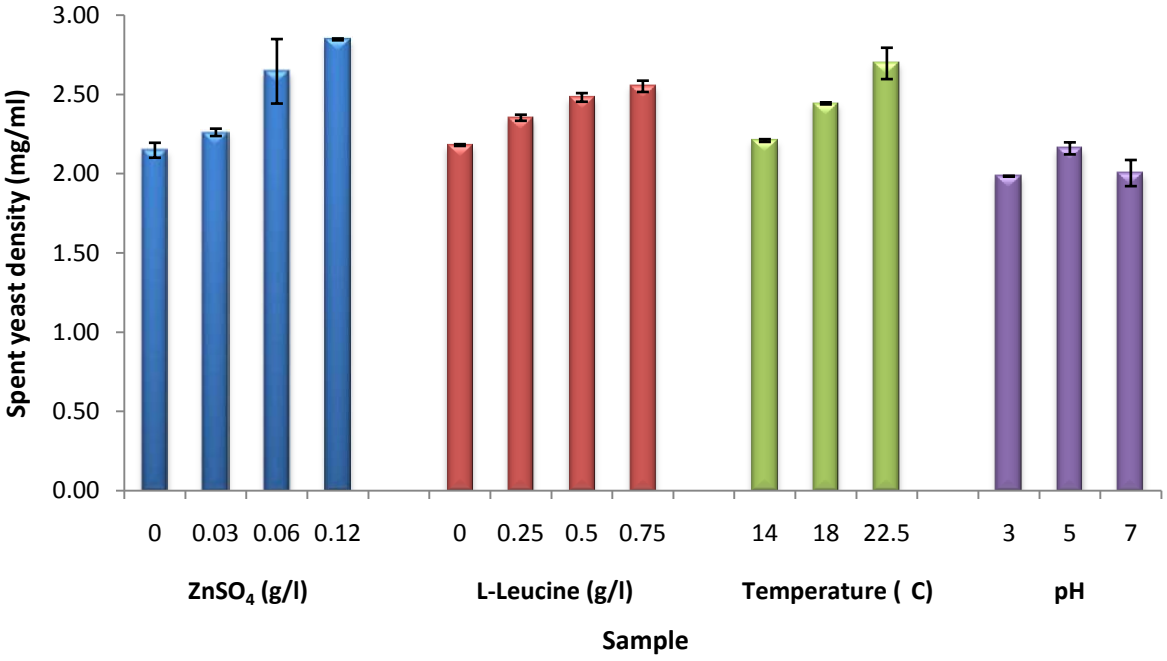


Figure 3.5: Spent yeast density produced under the various nutritional and fermentation conditions

3.3.2.2 Beer colour and foam head stability

Beer colour was measured after bottle conditioning to determine the colour intensities in the beers produced under the different conditions. The differences in colour intensity between the different experimental beers were similar. A commercial beer which served as a control had the deepest colour intensity as its absorbance at 430 nm was 0.862 (Table 3.1). The experimental beer that was supplemented with 0.75 g/l L-leucine resulted in the deepest colour intensity producing an absorbance of 0.683 at 430 nm.

The commercial beer had the best foam head stability compared to all the experimental beers. The best foam head stability was produced in the experimental beers with 0.75 g/l L-leucine, retaining as high as 53.4% foam head stability compared to the commercial beer, while those prepared at pH 7 and at RT had the least foam head stability rating (Table 3.1). An increase in $ZnSO_4$ in wort from 0.03 g/l to 0.12 g/l resulted in a decrease in foam head stability by 14.16% while increasing L-leucine concentration in the wort resulted in an increase in foam head stability. Wort supplemented with 0.75 g/l L-leucine had 14.59% better foam head stability than the unsupplemented control. Alteration of fermentation temperature resulted in a decrease on foam head stability at RT decreasing by 28.33%. However, increasing pH of the fermentation medium resulted in a decrease in foam head stability.

Table 3.1: Colour profiles and foam head stability of ale beer produced under the various nutritional and fermentation conditions

Sample	Beer colour (430nm)	Foam head stability rating
Zinc sulphate (g/l)		
0.00	0.655 ± 0.00	2.33 ± 0.58
0.03	0.653 ± 0.00	2.33 ± 0.58
0.06	0.655 ± 0.01	2.00 ± 0.00
0.12	0.647 ± 0.00	2.00 ± 0.00
L-leucine (g/l)		
0.00	0.654 ± 0.01	2.33 ± 0.58
0.25	0.665 ± 0.00	2.33 ± 0.58
0.50	0.674 ± 0.00	2.33 ± 0.58
0.75	0.683 ± 0.00	2.67 ± 0.58
Temperature (°C)		
14	0.661 ± 0.00	2.33 ± 0.58
18	0.666 ± 0.01	2.33 ± 0.58
Room temperature (22.5)	0.678 ± 0.17	1.67 ± 0.58
pH		
3	0.653 ± 0.01	1.33 ± 0.58
5	0.648 ± 0.00	2.33 ± 0.58
7	0.638 ± 0.01	1.67 ± 0.58
Commercial beer	0.862 ± 0.00	5.00 ± 0.00

Values are averages of triplicate results ± standard deviation

3.3.2.4 Detection of beer volatile ester compounds

The volatile ester compounds produced during the fermentations were quantified by Gas Chromatographic analysis of the head space samples and results shown in Table 3.2. Addition of 0.12 g/l $ZnSO_4$ into the fermentation medium resulted in a 27.70% increase in total acetate esters and 123.02% increase in total ethyl esters compared to the unsupplemented sample (Table 3.2). Ethyl acetate constituted roughly 72.5% of the total esters, while ethyl octanoate was present in very small amounts. Supplementation of wort with 0.120 g/l $ZnSO_4$ resulted in an increase in all ester concentrations with the highest increase of 145.85% in ethyl decanoate concentration obtained. This was followed by isoamyl acetate, phenyl ethyl acetate, ethyl acetate, ethyl hexanoate and ethyl octanoate increasing by 145.78%, 43.06%, 18.08%, 15.26% and 14.76%, respectively, compared to the unsupplemented control. There is a good correlation between total acetate ester concentration and $ZnSO_4$ concentration (R^2= 0.953) (Figure 3.6 a). Of these acetate esters, isoamyl acetate showed a good correlation with $ZnSO_4$ concentration (R^2= 0.980). There

was also a good correlation with $ZnSO_4$ concentration and ethyl ester concentration (R^2= 0.988) specifically ethyl decanoate (R^2= 0.985) and ethyl octanoate (R^2= 0.980). All acetate esters and ethyl decanoate exceeded their threshold level under all $ZnSO_4$ concentrations.

Addition of L-leucine (0.750 g/l) into the fermentation medium resulted in a 41.27% increase in total acetate ester concentration and 83.76% increase in total ethyl ester concentration compared to the control which was not supplemented with L-leucine (Table 3.2). The highest increase was observed for isoamyl acetate which increased by 605.21% compared to the unsupplemented control. This was followed by ethyl decanoate, phenyl ethyl acetate, ethyl acetate, ethyl hexanoate, and ethyl octanoate increasing by 96.46%, 52.86%, 19.02%, 6.96% and 6.57%, respectively. There was a good correlation between increasing L-leucine concentration and phenyl ethyl acetate (R^2= 0.961) (Figure 3.6 b). All acetate esters and ethyl decanoate exceeded their threshold level under all L-leucine concentrations.

Increasing the fermentation temperature from 18 °C to room temperature (22.5 °C) resulted in an increase in total acetate ester concentration by 14.42%, and total ethyl ester concentration by 62.82% (Table 3.2). The highest increase of 89.25% was observed for ethyl decanoate, followed by isoamyl acetate and ethyl acetate increasing by 22.80%, and 17.46%, respectively, compared to the control. However, there was a decrease in phenyl ethyl acetate, ethyl octanoate and ethyl hexanaote. There was a good correlation between total acetate esters (R^2= 0.999), specifically isoamyl acetate (R^2= 0.965) and the fermentation temperature (Figure 3.6c). Of the ethyl esters, there was a good correlation between fermentation temperature and ethyl decanoate (R^2= 0.982). All acetate esters and ethyl decanoate exceeded their threshold level under all fermentation temperature.

Fermentation pH that produced the highest concentration of esters was 7 resulting in a 13.08% increase in total acetate ester and 6.76% total ethyl ester production when compared to the control (pH 5) (Table 3.2). There was a 60.17% increase in isoamyl ester production at pH 7, followed by phenyl ethyl acetate, ethyl octanoate, ethyl acetate, ethyl decanoate, and ethyl hexanoate increasing by 14.56%, 9.60%, 9.21%, 6.27% and 1.13%, respectively. There was an 18.40% decrease in total ester concentration at pH 3, with isoamyl acetate decreasing by 43.64%. There was a good correlation between fermentation pH and total acetate ester concentration (R^2= 0.986) (Figure 3.6d). All acetate esters and ethyl decanoate exceeded their threshold level under all fermentation pH.

Table 3.2: Ester concentrations in ale beer produced under different nutritional and fermentation conditions

Sample	Acetate ester concentration (mg/l)			Ethyl ester concentration (mg/l)		
	Ethyl acetate	Isoamyl acetate	Phenyl ethyl acetate	Ethyl decanoate	Ethyl hexanoate	Ethyl octanoate
Zinc concentration (g/l)						
0.00	34.45 ± 1.78	2.56 ± 0.04	2.09 ± 0.02	1.96 ± 0.04	0.255 ± 0.01	0.159 ± 0.00
0.03	36.34 ± 1.64	3.93 ± 0.02	2.52 ± 0.08	2.48 ± 0.10	0.287 ± 0.00	0.168 ± 0.01
0.06	39.54 ± 0.10	4.75 ± 0.02	2.39 ± 0.07	3.59 ± 0.10	0.266 ± 0.03	0.172 ± 0.01
0.12	40.68 ± 0.12	6.29 ± 0.10	2.99 ± 0.09	4.82 ± 0.14	0.293 ± 0.01	0.183 ± 0.00
L-leucine concentration (g/l)						
0.00	31.46 ± 0.11	1.15 ± 0.01	4.44 ± 0.06	2.53 ± 0.03	0.259 ± 0.00	0.158 ± 0.00
0.25	32.55 ± 0.03	6.02 ± 0.01	5.15 ± 0.06	2.31 ± 0.02	0.262 ± 0.00	0.156 ± 0.00
0.50	32.64 ± 0.03	6.66 ± 0.14	5.59 ± 0.02	4.21 ± 0.04	0.254 ± 0.00	0.166 ± 0.00
0.75	32.36 ± 1.06	8.11 ± 0.05	6.79 ± 0.04	4.97 ± 0.20	0.276 ± 0.00	0.169 ± 0.00
Fermentation temperature (°C)						
14	28.64 ± 0.08	3.24 ± 0.02	3.85 ± 0.05	1.17 ± 0.00	0.259 ± 0.00	0.566 ± 0.01
18	30.59 ± 0.02	5.35 ± 0.02	5.87 ± 0.04	2.28 ± 0.05	0.253 ± 0.01	0.569 ± 0.00
Room temp (± 22.5)	35.93 ± 0.66	6.57 ± 0.07	5.34 ± 0.03	4.32 ± 0.04	0.231 ± 0.01	0.504 ± 0.00
Fermentation pH						
3	25.52 ± 0.02	1.33 ± 0.03	3.59 ± 0.05	2.54 ± 0.02	0.157 ± 0.00	0.297 ± 0.02
5	30.66 ± 0.05	2.36 ± 0.00	4.93 ± 0.01	2.43 ± 0.01	0.155 ± 0.00	0.439 ± 0.00
7	33.47 ± 0.04	3.73 ± 0.11	5.65 ± 0.04	2.58 ± 0.02	0.170 ± 0.00	0.444 ± 0.00
Threshold	30	1.2	3.8	1.5	0.21	0.9

Values are averages of triplicate results ± standard deviation

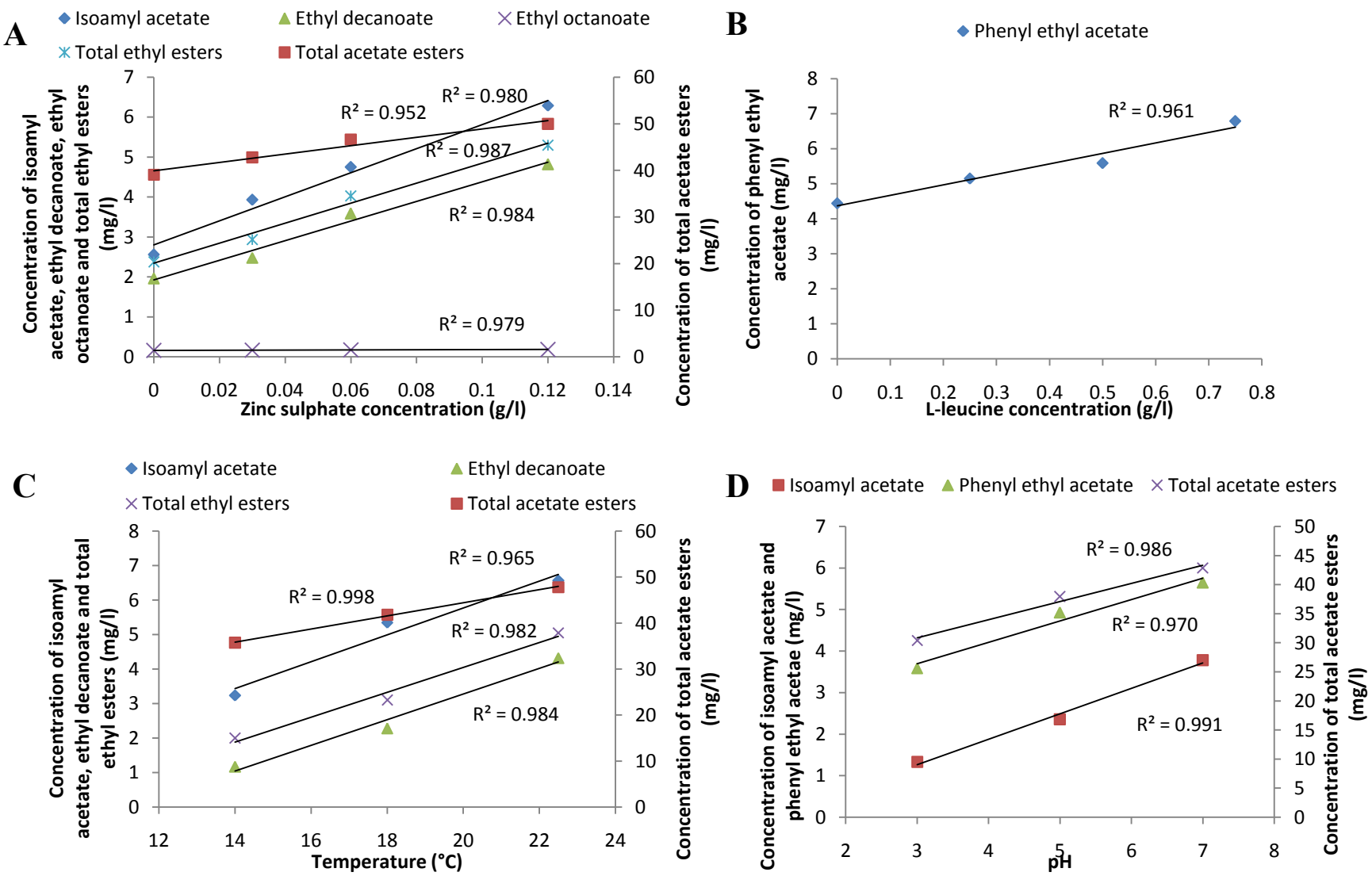


Figure 3.6: Profiles of esters in ale beer showing strong correlations with (a) concentrations of zinc sulphate, (b) concentrations of L-leucine (c) fermentation temperature and (d) fermentation pH

3.3.2.5 Stability of volatile ester compounds in ale beer over time

Stability of aroma-active esters was monitored in beer stored at 4 °C and room temperature over a three month period. Generally, beer produced in this study was more stable at 4 °C compared to room temperature after three months (Table 3.3). At 4 °C, there was a decrease in total ester concentration by 6.93% with ethyl octanoate being the least stable compound decreasing by 18.83%. Phenyl ethyl acetate was the most stable ester compound deceasing by only 2.23% after three months. Acetate esters were more stable than ethyl esters decreasing by 6.88% compared to 7.46% observed for ethyl esters after three months. By week 10, ethyl acetate concentration decreases below the threshold level. After 12 weeks of storage at 4 °C, phenyl ethyl acetate and ethyl decanoate concentrations remained above the threshold level while, ethyl octanoate and ethyl hexanoate remained below the flavour threshold. At room temperature, there was a 16.90% decrease in total ester concentration. The least stable compound was ethyl octanoate decreasing by 32.47% while the most stable compound at room temperature was phenyl ethyl acetate decreasing by 9.82% after three months. Ethyl esters were more stable at room temperature than acetate esters as it decreased by 15.64% compared to acetate esters that decreased by 16.99%. Ethyl acetate and phenyl ethyl acetate decreased below the threshold level on week 6 and 4, respectively. Ethyl decanoate remained above the threshold level after 12 weeks of storage at room temperature. Isoamyl acetate, ethyl octanoate and ethyl hexanoate remained below the threshold level after 12 weeks.

Table 3.3: Stability of aroma-active esters in ale beer during storage at 4 °C and room temperature (±22.5 °C)

Storage temperature	Storage time (weeks)	Acetate esters (mg/l)			Ethyl esters (mg/l)		
		Ethyl acetate	Isoamyl acetate	Phenyl ethyl acetate	Ethyl decanoate	Ethyl octanoate	Ethyl hexanoate
4 °C	0	31.88 ± 0.72	1.24 ± 0.02	4.48 ± 0.03	2.55 ± 0.03	0.154 ± 0.00	0.262 ± 0.01
	2	31.85 ± 0.00	1.22 ± 0.01	4.44 ± 0.01	2.54 ± 0.01	0.150 ± 0.00	0.261 ± 0.00
	4	31.27 ± 0.01	1.22 ± 0.01	4.43 ± 0.01	2.54 ± 0.01	0.147 ± 0.00	0.261 ± 0.00
	6	31.24 ± 0.09	1.22 ± 0.01	4.40 ± 0.01	2.51 ± 0.01	0.142 ± 0.00	0.260 ± 0.00
	8	30.29 ± 0.06	1.21 ± 0.01	4.44 ± 0.03	2.43 ± 0.00	0.138 ± 0.00	0.257 ± 0.00
	10	29.54 ± 0.06	1.15 ± 0.01	4.41 ± 0.07	2.40 ± 0.01	0.131 ± 0.00	0.255 ± 0.00
	12	29.51 ± 0.01	1.12 ± 0.01	4.38 ± 0.00	2.37 ± 0.00	0.126 ± 0.00	0.249 ± 0.00
Room temperature (± 22.5 °C)	0	31.88 ± 0.10	1.24 ± 0.01	4.48 ± 0.01	2.55 ± 0.00	0.154 ± 0.00	0.262 ± 0.00
	2	30.45 ± 0.01	1.21 ± 0.01	4.46 ± 0.00	2.50 ± 0.00	0.153 ± 0.00	0.260 ± 0.00
	4	30.21 ± 0.01	1.19 ± 0.01	4.40 ± 0.01	2.47 ± 0.04	0.149 ± 0.00	0.254 ± 0.00
	6	29.45 ± 0.00	1.14 ± 0.01	4.35 ± 0.02	2.42 ± 0.01	0.137 ± 0.00	0.254 ± 0.00
	8	28.39 ± 0.03	1.09 ± 0.01	4.28 ± 0.01	2.36 ± 0.01	0.131 ± 0.00	0.251 ± 0.00
	10	27.21 ± 0.01	1.04 ± 0.00	4.21 ± 0.01	2.33 ± 0.00	0.125 ± 0.00	0.235 ± 0.00
	12	26.28 ± 0.52	0.89 ± 0.00	4.04 ± 0.01	2.18 ± 0.00	0.104 ± 0.00	0.218 ± 0.00

3.4 Discussion

In this study, the influence of fermentation temperature and pH and wort composition on fermentation performance and the production of important aroma active esters were assessed. Fermentation performance under the various conditions was monitored for a six day fermentation period. It was not surprising that wort supplemented with $ZnSO_4$ or L-leucine lead to an increase in utilization of nutrients and a higher ethanol production, as metal ions such as zinc and amino acids such as L-leucine are essential for yeast growth and metabolism (Lodolo *et al*., 2008). Metal ions act as co-factors for important fermentation enzymes and also as modulators of environmental stress. In general, metal ions can impact on the metabolic processes during fermentation by influencing several important parameters including yeast growth, viability, enzyme activities, alcohol fermentation, stress tolerance, etc. (Venkateshwar *et al*., 2010). Amino acids play a crucial role in yeast nutrition as it is utilized by yeast for protein formation (structural and enzymic) required for growth (Pierce, 1987). Of the different fermentation temperatures and pHs investigated in this study optimum yeast performance was achieved at room temperature and at pH 5, respectively. Increasing fermentation temperature from 18 °C to room temperature (22.5 °C) resulted in a faster fermentation and a higher utilization of nutrients. Fermentation temperature affected not only the fermentation kinetics (rate of fermentation) but also the yeast metabolism which determined the chemical composition of the beer. Similar results have been reported by Torija *et al*. (2003).

The appearance of beer is an important quality in the final product therefore, colour and foam head stability was analysed in the ale beer in this study. Colour arises in raw materials primarily as a result of the Maillard reaction also called, descriptively, nonenzymic or nonoxidative browning (Lewis and Bamforth, 2006). Since all wort was prepared with the same raw ingredients, the colour profiles were similar. Beer foam head stability was increased with the addition on L-leucine into the fermentation medium, whereas the addition of $ZnSO_4$ and increase in the fermentation temperature resulted in a decrease in foam head stability. Since amino acids are a building block for proteins, increase in L-leucine concentration allows for more proteins to interact with Protein Z, the protein responsible for the stability of beer foam head, therefore resulting in an increase in foam head stability (Asano and Hashimota, 1980). Brewers' spent yeast is usually sold for use as food and feed due to its high vitamin content (Ingledew *et al*., 1977), production of a high spent yeast density is therefore beneficial. Addition of $ZnSO_4$ or L-

leucine into the fermentation medium resulted in an increase in spent yeast density. This is due to the fact that these supplements stimulated yeast growth, thus resulting in a higher spent yeast density yield. A similar trend was observed with elevated fermentation temperature.

Volatile esters are of major industrial interest because the presence of these compounds determines the fruity aroma of beer (Saerens *et al*., 2008a). Addition of $ZnSO_4$ (0.120 g/l) into the fermentation medium resulted in an increase in acetate and ethyl esters. This could be due to the stimulation of higher alcohol production by zinc, which can subsequently be converted to esters (Hodgson and Moir, 1990 cited by Verstrepen *et al*., 2003a). Similarly, addition of L-leucine (0.750 g/l) into the fermentation medium resulted in an increase in acetate and ethyl esters. The concentration and composition of wort FAN have an impact on the production of higher alcohol and esters, due to the role of amino acid metabolism in the formation of these flavour compounds (Pierce, 1987; O'Connor-Cox and Ingledew, 1989). Increasing the fermentation temperature from 18 °C to room temperature (22.5 °C) resulted in an increase in acetate and ethyl ester concentration in the beer. According to Suomaleinen (1981), an increase in the fermentation temperature releases higher levels of esters through more efficient excretion and/or enhanced autolysis of yeast. The fermentation pH that resulted in the highest ester production in his study was pH 7, resulting in higher acetate and ethyl ester production when compared to the control (pH 5). Horton *et al*. (2003) studied the effect of pH on the enzyme alcohol acetyltransferase (AATase) in brewer's yeast and they found that the enzyme was most active in the pH range of 7 to 8. Generally, ale yeast strains produce a higher concentration of higher alcohols than lager strains (Saerens *et al*., 2008b). Since there is a higher concentration of higher alcohols, more esters can be formed. This explains the higher ester concentration that was obtained for ale beer compared to lager beer.

In ale beers, the intensity of ester aroma character is regarded as an important component of sensory quality, having a more intense fruity aroma than lagers (Verstrepen *et al*., 2003a). The concentration of the various ester compounds gradually decreased over the three month period possibly due to ester hydrolysis with beers stored at room temperature resulting in a much faster decrease in ester concentration than those stored at 4 °C. Chemical hydrolysis is an acid-catalysed process, but the activity of the enzyme with esterase activity, sometimes detected in beer, can affect the ester profile. Neven *et al*. (1997) showed that some esterases are released by yeast into beer as a result of cell autolysis during fermentation and maturation. Such esterase

activity is strain dependent and top-fermenting (ale) yeasts are more active than bottom fermenting (lager) yeasts. This could explain why there was a higher decrease in ester concentration after three months of storage at room temperature in ale beer than in lager beer.

Fermentation conditions and nutritional supplements are important in beer brewing due to its influence on fermentation performance and in final product characteristics. Control of flavour ester levels in alcoholic beverages is a sensitive process. Supplementing wort with essential nutrients and altering important fermentation conditions can be used to alter ester formation in beer. Moreover, since these conditions increase the rate of fermentation, allowing yeast to utilize nutrients faster and producing ethanol faster, the fermentation time can be reduced by two to three days. This is promising for a reduced operational cost for the production of ale beer with the desired flavour properties.

CHAPTER 4: Expression levels of ester biosynthetase genes in ale and lager yeast strains under the optimum nutritional and fermentation conditions

4.1 Introduction

The yeast *Saccharomyces cerevisiae* produces a broad range of aroma-active substances during fermentation, which are vital for the complex flavour of beer. Of these, aroma active substances, volatile esters are rather significant. Volatile esters are formed intracellularly and are the product of an enzyme-catalysed condensation reaction formed when alcohol and carboxylic acid functional groups react, and a water molecule is eliminated (Sumby *et al*., 2010; Verstrepen *et al.,* 2003b). The ethyl esters comprise of an alcohol group (ethanol) and an acid group (medium-chain fatty acid) (Saerens *et al*., 2008b), and include ethyl hexanoate, ethyl octanoate and ethyl decanoate. The acetate esters are comprised of an acid group (acetate) and an alcohol group which is either ethanol or a complex alcohol derived from amino acid metabolism (Saerens *et al*., 2008b), and includes ethyl acetate and isoamyl acetate (Sumby *et al*., 2010). This condensation reaction is catalysed by alcohol acetyltransferases (Verstrepen *et al.,* 2003b), a membrane associated sulfhydryl enzyme (Rojas *et al*., 2002; Lilliy *et al*., 2000). At present, three different alcohol acetyltransferases have been identified in yeast: Atf1, its closely related homologue Lg-Atf1, and Atf2. Atf1 and Atf2 are present in both *S. cerevisiae*, whereas Lg-Atf1 is found only in *S. pastorianus.* Homology-based searches of the *S. cerevisiae* genome have not revealed any other gene encoding a putative ester-synthesizing enzyme with sequence similarity to Atf1 and/or Atf2 (Saerens *et al*., 2006). The expression levels of *ATF1* and *ATF2* significantly affect the production of ethyl and isoamyl acetate (Verstrepen *et al.,* 2003a). In addition to these alcohol acetyltransferases, two other enzymes, encoded by *EHT1* and *EEB1*, have been described as being responsible for the production of ethyl esters (Mason and Dufour 2000; Saerens *et al*., 2006). Eeb1 is the main enzyme, while Eht1 plays only a minor role.

Of all known ester synthases, Atf1 is the most important for the production of flavour active acetate esters. The Atf1 protein, encoded by the *ATF1* gene, consists of 525 amino acids with a calculated molecular weight of 61 kDa (Fujii *et al*., 1994). Evidence from gene disruption and expression analysis of members of the *ATF* gene family indicate that different ester synthases are involved in the synthesis of esters during alcoholic fermentation (Lodolo *et al*., 2008). Deletion analysis has shown that *ATF1* is responsible for 80% of the isoamyl acetate

formation, 75% of the phenyl ethyl acetate production, and about 40% of the ethyl acetate synthesis. In addition, overexpression of the Atf1-encoding gene, *ATF1*, results in more than 100-fold increase in isoamyl acetate, as well as a 10–200-fold increase in other esters, such as ethyl acetate, phenyl ethyl acetate and C_3–C_8 acetate esters (Fujii *et al*., 1994; Lilly *et al*., 2000; Verstrepen *et al*., 2003a). A second alcohol acetyltransferase, *ATF2* has also been characterised. *ATF2*, along with *ATF1*, is responsible for the production of ethyl acetate and 3-methylbutyl acetate during fermentation (Lilly *et al*., 2006; Malcorps and Dufour, 1992; Yoshioka and Hashimoto, 1983). A study using over-expression of a recombinant *ATF2* in *E. coli*, with mutations in other pathways that compete for acetyl-CoA, showed that 3-methylbutyl acetate production was linked to intracellular acetyl-CoA levels (Sumby *et al.,* 2010).

More recently, two new proteins with both medium-chain fatty acid (MCFA) ethyl ester synthase and esterase activity have been reported (Saerens *et al*., 2006). However, how the balance between MCFA ethyl ester synthesis and hydrolysis is regulated by these proteins *in vivo* has not been determined. *EHT1* has been suggested to encode an ethanol hexanoyl transferase, which generates ethyl hexanoate from ethanol and hexanoyl-CoA, playing a minor role in MCFA ethyl ester biosynthesis and possessing short-chain esterase activity (Saerens *et al*., 2006). *EEB1* encodes an ethanol acyltransferase responsible for the major part of MCFA ethyl ester biosynthesis during fermentation. *EEB1* also possesses short-chain esterase activity and may be involved in lipid metabolism and detoxification. Saerens *et al*. (2006) also hypothesised that yeast cells must have one or more additional enzymes responsible for MCFA ethyl ester synthesis, due to a double deletion mutant retaining some MCFA ethyl ester synthesis ability (dependent on the substrate).

Some of these acetate esters, like ethyl acetate and isoamyl acetate, are important flavour components of fermented beverages, such as beer and wine. In modern beer and wine production processes, the delicate balance of acetate esters is often disturbed, leading to severe undesired off flavours. Much attention has therefore been drawn to the different factors that control the formation of volatile acetate esters (Verstrepen *et al.,* 2003b). The need to understand and control ester synthesis is driven by problems encountered in brewing procedures, such as high-gravity brewing (production of disproportionate amounts of ethyl acetate and isoamyl acetate), the use of large scale cylindroconical fermenters (reduction of ester levels) or the production of reduced-alcohol beers (lack of flavour compounds). An understanding of the molecular basis of

ester synthesis should allow for better control of ester production in these processes (Mason *et al*., 2000).

In order to obtain insight into the influence of fermentation temperature, pH and wort supplements on flavour formation, this study investigated the expression level of the ester biosynthesis genes (*ATF1, ATF2, EHT1* and *EEB1)* during fermentation under conditions that resulted in the highest ester production, as described in Chapters two and three using a lager and ale brewing yeast strain. This will give information regarding the propensity of different yeast strains to produce specific biosynthesis genes associated with flavour compounds and therefore assist in predicting the effect of fermentation paramenters and nutritional supplements on beer flavour profiles. This study aims to investigate a possible correlation between the gene expression levels of the ester biosynthetase genes and the concentration of esters produced under the different conditions.

4.2 Materials and Methods

4.2.1 Wort preparation and fermentation conditions

The fermentation medium (wort) was prepared as described in chapter two. Fermentations were performed in duplicate, a sample was taken from each fermentation vessels and duplicate samples were pooled. This was followed by RNA extraction. A control fermentation was carried out at 14 °C for the lager strain and 18 °C for the ale strain at pH 5 with no supplements. Conditions that resulted in the highest ester concentration found in Chapter two and three were tested separately. These conditions were: a. wort supplementation with 0.120 g Zinc Sulphate ($ZnSO_4$), b. wort supplementation with 0.75 g L-leucine, c. fermentation temperature of 22.5 °C (for both ale and lager beer) and d. fermentation pH of 7.

4.2.2 RNA Extraction

One milliliter samples was collected from the fermentation vessel on each day of fermentation and centrifuged at 3,000 rpm for 5 min. RNA extraction from pelleted cells was performed with Trizol (Invitrogen), according to the manufacturer's instructions. Pelleted cells were lysed in TRIZOL Reagent by repetitive pipetting using 1 ml of the reagent per 5-10 $\times$ 10^6 of yeast cells. The homogenized samples were incubated for 5 min at 30 °C to permit the complete dissociation of nucleoprotein complexes. Two hundred microlitres of chloroform per 1 ml of TRIZOL Reagent was added. Tubes were hand shaken vigorously for 15 s and incubated at 30 °C for 3 min. The samples were centrifuged at 12,000 rpm for 15 min at 4 °C. Following centrifugation, the mixture separated into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The RNA remained exclusively in the aqueous phase. The volume of the aqueous phase was about 60% of the volume of TRIZOL Reagent used for homogenization. The aqueous phase was transferred to a fresh tube and the RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. A 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL Reagent used for the initial homogenization was added. Samples were incubated at 30 °C for 10 min and centrifuged at 12,000 rpm for 10 min at 4 °C. The RNA precipitate, often invisible before centrifugation, formed a gel-like pellet on the side and bottom of the tube. The supernatant was removed and the RNA pellet was washed once with 1 ml 75% ethanol per 1 ml of TRIZOL reagent used for the initial homogenization. The sample was mixed by vortexing and centrifuged at 7,500 rpm for 5 min at 4 °C. At the end of the procedure, the

RNA pellet was air dried for 10 min. RNA was dissolved in RNase-free water by passing the solution a few times through a pipette tip, and incubated for 10 min at 55 °C. RNA concentrations were determined by the NanoDrop 2000c (Thermo Scientific) using 1 µl of RNA sample and was then stored at -70 °C until further use.

4.2.3. Reverse transcription

For each sample, 1 µg of total RNA were subjected to reverse transcription (RT) using the Reverse Transcription System (Promega) according to manufacturer's instructions. RNA was combined with 0.1 µl of cDNA random primers in nuclease-free water to give a final volume of 5 µl per reaction in thin walled sterile tube. Tubes were placed in a preheated 70 °C heat block for 5 min and then chilled on ice for 5 min. This was followed by tubes being spun down in a microcentrifuge for 10 s to collect the condensate and to maintain the original volume. The reverse transcription reaction mix was prepared by combining 4.0 µl ImProm-II™ 5X reaction buffer, 1.2 µl $MgCl_2$ (final concentration 0.5 mM), 0.5 µl dNTP Mix, 0.5 µl Recombinant RNasin® Ribonuclease Inhibitor, 1.0 µl ImProm-II™ Reverse Transcriptase and 7.8 µl of nuclease free water in a sterile 1.5 ml microcentrifuge tube on ice to give a final volume of 15 µl. Fifteen microlitre aliquots of the reverse transcription reaction mix was added to a reaction tube on ice that contained 5 µl of RNA and primer mix for a final reaction volume of 20 µl per tube. The tubes were placed in a temperature controlled heat block equilibrated at 25 °C, and incubated for 5 min to allow for annealing. The tubes were then incubated at 42 °C for one hour for extension. Reverse transcriptase was thermally inactivated prior to amplification by incubating the reaction tubes at 70 °C for 15 min. Concentrations of cDNA were measured using the NanoDrop 2000c (Themo Scientic) and samples diluted to 70 ng/µl before storage at -70 °C.

4.2.4. Detection of gene expression levels by Quantitative PCR (qPCR)

The expression levels of *ATF1*, *ATF2*, *EHT1* and *EEB1* was determined using quantitative-PCR (qPCR). The 25 µl PCR reaction was composed of 12.5 µl Power SYBR Green PCR Master Mix with ROX (Applied Biosystems), 1.25 µl of each primer (500 nM), 7.5 µl ddH_2O and 2.5 µl of cDNA. The PCR program consisted of an initial denaturation for 10 min at 95 °C, amplification by 40 cycles of 15 s at 95 °C and 1 min at the optimal annealing temperature for the specific primer pair under qPCR conditions: 58 °C for *RDN18, ATF1, ATF2*

and 56 °C for *EHT1* and *EEB1*. Primers for the selected genes were designed to anneal close to the 3'-end of each gene. Primer sequences that were used for qPCR analysis (from 5′ to 3′) are shown in Table 4.1.

Table 4.1: Primer sequences used for qPCR analysis (Saerens *et al.*, 2008b).

Gene	Primer sequence
RDN18-F	CGGCTACCACATCCAAGGAA
RDN18-R	GCTGGAATTACCGCGGCT
ATF1-F	GTACGAGGAGGATTACCA
ATF1-R	ATGATCTCGGTGACAAC
ATF2-F	AAGCCGTACTACGTTC
ATF2-R	CGCTCATGTCCATGTTC
EHT1-F	TGGCTCTCCCCGAT CA
EHT1-R	AGGCGTGAACATATAGAAAGATGGA
EEB1-F	TCGTACACACTTGGGACAAGTTG
EEB1-R	CAGTCCTTGTTAGAAATTGTGTTAAAGTTC

Reactions were run in a 7500/7000 Fast Real Time PCR System (Applied Biosystems). Gene expression was quantified using the $2^{-\Delta\Delta Ct}$ Method (Pfaffl model) which combines gene quantification and normalization. For this mathematical model, it was essential to determine the crossing threshold (C_t) value of each transcript. The C_t values of both the control and the genes of interest were normalized to an appropriate housekeeping gene (ΔC_t) (4.2.4.1). 18S rRNA gene (*RDN18*) was used as the housekeeping gene because its expression is stable under experimental conditions. By using this normalization with *RDN18*, it was possible to avoid a series of well-known sources of error in the gene expression quantification, which include different RNA extraction efficiencies depending on the growth stage, different mRNA stabilities and different mRNA cellular contents at different stages of fermentation (Bustin 2009). Once the ΔC_t between the target and housekeeping gene is calculated, then the $2^{-\Delta\Delta Ct}$ was determined (4.2.4.2). The relative expression of a target gene is based on static PCR efficiency of 2. A control fermentation was carried out at 14 °C for the lager strain and 18 °C for the ale strain at pH 5 with no supplements. The quantified expression of the control was calculated as 1 and gene expression of the experimental samples was reported as fold expression relative to the control to determine how the wort supplements and altering fermentation conditions affected the gene expression.

ΔC_t sample = C_t sample – C_t reference

ΔC_t control = C_t control – C_t reference (4.2.4.1)

Relative Quantification (RQ) = $2^{-(\Delta Ct \text{ sample} - \Delta Ct \text{ control})} = 2^{-\Delta\Delta Ct}$ (4.2.4.2)

4.3 Results (Data for results presented in Chapter is shown in Appendix C)

4.3.1 Synthesis and measurement of cDNA

The RNA concentration ranged from 97.0 to 1047.0 ng/µl for samples extracted from the ale strain and 110.7 to 592.5 ng/µl for samples extracted from the lager strain throughout the fermentation period. The A_{260}/ A_{280} ratio obtained ranged from 1.30 to 1.72 for the ale fermentation and 1.26 to 1.80 for the lager fermentation (Tables 4.2). The A_{260}/ A_{280} ratio for samples from the lager brewing strain were higher than those of the ale brewing strain. As RNA cannot serve as a template for PCR, the first step in a qPCR assay is the synthesis of cDNA from template RNA. Synthesis of cDNA was accomplished by reverse transcriptase PCR and cDNA was quantified using the Nanodrop. The A_{260}/ A_{280} ratio ranged from 1.25 to 1.47 for the ale strain and 1.14 to 1.6 for the lager strain (Tables 4.3).

Table 4.2: RNA concentration and spectrophotometric reading of samples extracted during the fermentation period

Sample	Ale yeast strain		Lager yeast strain	
	RNA Concentration (ng/µl)	A_{260}/ A_{280} ratio	RNA Concentration (ng/µl)	A_{260}/ A_{280} ratio
Control				
Day 1	223.3	1.43	280.7	1.74
Day 2	686.0	1.54	337.3	1.66
Day 3	333.8	1.67	411.7	1.72
Day 4	238.8	1.61	148.2	1.73
Day 5	127.6	1.58	134.9	1.56
Day 6	282.9	1.65	403.2	1.76
Zinc[a]				
Day 1	403.1	1.65	486.1	1.45
Day 2	443.6	1.70	411.3	1.80
Day 3	499.9	1.30	463.6	1.47
Day 4	226.7	1.58	363.2	1.66
Day 5	159.0	1.63	255.3	1.57
Day 6	204.8	1.69	420.4	1.77
L-leucine[b]				
Day 1	401.8	1.56	198.1	1.32
Day 2	1047.0	1.52	556.5	1.42
Day 3	249.0	1.61	508.3	1.37
Day 4	251.4	1.64	357.8	1.65
Day 5	211.4	1.61	105.6	1.26
Day 6	231.6	1.64	110.7	1.54
Temperature[c]				
Day 1	97.70	1.50	409.7	1.45
Day 2	557.9	1.33	304.9	1.74
Day 3	299.5	1.61	544.9	1.32
Day 4	250.8	1.62	385.5	1.80
Day 5	196.5	1.65	295.7	1.69
Day 6	338.9	1.72	592.5	1.44
pH[d]				
Day 1	222.1	1.55	268.0	1.67
Day 2	486.8	1.44	309.2	1.72
Day 3	483.6	1.37	439.6	1.75
Day 4	244.8	1.59	302.1	1.69
Day 5	134.4	1.57	222.5	1.64
Day 6	233.4	1.65	333.0	1.71

[a] Wort supplemented with of zinc sulphate (0.120 g/l)
[b] Wort supplemented of L-leucine (0.075 g/l)
[c] Fermentation temperature (22.5 °C)
[d] Fermentation pH (pH 7)

Table 4.3: cDNA yield and A_{260}/ A_{280} ratio

Sample	Ale yeast strain		Lager yeast strain	
	DNA Concentration (ng/µl)	**A_{260}/ A_{280} ratio**	**DNA Concentration (ng/µl)**	**A_{260}/ A_{280} ratio**
Control				
Day 1	786.7	1.46	579.2	1.24
Day 2	872.1	1.39	566.8	1.31
Day 3	1097.8	1.47	1754.3	1.60
Day 4	943.9	1.42	661.1	1.34
Day 5	486.9	1.38	817.4	1.46
Day 6	918.4	1.46	1059.5	1.46
Zinc[a]				
Day 1	866.0	1.36	789.4	1.42
Day 2	879.6	1.39	742.6	1.38
Day 3	627.8	1.35	547.5	1.25
Day 4	692.9	1.31	479.8	1.14
Day 5	668.0	1.36	904.5	1.43
Day 6	1134.9	1.44	1042.6	1.46
L-leucine[b]				
Day 1	467.1	1.31	927.7	1.42
Day 2	414.8	1.25	958.9	1.46
Day 3	993.2	1.46	791.9	1.43
Day 4	1062.9	1.43	656.4	1.27
Day 5	746.3	1.39	615.0	1.37
Day 6	926.3	1.41	793.4	1.39
Temperature[c]				
Day 1	907.6	1.44	808.0	1.39
Day 2	861.1	1.39	872.5	1.39
Day 3	1052.2	1.42	874.3	1.40
Day 4	792.4	1.36	853.5	1.40
Day 5	755.5	1.40	872.9	1.42
Day 6	617.9	1.31	710.6	1.37
pH[d]				
Day 1	994.7	1.44	974.6	1.41
Day 2	837.2	1.40	645.2	1.33
Day 3	1011.1	1.42	842.4	1.39
Day 4	662.6	1.30	1051.0	1.47
Day 5	589.8	1.43	654.0	1.45
Day 6	1046.4	1.42	646.7	1.29

[a] Wort supplemented with of zinc sulphate (0.120 g/l)
[b] Wort supplemented of L-leucine (0.075 g/l)
[c] Fermentation temperature (22.5 °C)
[d] Fermentation pH (pH 7)

4.3.2 Ester biosynthetic gene expression

The expression levels of four genes involved in the synthesis of flavour active ester compounds was quantified during fermentation under the different nutritional supplement or fermentation condition. The names and functions of the genes selected for this study are detailed in Table 4.4.

Table 4.4: Genes involved in aroma biosynthetic pathways whose expression was quantified in this study

Gene	Name	Metabolic pathway
RDN18	18S ribosomal RNA	Component of the 40S ribosomal subunit
ATF1	Alcohol acetyltransferase	Acetate ester production
ATF2	Alcohol acetyltransferase	Acetate ester production
EEB1	Acyl-coenzymeA:ethanol *O*-acyltransferase	Ethyl ester production
EHT1	Ethanol hexanoyl transferase	Ethyl ester production

The expression profiles of the various genes were studied, and similar trends were observed (Figure 4.1). The expression profile of *EEB1* during the fermentation period was similar under the different conditions showing the highest expression on Day 2, thereafter decreased gradually throughout the fermentation period. The maximum expression of this gene was observed with the addition of L-leucine into the fermentation medium (3.17-fold higher expression compared to the control). The expression profile of *EHT1* under all parameters showed maximum expression on Day 3 of fermentation thereafter progressively decreasing. The addition of $ZnSO_4$ into the fermentation medium resulted in the highest expression (3.27-fold) of *EHT1* when compared to the other parameters. The expression profile of *ATF2* showed two peaks during the fermentation period which was observed on Days 1 and 4. The highest expression (133.48-fold) of this gene was the resultant of L-leucine supplementation in the wort. The expression profile for *ATF1* however was not similar among the different conditions. Optimum fermentation temperature and pH resulted in maximum expression of *ATF1* on Day 2 whereas supplementation with zinc yielded maximum expression on Day 3, while addition of L-leucine showed maximum expression on Day 5.

The expression of four of the genes in the ale strain varied during fermentation and showed different expression profiles depending on the fermentation condition and nutritional supplement (Figure 4.1). Among the four genes monitored, *ATF2* was the most highly expressed gene (up to 133.48-fold relative to the control on first day of fermentation) when the fermentation medium was supplemented with 0.75 g L-leucine (Figure 4.1c). This was followed by *EEB1* and *ETH1* (up to 3.31 and 1.28-fold relative to the control, respectively) under the same conditions. The maximum expression of these genes was achieved on Day 2 of fermentation. The lowest expressed gene was *ATF1* whose expression did not exceed that of the control throughout the fermentation. Supplementing the fermentation medium with 0.12 g $ZnSO_4$ resulted in the maximum expression of *EHT1* on Day 3 (up to 3.27-fold relative to the control). The expression of *ATF1* and *EEB1* varied during the fermentation period but did not exceed that of the control. The increase in fermentation temperature from 18 °C to 22.5 °C resulted in *ATF2* being the most highly expressed gene (up to 37.30-fold relative to the control on day 4 of fermentation) (Figure 4.1c). This was followed by *EHT1* showing a maximum expression of 2.43-fold on Day 3 and *EEB1* showing a maximum expression of 1.58-fold on Day 2 of fermentation relative to the control. Expression of *ATF1* did not exceed that of the control, exhibiting the lowest expression. Alteration of the fermentation pH from 5 to 7 lead to a maximum expression of *ATF2* on Day 4, showing an expression of up to 7.73 times relative to the control. This was followed by *ATF1* (up to 7.07-fold relative to the control), *EEB1* (up to 3.31-fold relative to the control) and *EHT1* (up to 2.31-fold relative to the control).

Expression profiles of the different genes involved in ester synthesis in the lager yeast strain varied widely when compared to the ale strain (Figure 4.2), and did not display a similar trend. The expression profile of *EEB1* showed maximum expression at different times during fermentation under the different conditions (Figure 4.2a). Addition of L-leucine and alteration of fermentation temperature displayed a similar trend, showing minimal gene expression during the first four days of fermentation, then peaking on Day 5 followed by a rapid decrease. However, when $ZnSO_4$ was added, a maximum expression was observed on Day 4 before rapidly decreasing. The gene expression profile where fermentation pH was altered was relatively low when compared to the other parameters tested. The expression profile for *EHT1* showed similar trends exhibiting maximum expression on Day 5 of fermentation when both fermentation temperature and pH were altered.

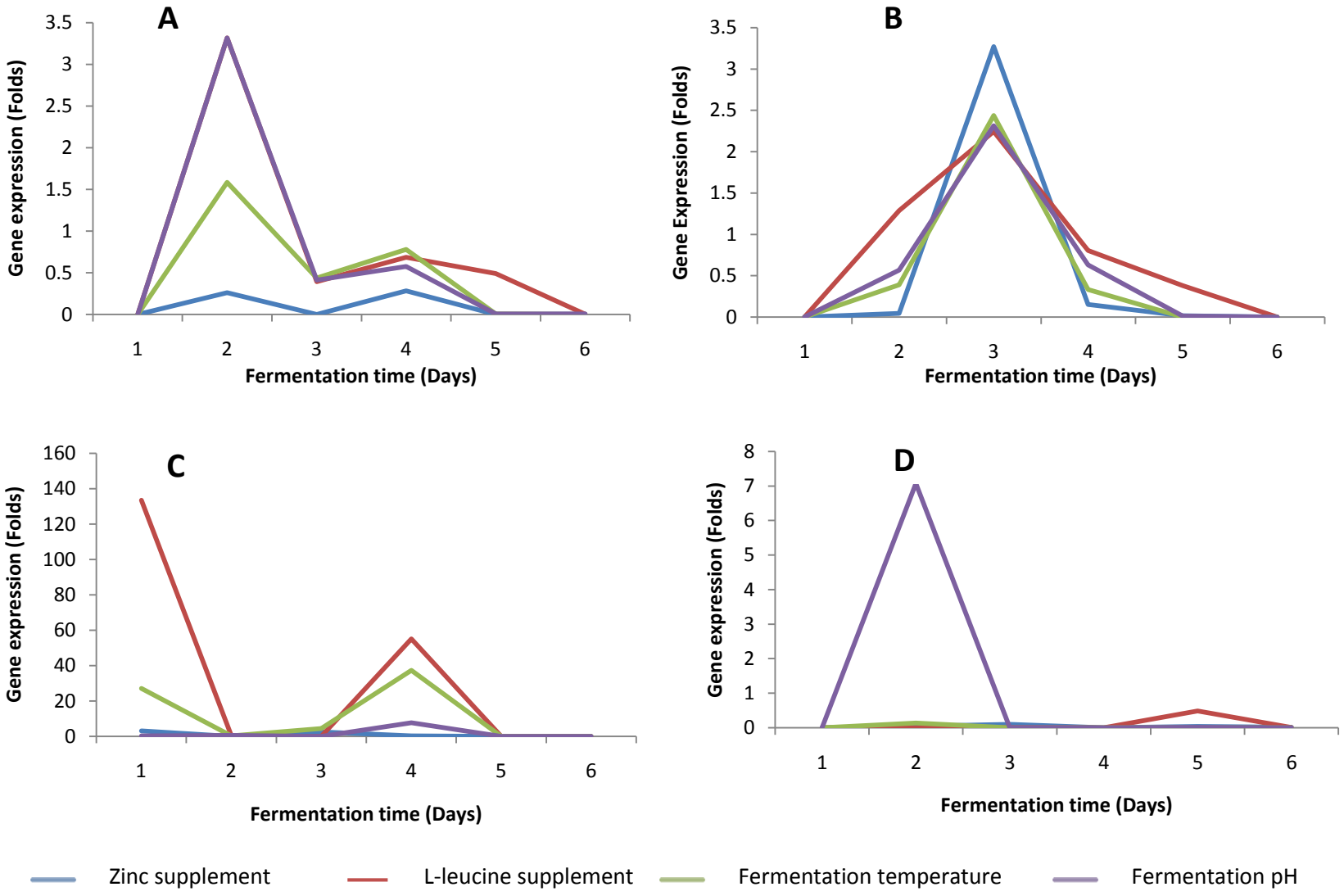


Figure 4.1: Relative expression of *EEB1* (a), *EHT1* (b), *ATF2* (c), and *ATF1* (d) genes involved in the synthesis of esters in an ale brewing yeast strain under the fermentation conditions and nutritional supplementation produced the highest ester production

Addition of L-leucine resulted in the maximum expression of *EHT1* on Day 4 followed by a rapid decrease whereas the addition of $ZnSO_4$ resulted in a relatively low expression (Figure 4.2b). The expression profile for *ATF2* displayed different trends for all conditions (Figure 4.2c). The addition of L-leucine into the wort resulted in minimal expression of this gene over the first four days of fermentation followed by a continual increase until the end of fermentation. Gene expression for zinc supplements and fermentation temperature peaked on Day 4 and 5, respectively. The expression profile for *ATF1* displayed a similar trend as *EHT1* when the fermentation conditions were altered, showing maximum expression on day 5. However, the expression of this gene when wort was supplemented with nutrients was relatively low.

Addition of 0.120 g $ZnSO_4$ into the fermentation medium resulted in the maximum expression of *ATF2* and *EHT1* on day 3 (up to 121.02 and 68.94 times relative to the control, respectively) (Figure 4.2b and c). The expression level of *ATF1* and *EHT1* was lower than the control throughout the fermentation. A similar trend can be seen with *ATF1* when 0.075 g of L-leucine was added into the fermentation medium. However, expression of *ATF2* and *EEB1* was much higher with a maximum of 1500.00 and 2125.20 times expression obtained, relative to the control, respectively. Expression of *EHT1* was up to 1.39 times that of the control. An alteration in the fermentation temperature from 14 °C to 22.5 °C resulted in a substantial increase in the expression levels of the genes. All genes on day 5 showed an expression profile of up to 1134.7 times higher when compared to the control. Altering the fermentation pH from 5 to 7 resulted in *EHT1* being the most highly expressed gene on day 1 (up to 6.88 times relative to the control). This was followed by *ATF2*, *ATF1* and *EEB1* (up to 2.76, 1.77 and 1.76 times relative to the control, respectively).

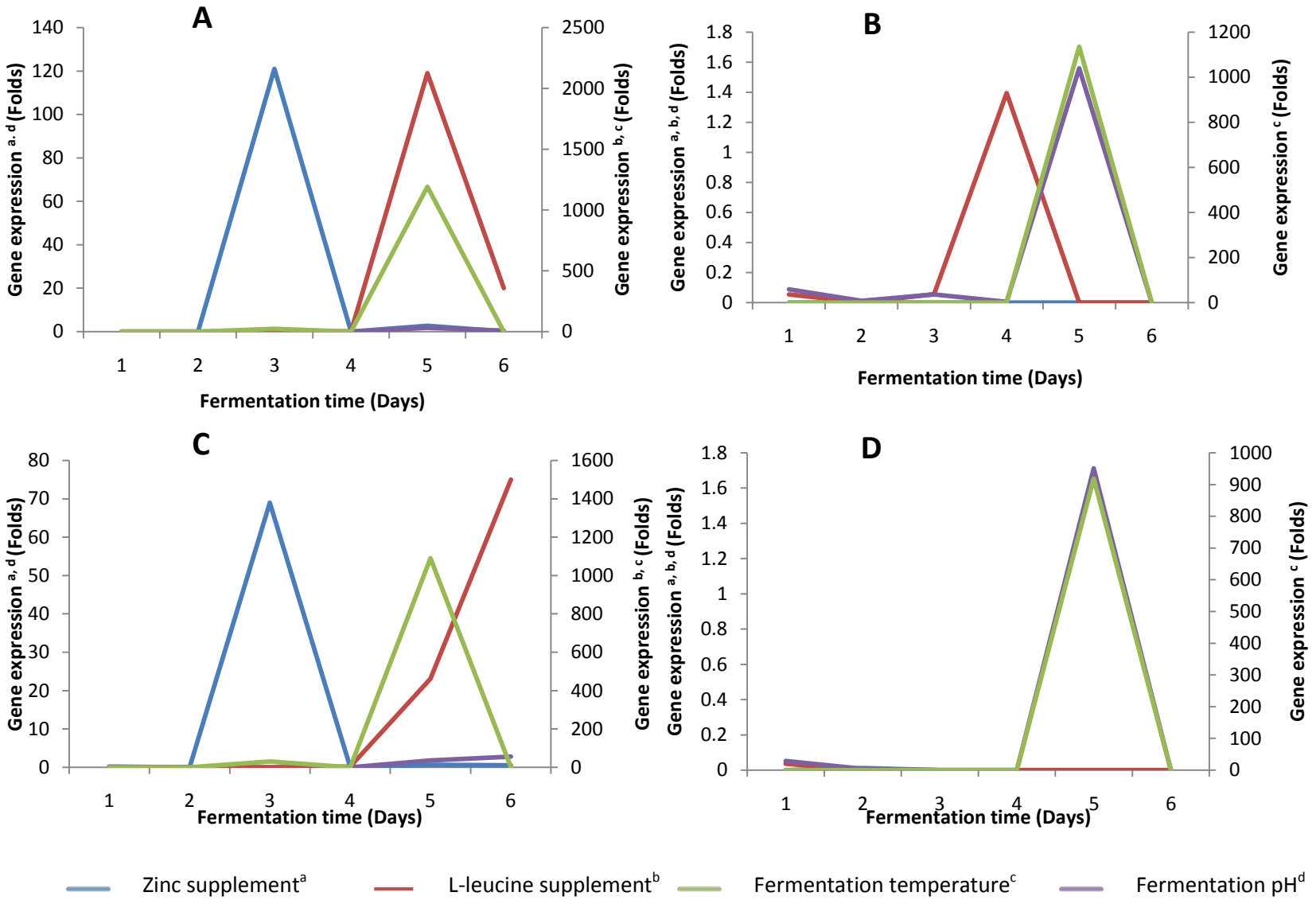


Figure 4.2: Relative expression of *EEB1* (a), *EHT1* (b), *ATF2* (c), and *ATF1* (d) genes involved in the synthesis of esters in a lager brewing yeast strain under the fermentation conditions and nutritional supplementation that produced the highest ester production

4.4 Discussion

In this study, the effects of zinc and amino acid supplementation into wort and fermentation temperature and pH on the expression level of the genes that encode the enzymes responsible for the production of volatile esters were studied in wort fermented separately with an ale and a lager yeast strain. A variety of yeast synthesized volatile aroma ester compounds were analyzed after fermentation and the expression of four genes related to the biosynthesis of these ester compounds were monitored to determine how fermentation and nutritional conditions affected their expression. Parameters that resulted in a substantial increase in total ester concentration in the final beer when compared to the control (Chapter 2 and 3) were used in this chapter. This chapter, therefore, focused on identifying whether the expression profiles of the genes responsible for the production of these esters can be used as a predictive tool to determine the ester concentration or to control the final concentration.

The expression profiles of *ATF1* and *ATF2* were analyzed to determine if there was a correlation with the concentration of acetate esters in ale beer. $ZnSO_4$ supplementation, L-leucine supplementation, increased fermentation temperature and fermentation pH resulted in an increase in total acetate ester concentration by 27.7%, 41.27%, 14.42% and 13.07% respectively, in ale beer when compared to the control (Chapter 3). This increase corresponded to the expression level of *ATF2* under these conditions. The maximum expression of *ATF2* was a result of L-leucine supplements, which correlates with the high increase in acetate ester concentration. However, the maximum expression of *ATF1* as a result of a neutral pH did not correlate with the final concentration of acetate esters in the finished product. Furthermore, the maximum expression level of *ATF2* was much higher than that of *ATF1*. Similar results have been reported by Saerens *et al*. (2008a), where it was found that *ATF2* displayed a better correlation with acetate ester concentration than *ATF1*. Therefore, the expression of *ATF2* could be used as a tool to control acetate ester concentration in ale beer. $ZnSO_4$ supplementation, L-leucine supplementation, optimum fermentation temperature and optimum fermentation pH resulted in an increase in total acetate ester concentration by 9.51%, 21.24%, 50.68% and 7.30% respectively, in lager beer when compared to the control (Chapter 2). The highest concentration of acetate esters was a result of elevated fermentation temperature. Under this condition there was strong expression of *AFT1* and *ATF2*. Once again *AFT2* was maximally expressed as a result of L-leucine supplementation. These findings corroborate earlier results, obtained by Verstrepen

et al. (2003b), which showed that *ATF* transcription is a limiting factor for acetate ester synthesis. Thevelein and de Winde (1999) and Verstrepen *et al*. (2003a) explained this increase in *ATF1* expression at higher amino acid concentration and the resulting increase in acetate ester concentration. They showed that *ATF1* is induced by glucose and nitrogen compounds as a target of the Ras/cAMP/PKA and the fermentable growth medium-induced (FGM) pathway. In contrast, Molina *et al*. (2007) found no correlation between expression of *ATF1* and *ATF2* and the production of any ester compound during wine fermentation. They concluded that under wine fermentation conditions, ester production was largely regulated at the posttranscriptional level.

ATF1 and *ATF2* were deleted and overexpressed by Fujii *et al*. (1994) and Nagasawa *et al.* (1998). It was found that deletion of *ATF1* reduces isoamyl acetate production by 80% and ethyl acetate production by 30%. *ATF2* deletion has similar but smaller effects on ester production (Nagasawa *et al.,* 1998). Accordingly, overexpression of these genes in sake yeast led to a 10-fold increase in ethyl acetate production and a 30-fold increase in isoamyl acetate formation. Similarly, Lilly *et al.* (2000) showed increased ester concentrations in wines produced with genetically modified yeast overexpressing the *ATF1* gene. Verstrepen *et al*. (2003b) overexpressed *ATF1* and *ATF2* in a commercial brewer's yeast strain and found that the pilot-scale beers produced with an *ATFI*-overexpressing strain contained five times more acetate esters than the beers produced with the wild-type strain. Overexpression of *ATF2* led to smaller increases in isoamyl acetate formation and no significant changes in ethyl acetate levels (Verstrepen *et al*., 2003b). These results indicate that it is possible to use genetic modification in order to create new yeast strains with desirable ester production characteristics and since there is a correlation between *ATF* expression and acetate ester concentration, it can be used as a predictive tool. In addition, the highly elevated ester levels obtained with the overexpression by yeast strains clearly indicate that ester synthesis during brewery fermentations is not strictly limited by substrate availability and concentration, but rather the expression level of the *ATF* genes.

EEB1 and *EHT1* have been described to be responsible for the production of ethyl esters (Saerens *et al.,* 2006). $ZnSO_4$ supplementation, L-leucine supplementation, increased fermentation temperature and fermentation pH resulted in an increase in total ethyl ester concentration by 123.02%, 83.73%, 62.81% and 6.76% respectively, in ale beer when compared to the control (Chapter 3). The maximum expression of *EEB1* was a result of a pH change.

However, the maximum expression of *EHT1* appears to be a result of zinc supplementation followed by L-leucine supplementation. Therefore, it appears that *EHT1* expression correlated with the final ethyl ester concentration. *EHT1* appears to be the most important gene for ethyl ester synthesis during ale fermentation. $ZnSO_4$ supplementation, L-leucine supplementation, optimum fermentation temperature and optimum fermentation pH resulted in an increase in total ethyl ester concentration by 80.06%, 31.29%, 87.19% and 25.96% respectively, in lager beer when compared to the control (Chapter 2). However, the concentrations of the ethyl esters were considerably lower than acetate esters. The maximum expression of *EEB1* as a result of L-leucine supplementation correlates with the elevated ethyl ester concentration obtained with this supplement, while the maximum expression of *EHT1* appeared to be a result of elevated fermentation temperature. Thus, changes in expression of *EEB1* do not initiate changes in ethyl acetate concentration. The lack of correlation between *EEB1* expression and ethyl ester concentration suggests that it is not a primary factor for ethyl ester production. Saerens *et al*. (2008b) showed contradictory results, reporting that there was a strong negative correlation between *EHT1* expression levels and the concentration of ethyl esters. Molina *et al.* (2007) reported that *EHT1* expression level remained high at the end of wine fermentation as a response to high levels of ethyl esters present. In other studies, enhancing enzyme activity by overexpression of the ethyl ester synthesis genes did not affect ethyl ester production, since overexpression of the *EHT1* or *EEB1* allele derived from the industrial ale strain CMBS SS01 did not result in an increase in the production of ethyl esters. This was also observed previously in a yeast strain overexpressing the *EHT1* or *EEB1* allele derived from a laboratory strain (Saerens *et al*., 2006). On the other hand, both *EHT1* and *EEB1* also displayed esterase activity in addition to ester synthesis activity (Saerens *et al*., 2006). This might provide an alternative explanation for these contradictory results.

The control of flavour ester levels in alcoholic beverages is often problematic. Insufficient flavour ester synthesis or aberrant flavour ester profiles are quite common in beer and wine fermentations (Verstrepen *et al*., 2003c). Results from this study indicate that this effect appears to be due, at least in part, to differential expression levels of specific genes involved in the biosynthesis of aroma compounds. This suggests that analysis of gene expression levels may help to control ester synthesis and allow brewers to identify or construct yeast strains

that would produce the desired amounts of esters in accordance with specific consumer preferences.

CHAPTER FIVE: General Discussion and Conclusion

5.1 The research in perspective

When considering brewing of beer in its most simplistic form, it probably represents mankind's oldest biotechnology. Numerous inventions have led to improved technologies and capabilities to optimize fermentation technology on an industrial scale. Yeast metabolism makes an important contribution to beer flavour (Smogrovicova *et al*., 1999), giving beer its distinctive personality by producing flavour active volatile compounds. According to Verstrepen *et al.* (2003a), controlling the concentration of these volatile esters is achieved by controlling the substrate concentration, acyl CoA and fusel alcohol and the total activity of the enzymes involved in the synthesis and breakdown of the respective esters. Generally, ester formation is a sensitive process, which is rather difficult to control due to numerous influencing factors involved (Branyik *et al.,* 2008). Insufficient flavour ester synthesis or aberrant flavour ester profiles are quite common in beer. Ester production is mainly controlled by the expression level of alcohol acetyltransferase genes and Acyl-coenzymeA:ethanol *O*-acyltransferase genes. Thus, any factor that influences the expression of the ester synthase genes and/or the concentrations of substrates will affect ester production accordingly. In this study, the influence of important fermentation and nutritional parameters on the production of both acetate and ethyl esters by an ale and a lager brewing yeast strain were investigated. The expression levels of the biosynthesis genes responsible for the production of esters during fermentation under the different conditions were also investigated to allow for the prediction of the effect of fermentation parameters on flavour beer profiles.

Results from this study have shown that different yeast strain type, wort composition and fermentation conditions affect fermentation performance, beer characteristics and ester profiles. These parameters are important as they affect the biochemical pathways of yeast leading to different flavour profiles and beer characteristics. Supplementing wort with $ZnSO_4$ or L-leucine increased nutrient utilization and ethanol production in both ale and lager beer. In lager beer, wort that contained the highest concentration of zinc sulphate (0.12 g/l) produced a similar amount of ethanol (4.31% v/v) on Day 4 of fermentation compared to the control (4.06% v/v) on Day 6. In addition, a similar amount of reducing sugars was utilized on Day 3 of fermentation (33.88%) compared to that of the control which utilized 27.67% by Day 6. A similar trend was observed for ale beer. Therefore, it would be possible to decrease the fermentation time if supplements are added to the wort. The economic benefits of faster fermentation are advantageous and can be achieved by supplementation with $ZnSO_4$ or L-

leucine. Mineral nutrients should be given careful attention, because efficient conversion of carbon sources to desired product by fermentation depends not solely on the available fermentable carbon but also on the bioavailability of essential metal ions. Nitrogen compounds in wort are fundamental for brewing processes, beer quality and stability and they affect the rate of fermentation and the formation of active flavour compounds. This study revealed that the addition of L-leucine in wort allowed for the production of more ethanol and higher utilization of nutrients. Furthermore, increasing temperature accelerates fermentation allowing higher nutrient utilization and ethanol production, while the optimum pH for fermentation performance was 5 for both ale and lager beer. The tested parameters had no effect on beer colour as beer was prepared using the same ingredients. A major positive attribute of beer is its foam head stability which was found in this study to be enhanced when wort was supplemented with L-leucine by 33.5% and 14.6%, respectively, for lager and ale beer. This indicated that L-leucine plays an important role in stability and lacing of beer foam.

The unique flavour profiles of beer can largely be attributed to the biochemical activities within the yeast cell during fermentation. Brewers therefore have a broad range of options at their disposal to control acetate and ethyl ester production. While the optimal technique to control ester synthesis is dependent on many factors and may therefore differ from case to case, some parameters are more easily adapted and allow for a more selective control than others. This study aimed at identifying whether altering fermentation conditions or supplementing wort with nutrients could be used to control ester concentration in ale and lager beers. Results demonstrated that supplementations with $ZnSO_4$ and L-leucine and higher temperature and pH produced a higher ester concentration in all beers. This effect is due to the influence of these conditions on substrate availability. The two substrates that is responsible for ester formation is acetyl-CoA and higher alcohols (Verstrepen *et al*., 2003). Zinc and nitrogen concentration and fermentation temperature is known to affect higher alcohol formation (Hodgson and Moir, 1990, cited by Verstrepen *et al*., 2003; Verstrepen *et al*., 2003; Calderbank and Hammond, 1994), while acetyl-CoA is influenced by nitrogen concentration (Boulton *et al*., 1996 cited by Torrea *et al*., 2003). Based on results of R^2 values obtained in this study, the best method to control acetate and ethyl ester synthesis in a lager strain would be careful regulation of wort pH as it produced the highest R^2 coefficient of 0.994 and 0.961 for acetate and ethyl esters, respectively. Increasing fermentation pH from 5 to 7 resulted in a 4.02% and 16.14% increase in total acetate and ethyl ester concentration, respectively. Supplementation with $ZnSO_4$ and alteration of temperature shows promise but

only for the control of acetate ester production, obtaining R^2 coefficient of 0.985 and 0.998, respectively. Addition of 0.12 g/l $ZnSO_4$ and elevating fermentation temperature from 14 °C to RT resulted in a 9.51% and 50.68%, respectively, increase in acetate ester concentration. Supplementation with L-leucine was not as effective as the other conditions tested in controlling ester production yielding a R^2 coefficient of 0.914 and 0.912 for acetate and ethyl esters, respectively. Results obtained when using an ale brewing strain differed from the lager strain. Supplementation with $ZnSO_4$ and alteration of temperature are the best methods that can be used to control or predict ester production. Addition of $ZnSO_4$ to wort increased the concentration of acetate and ethyl esters in beer by 27.7% and 123.02%, respectively; furthermore it produced a R^2 coefficient of 0.953 and 0.988, respectively. Alteration of the fermentation temperature can also be used to control ester concentration, since it produced R^2 coefficients of 0.998 and 0.985 for acetate and ethyl esters, respectively. Elevating fermentation temperature resulted in 14.42% and 62.82% increase in acetate and ethyl ester concentration, respectively. This demonstrates that aroma-active ester concentration in beer can be controlled by altering fermentation conditions or by supplementing wort with nutrients. Aroma-active esters in ale and lager beer were found to be more stable at 4 °C than at room temperature. Ale and lager beer displayed similar ester stability over the three month period at 4 °C decreasing by 6.93% and 7.92%, respectively. At room temperature, esters present in lager beer was found to be more stable than in ale beer decreasing by 13.32% and 16.90%, respectively.

An additional parameter that affects ester synthesis is the activity of alcohol acetyltransferases which are encoded by the genes *ATF1* and *ATF2*, ethanol hexanoyl transferase and acyl-coenzymeA:ethanol *O*-acyltransferase encoded by *EHT1* and *EEB1,* respectively. Despite the major advances in characterizing the genome of yeast *S. cerevisiae* in laboratory strains, there is a limited understanding of the expression of genes during fermentation. Real-Time quantitative PCR was used for the analysis of gene expression in yeast throughout the fermentation period. Analysis of the expression profiles of these genes revealed that ester synthesis cannot be explained solely by substrate availability but also by the expression levels of the ester biosynthesis genes. Results showed that high concentrations of acetate esters are produced when *ATF2* is highly expressed. In ale beer, L-leucine supplements resulted in the highest increase in acetate ester concentration by 41.27%, which correlates with the higher expression of *ATF2* (133.49-fold) under this condition. In lager beer, elevated temperature resulted in the highest increase in acetate ester concentration by 50.68% and can be linked to the high expression of *ATF1* (918.53-fold) and *ATF2* (1090.13-

fold) observed. The highest concentration of ethyl ester produced in ale beer was a result of $ZnSO_4$ supplementation which correlated with a high *EHT1* expression level. In lager beer, the highest ethyl ester concentration was a result of elevated fermentation temperature which correlates with a high *EHT1* expression level. *EEB1* expression level did not correlate with ethyl ester concentration indicating that it is not a primary factor for ester production. These results reveal that expression levels of the ester biosynthetase genes also play an important role in ester formation.

5.2 Potential for future development of the study

The results from this study hold promise for future application of the use of supplements or alteration of fermentation conditions to control or predict ester concentrations in ale and lager beer. Since these parameters accelerate fermentation of wort, the fermentation time can be shorten. This would therefore, be advantageous as it will save both time and cost. It would be interesting to investigate the effect of a shorter fermentation period on the final ester concentration in beer and whether or not the ester concentrations will fall within the threshold levels. Results obtained in this study, open up a lot interesting perspectives that will further investigate yeast metabolism during fermentation. Furthermore, since this study investigated the effect of a single parameter on fermentation performance, beer characteristics and ester synthesis, the effect of a combination of parameters could provide valuable information. Perhaps the most convenient and selective way to reduce ester production could be by using a (slightly) lower fermentation temperature, or lowering wort free amino nitrogen (FAN) and zinc concentrations. However, care has to be taken in order to avoid excessive yeast stress due to low FAN concentrations, as this may lead to decreased fermentation performance.

The stability of esters was monitored at different temperatures over time. Beers stored at room temperature resulted in a decrease in ester concentration by 13.32% and 16.90% for lager and ale beer, respectively. Further studies are needed to investigate the effects of these parameters on flavour stability as this could provide a method to improve the flavour stability of a beer. Decreases in ester concentration during storage are largely attributed to esterase activity in beer (Neven *et al*., 1997). Pasteurization of beer will inactivate these esterases that hydrolyse esters, thus preventing some ester decreases during storage. Optimisation of the brewing process with respect to flavour stability requires a clear insight of the types of flavour changes during storage and the nature of the molecules involved. This may, however, vary between beer types (e.g., ale and lager beers). Secondly, it is necessary to clarify the

reaction pathways in beer leading to the staling compounds. Finally, the influence of the production process on the staling reactions must be made clear. Knowledge of the aging phenomenon in a particular type of beer can be used to develop appropriate technological process improvements to control its particular flavour stability. Besides their relevance for flavour stability, the investment costs for suggested process modifications must be evaluated and a balance should be made between better and longer flavour stability and costs.

The easiest way to get a grip on beer fruitiness is perhaps by using genetically modified variants of the producing strain. Indeed, using strains with different expression profiles of the *ATF* and *EHT1* genes makes it possible to selectively enhance or decrease the production of aroma-active esters, by using overexpression or deletion of the ester biosynthetase genes to create new yeast strains with desirable ester production characteristics. However, this requires both further research and a drastic change in public perception concerning the use of genetically modified organisms in food production. In addition, there are limited data on the role of ester hydrolysing enzymes such as isoamyl acetate hydrolase (*IAH1*) which is an isoamyl acetate hydrolysing esterase produced by yeast during fermentation. Therefore, further studies should aim at investigating this.

S. cerevisiae plays a primary role in the beer brewing process. Arguably, one of the biggest breakthroughs in yeast research was the sequencing of the complete genome in 1996, the first complete sequence of a eukaryote (Goffeau *et al*., 1996). This knowledge supported the development of microarray technology. Transcript-level analysis with DNA microarrays has become a powerful tool to study gene expression and metabolic changes and has been applied to the brewing yeast transcriptome during production-scale lager beer fermentation (Olesen *et al*., 2002). Future developments in this field can provide clarity regarding the best brewing practices to optimize yeast performance during propagation, yeast handling and fermentation. The impact of wort composition fluctuations on yeast performance remains a complex area. The identification of specific gene activities linked to wort compositional changes will allow for more directed research. The recent developments in metabolomics have shown that future developments have the potential not only to differentiate but also to effectively select brewing strains for new brands. Progressive developments in yeast genetics and molecular biology of laboratory cultures have provided the scientific basis for genetic manipulation of industrial strains. Therefore, further studies should aim at genetically manipulating the yeast strains used in this study for optimal fermentation performance.

REFERENCES

Anderson, R. G. and B. H. Kirsop. 1974. The control of volatile ester synthesis during the fermentation of wort of high specific gravity Journal of the Institute of Brewing. **80**:48-55.

Anderson, R. G., and B. H. Kirsop. 1975. Oxygen as a regulator of ester accumulation during the fermentation of wort of high specific gravity. Journal of the Institute of Brewing. **81**:111–115.

Asano, K. and N. Hashimoto. 1980. Isolation and characterization of foaming proteins in beer. Journal of the American Society of Brewing Chemists. **38**:129–130.

Bamforth, C. W. 1985. The foaming properties of beer. Journal of the Institute of Brewing. **91:**370–383.

Bamforth, C. W. 1999. The science and understanding of the flavour stability of beer: a critical assessment. Brauwelt International. **17**:98–110.

Bamforth, C. W. 2000. Beer: An ancient yet modern biotechnology. Chemical Education. **5**:102–112.

Blieck, L., G. Toye, F. Dumurtier, K. J. Vertstrepen, F. R. Delvaux, J. M. Thevelein, and P. van Dijck, 2007. Isolation and characterization of brewer's yeast variants with improved fermentation performance under high-gravity conditions. Applied and Environmental Microbiology. **73**:815-824.

Branyik, T., A. A. Vicente, P. Dostalek and J. A. Teixeira. 2008. A review of flavour formation in continuous beer fermentations. Journal of the Institute of Brewing. **114:**3-13.

Branyik, T., and A. A. Vicente. 2004. Continuous primary fermentation of beer with yeast immobilized on spent grains – The effect of operation conditions. Journal of the American Society of Brewing Chemists. **62**:29-34.

Brown, A. K., and J. R. M. Hammond. 2003. Flavour control in small-scale beer fermentations. Chemical Engineering Research and Design. **81**:40-49.

Bustin, A. S., V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, G. L. Shipley, J. Vandesompele, and C. T. Wittwer. 2009. The MIQE guidelines: minimum information for publication of quantitative Real-Time PCR experiments. Clinical Chemistry. **55**:611:622.

Cabane, B., C. Ramos-Jeunnehornrne, N. Lapage, and C. A. Masschelein. 1974. Vicinal diketones – the problem and prospective solutions. Journal of the American Society of Brewing Chemists. **73**:94-99.

Calderbank, J., and J. R. M. Hammond. 1994. Influence of higher alcohol availability on ester formation by yeast. Journal of the American Society of Brewing Chemists. **52**:84-90.

Callemien, D., S. Dasnoy, and S. Collin. 2006. Identification of a Stale-Beer-like Odorant in Extracts of Naturally Aged Beer. Journal of Agricultural and Food Chemists. **54:**1409-1413.

Campo, E., J. Cacho and V. Ferreira. 2007. Solid phase extraction, multidimensional gas chromatography mass spectrometry determination of four novel aroma powerful ethyl esters Assessment of their occurrence and importance in wine and other alcoholic beverages. Journal of Chromatography A. **1140**:180–188.

Casey, G. P. 1996. Practical applications of pulsed field electrophoresis and yeast chromosome fingerprinting in brewing QA and R&D. Master Brewers Association of the Americas. **33**:1–10.

Casey, G. P., C. A. Magnus, and W. M. Ingledew. 1984. High gravity brewing: effects of nutrition on yeast composition, fermentative ability and alcohol production. Applied and Environmental Microbiology. **48**:639-646.

Clapperton, J. F. 1976. Ribes flavour in beer. Journal of the Institute of Brewing: **82**: 175–176.

Cortacero-Ramirez, S., M. H. de Castro,A. Segura-Carretero, C. Cruces-Blanco, and A. Fernández-Gutierrez. 2003. Analysis of beer components by capillary electrophoretic methods. Trends in Analytical Chemistry. **22**:440-455.

Cunningham, S., and G. G. Stewart. 1998. Effects of high-gravity brewing and acid washing on brewers' yeast. Journal of the American Society of Brewing Chemists**. 56**:12–18.

da Silva, G. A., F. Augusto, and R. J. Poppi. 2008. Exploratory analysis of the volatile profile of beers by HS–SPME–GC. Food Chemistry. **111**:1057–1063.

Dalgliesh, C. E. 1977. Flavour stability. p. 623–659. *In*: Proceedings of the European Brewery Convention Congress, Amsterdam, DSW: Dordrecht.

de Schutter, D. P., D. Saison, F. Delvaux, G. Derdelinckx, J. Rock, H. Neven and F. R. Delvaux. 2008. Optimisation of wort volatile analysis by headspace solid-phase microextraction in combination with gas chromatography and mass spectrometry. Journal of Chromatography A. **1179**:75–80.

de Smidt, O., J. C. du Preez, and J. Albertyn. 2008. The alcohol dehydrogenases of *Saccharomyces cerevisiae*: a comprehensive review. FEMS Yeast Research. **8**:967–978.

Depraetere, S. A., F. Delvaux, D. De Schutter, I. S. Williams, J. Winderickx, and F. R. Delvaux. 2008. The influence of wort aeration and yeast preoxygenation on beer staling processes. Food Chemistry. **107**:242–249.

Dickinson, J. R., and W. Dawes. 1992. The catabolism of branched-chain amino acids occurs via 2-oxoacid dehydrogenase in *Saccharomyces cerevisiae*. Journal of General Microbiology. **138**:2029-2033.

Dombek, K. M., and L. O. Ingram. 1986. Magnesium limitation and its role in apparent toxicity of ethanol during yeast fermentation. Applied and Environmental Microbiology. **52:**975–981.

Dragone, G., D. P. Silva, and J. B. de Almeida. 2004. Factors influencing ethanol production rates at high-gravity brewing. Swiss Society of Food Science and Technology. **37**:790–802.

Edelen, L., L. J. Miller, and H. Patino. 1996. Effects of yeast pitch rates on the fermentation performance and beer quality. Master Brewers Association of the Americas. **33:**30–32.

Eide, D. J. 2006. Zinc transporters and the cellular trafficking of zinc. Biochimica et Biophysica Acta. **1763**:711–722.

Eide, D. J. 1998. The molecular biology of metal ion transport in *Saccharomyces cerevisiae*. Annual Review of Nutrition. **18**:441–469.

Engan, S. 1970. Wort composition and beer flavour I: the influence of some amino acids on the formation of higher aliphatic alcohols and esters. Journal of the Institute of Brewing. **76**:254-261.

Erten, H., H. Tanguler., and H. Cakiroz. 2007. The effect of pitching rate on fermentation and flavour compounds in high gravity brewing. Journal of the Institute of Brewing. **113**:75–79.

Etievant, P. X., G. Callement, D. Langlois, S. Issanchou, and N. Coquibus. 1999. Odour intensity evaluation in gas chromatography–olfactometry by finger span method. Journal of Agricultural and Food Chemistry. **47**:1673–1680.

Evans, D. E., M. C. Sheehan, and D. C. Stewart. 1999. The impact of malt derived proteins on beer foam quality. Part II: The influence of malt foam-positive proteins and non-starch polysaccharides on beer foam quality. Journal of the Institute of Brewing. **105**:171–177.

Ferreira, I. M. P. L. V. O., O. Pinhoa, E. Vieiraa, and J. G. Tavarela. 2010. Brewer's *Saccharomyces* yeast biomass: characteristics and potential applications. Trends in Food Science & Technology. **21**:77-84.

Fujii, T., N. Nagasawa, A. Iwamatsu, T. Bogaki, Y. Tamai, and M. Hamachi. 1994. Molecular cloning, sequence analysis, and expression of the yeast alcohol acetyltransferase gene. Applied Environmental Microbiology. **60**:2786-2792.

Furusho, S., N. Kobayashi, N. Nakae, M. Takashio, T. Tamaki, and K. Shinotsuka. 1999. A developed descriptive sensory test reveals Beer flavor changes during storage. Master Brewers Association of the Americas. **36**:163–166.

Gee, D. A. and W. F. Ramirez. 1994. A flavour model for beer fermentation. Journal of the Institute of Brewing. **100**:321-329.

Goldammer, T. 2008. The Brewer's Handbook - The Complete Book to Brewing Beer, p. 337-340. 2nd ed.

Grosch, W. 1993. Detection of potent odorants in foods by aroma extract dilution analysis. Trends in Food Science and Technology. **4**:68–73.

Guido, L. F., Rodrigues, P. G., Rodrigues, J. A., Gonçalves, C. R., and A. A. Barros. 2004. The impact of the physiological condition of the pitching yeast on beer flavour stability: an industrial approach. Food Chemistry. **87**:187–193.

Guyot-Declerck, C., N. Francois, C. Ritter, B. Govaerts and S. Collin. 2005**.** Influence of pH and ageing on beer organoleptic properties - A sensory analysis based on AEDA data. Food Quality and Preference. **16**:157-162.

Haddad, P. R. P. Doble and M. Macka. 1999. Developments in sample preparation and separation techniques for the determination of inorganic ions by ion chromatography and capillary electrophoresis. Journal of Chromatography A. **856** : 145–177.

Hammond, J. R. M. 1993. Brewing yeast, The Yeasts, p. 5–67. Rose, A. H and J. S. Harrison (eds), 2nd edn. Academic Press, London.

Hansen, J., and M. C. Kielland-Brandt. 1996. Modification of biochemical pathways in industrial yeasts. Journal of Biotechnology. **49**:1-12.

Hohmann, S. 2002. Osmotic stress signalling and osmoadaptation in yeasts. Microbiology and Molecular Biology Reviews. **66**:300–372.

Horton, C. E., K. Huang, G. N. Bennett and F. B. Rudolph. 2003. Heterologous expression of the *Saccharomyces cerevisiae* alcohol acetyltransferase genes in *Clostridium acetobutylicum* and *Escherichia coli* for the production of isoamyl acetate. Journal of Industrial Microbiology and Biotechnology. **30**:427-432.

Hu, C. K., F. W. Bai, and L. J. An. 2003. Enhancing ethanol tolerance of a self-flocculating fusant of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* by Mg^{2+} via reduction in plasma membrane permeability. Biotechnology Letters. **25**:1191–1194.

Ingledew, W. M., L. A. Langille, G. S. Menegazzi, and M. H. Mok. 1977. Spent brewer's yeast - analysis, improvement, and heat processing considerations. Master Brewers Association of the Americas. **14:**231-237.

Jentsch, M. 2007. Top-fermented beer specialities in focus. Brauwelt International. **5**:332–334.

Jespersen, L., and M. Jakobsen. 1996. Specific spoilage organisms in breweries and laboratory media for their detection. International Journal of Food Microbiology. **33**:139-155.

Jiménez, J. and T. Benítez. 1987. Adaptation of yeast cell membranes to ethanol. Applied and Environmental Microbiology. **53**:1196–1198.

Jones, R. P. and P. F. Greenfield. 1984. A review of yeast ionic nutrition: growth and fermentative requirements. Process Biochemistry. **4**:48–59.

Jones. R. P. and G. M. Gadd. 1990. Ionic nutrition of yeast – the physiological mechanisms involved and applications for biotechnology. Enzyme Microbial Technology. **12**:402-418.

Kim, T. H., S. M. Lee, Y. Kim, K. H. Kim, S. Oh and H. J. Lee. 2003. Aroma dilution method using GC injector split ratio for volatile compounds extracted by headspace solid phase microextraction. Food Chemistry. **83**:151–158.

Kobayashi, M., H. Shimizu and S. Suteaki. 2008. Beer volatile compounds and their application to low-malt beer fermentation. Journal of Bioscience and Bioengineering. **106:**317-323.

Kobayashi, M., K. Nagahisa, H. Shimizu and S. Shioya. 2006. Simultaneous control of apparent extract and volatile compounds concentrations in low-malt beer fermentation. Applied Microbiology and Biotechnology. **73**:549–558.

Kobayashi, N., M. Sato, and S. Fukuhara. 2007. Application of shotgun DNA microarray technology to gene expression analysis in lager yeast. Journal of the American Society of Brewing Chemists. **65**:92–98.

Kodama, Y., F. Omura, K. Miyajima, and T. Ashikari. 2001. Control of higher alcohol production by manipulation of the *BAP2* gene in brewing yeast. Journal of the American Society of Brewing Chemists. **59**:157–162.

Kopsahelis, N., M. Kanellaki, and A. Bekatorou. 2007. Low temperature brewing using cells immobilized on brewer's spent grains. Food Chemistry. **104**:480-488.

Kronlof, J., and M. Linko. 1992. Production of beer using immobilized yeast encoding α – acetolactate decarboxylase. Journal of the Institute of Brewing. **98**:479-491.

Kruger, L., A. T. W. Pickerell and B. Axcell. 1992. The sensitivity of different brewing yeast strains to carbon dioxide inhibition: fermentation and production of flavour-active volatile compounds. Journal of the Institute of Brewing. **98**:133-138.

Lambrechts, M. G., and I. S. Pretorius. 2000. Yeast and its importance to wine aroma - A review. South African Journal of Enology and Viticulture. **21**:97−129.

Lawrence, D. R. 1988. Spoilage organisms in beer, p. 1-48. *In*: Developments in Food Microbiology. R. K. Robinson (ed), Elsevier, London.

Lekkas, C., G. G. Stewart, A. E. Hill, B. Taidi and J. Hodgson. 2007. Elucidation of the role of nitrogenous wort components in yeast fermentation. Journal of the Institute of Brewing. **113:**3–8.

Lewis, M. J. and C. W. Bamforth. 2006. Essays in brewing science. Springer Science and Business Media.

Lilly, M., M. G. Lambrachts and I. S. Pretorius. 2000. Effect of increased yeast alcohol acetyltransferase activity on flavor profiles of wine. Applied and Environmental Microbiology. **66**:744–753.

Lodolo, E. J., J. L. F. Kock, B. C. Axcell and M. Brooks. 2008. The yeast *Saccharomyces cerevisiae* the main character in beer brewing. FEMS Yeast Research. **8**:1018–1036.

Lucero, P., E. Peñalver, E. Moreno, and R. Lagunas. 2000. Internal trehalose protects endocytosis from inhibition by ethanol in *Saccharomyces cerevisiae*. Applied and Environmental Microbiology. **66:**4456– 4461.

Lusk, L. T., H. Goldstein, and D. Ryder. 1998. Independent role of beer proteins, melanoidins and polysaccharides in foam formation. Journal of the Institute of Brewing Chemistry. **53**:93–103.

Mason, A. B., and J. Dufour. 2000. Alcohol acetyltransferases and the significance of ester synthesis in yeast. Yeast. **16**:1287-1298.

Meilgaard, M. 1972. Stale flavor carbonyls in brewing. Brewers Digest: **47**:48–57.

Meilgaard, M. 1975. Flavour chemistry of beer. Part 2. Flavour and threshold of 239 aroma volatiles. Master Brewers Association of the Americas. **12**:151–168.

Moir, M. 1992. The desideratum for flavour control. Journal of the Institute of Brewing. **98**:215-220.

Molina, A. M., J. H. Swiegers, C. Varela, I. S. Pretorius, and E. Agosin. 2007. Influence of wine fermentation temperature on the synthesis of yeast-derived volatile aroma compounds. Applied Microbiology and Biotechnology. **77**:675–687.

Nabais, R. C., I. Sa-Correia, C. A. Viegas, J. M. Novais. 1988. Influence of calcium ion on ethanol tolerance of *Saccharomyces bayanus* and alcoholic fermentation by yeasts. Applied and Environmental Microbiology. **54:**2439–2446.

Nagasawa, N., T. Bogaki, A. Iwamatsu, M. Hamachi, and C. Kumagai. Cloning and nucleotide sequence of the alcohol acetyltransferase II gene (*ATF2*) from *Saccharomyces cerevisiae*. Bioscience, Biotechnology, and Biochemistry. **62**:1852-1857.

Nagodwithana, T. W., C. Castellano, and K. H. Steinkraus. 1974. Effect of dissolved oxygen, temperature, initial cell count and sugar concentration on the viability of *Saccharomyces cerevisiae* in rapid fermentations. Applied and Environmental Microbiology. **28**:383-391.

Neven, H., F. Delvaux, and G. Derdelinckx. 1997. Flavor evolution of top fermented beers. Master Brewers Association of the Americas. **34**:115–118.

Nordström, K. 1963. Formation of ethyl acetate in fermentation with brewer's yeast - metabolism of acetyl-CoA. Journal of the Institute of Brewing. **69**:142–153.

Nordström, K. 1964. Formation of esters from acids by brewer's yeast. IV. Effect of higher fatty acids and toxicity of lower fatty acids. Journal of the Institute of Brewing. **70**:233–242.

O'Connor-Cox, E. S. C. and W. M. Ingledew. 1989. Wort nitrogenous sources – their use by brewing yeasts: A review. Journal of the American Society of Brewing Chemists. **47:**102–108.

O'Connor-Cox, E. S. C., J. Paik, and W. M. Ingledew. 1991. Improved ethanol yields through supplementation with excess assimilable nitrogen. Journal of Industrial Microbiology. **8**:45-52.

Olesen, K., T. Felding, C. Gjermansen, and J. Hansen. 2002. The dynamics of the *Saccharomyces carlsbergensis* brewing yeast transcriptome during a production-scale lager beer fermentation. FEMS Yeast Research. **2**:563–573.

Ough, C.S. 1966. Fermentation rates of grape juice. III. Effects of initial ethyl alcohol, pH and fermentation temperature. American Journal of Enology and Viticulture. **17**:74-81.

Palamand, S. R., and J. M. Aldenhoff. 1973. Bitter tasting compounds of beer: chemistry and taste properties of some hop resin compounds. Journal of Agriculture and Food Chemistry. **21**:535-543.

Patel, S., and T. Shibamoto. 2003. Effect of 20 different yeast strains on the production of volatile components in Symphony wine. Journal of Food Composition and Analysis. **16**:469–476.

Peddie, H. A. B. 1990. Ester formation in brewery fermentations. Journal of the Institute of Brewing. **96**:327–331.

Petka, J., Ferreira, V. Gonzales-Vinas, M. A., and J. Cacho. 2006. Sensory and chemical characterization of the aroma of a White wine made with Devin grapes. Journal of Agricultural and Food Chemistry. **54**:909–915.

Pham, T. K., P. K. Chong, C. S. Gan, and P. C. Wright. 2006. Proteomic analysis of *Saccharomyces cerevisiae* under high gravity fermentation conditions. Journal of Proteome Research. **5**:3411-3419.

Pierce, J. S. 1987. The role of nitrogen in brewing. Journal of the Institute of Brewing Chemsitry. **93**:378–381.

Pinho, O., I. M. P. L. V. O. Ferreiro and L. H. M. L. M. Santos. 2006. Method optimization by solid-phase microextraction in combination with gas chromatography with mass spectrometry for analysis of beer volatile fraction. Journal of Chromatography A. **1121**:145-153

Piper, P. W., K. Talreja, B. Panaretou, P. Morades-Ferreira, K. Byrne, U. M. Praekelt, P. Meacock, M. Recnacq and H. Boucherie. 1984. Induction of major heat-shock proteins of *Saccharomyces cerevisiae*, including plasma membrane Hsp30, by ethanol levels above a critical threshold. Microbiology. **140**:3031–3038.

Plutowska, B and W. Wardencki. 2008. Application of gas chromatography–olfactometry (GC–O) in analysis and quality assessment of alcoholic beverages – A review. Food Chemistry. **107**:449–463.

Polaina, J. 2002. Brewer's Yeast: Genetics and Biotechnology. Applied Mycology and Biotechnology. **2**:1-17.

Prasad, R. and A. H. Rose. 1986. Involvement of lipids in solute transport in yeasts. Yeast. **2:**205–220.

Pratt, P. L., J. H. Bryce, and G. G. Stewart. 2003. The effects of osmotic pressure and ethanol on yeast viability and morphology. Journal of the Institute of Brewing. **109**:218–228.

Priest, F. G. 1996. Gram-positive brewery bacteria, p. l27- 161. *In*: Brewing Micobiology. F. G. Priest and I. Campbell (eds). 2nd ed. Elsevier. London,

Quilter, M. G., J. C. Hurley, F. J. Lynch, and M. G. Murphy. 2003. The production of isoamyl acetate from amyl alcohol by *Saccharomyces cerevisiae*. Journal of the Institute of Brewing. **109**:34–40.

Ramos-Jeunehomme, C., R. Laub, and C. A. Masschelein. 1991. Why is ester formation in brewery fermentations yeast strain dependent?, p. 257-264. *In:* Proceedings of the 23rd European Brewery Convention Congress, Lisbon. Oxford University Press, Oxford, UK.

Rattray, J. B. M., A. Schibeci and D. K. Kidby. 1975. Lipids of yeasts. Bacteriological Reviews. **39**:197–231.

Reddy, L. V. A., and O. V. S. Reddy. 2010. Effect of fermentation conditions on yeast growth and volatile composition of wine produced from mango (*Mangifera indica L.*) fruit juice. Food and Bioproducts Processing. **89**:1-5.

Rees, E. M. R. and G. G. Stewart. 1998. Strain specific response of brewer's yeast strains to zinc concentrations in conventional and high gravity worts. Journal of the Institute of Brewing. **104**:221-228.

Renger, R. S., S. H. van Hateren, and K. C. A. M. Luyben. 1992. The formation of esters and higher alcohols during brewery fermentation; the effect of carbon dioxide pressure. Journal of the Institute of Brewing. **98:**509-513.

Rodrigues, F., M. Caldeira, and J. S. Camara. 2008. Development of a dynamic headspace solid-phase microextraction procedure coupled to GC–qMSD for evaluation the chemical profile in alcoholic beverages. Analytica Chimica Acta. **609**:82–104.

Rojas, V., J. V. Gila, P. Manzanares, R. Gavara, F. Pinaga, and A. Flors. 2002. Measurement of alcohol acetyltransferase and ester hydrolase activities in yeast extracts. Enzyme and Microbial Technology. **30**:224–230.

Royle, L., J. M. Ames, C. A. Hill, and D. S. J. Gardner. 2001. Analysis of hop acids by capillary electrophoresis. Food Chemistry. **74**:225–231.

Sablayrolles, J. M. and C. B. Ball. 1995. Fermentation kinetics and the production of volatiles during alcoholic fermentation. Journal of the American Society of Brewing Chemists. **53**:71-78.

SABMiller. 2011. Business overview: Global beer market trends. http://www.sabmiller.com/index.asp?pageid=39 (Accessed January 2012)

Sadasivam, S., and A. Manickum. 1996. Biochemical methods, chapter 1 carbohydrates, p. 6-8, 2nd ed. New age international publishers, India.

Saerens, S. M. G., F. Delvaux, K. J. Vertrepen, P. van Dijck, J. M. Thevelein, and F. R. Delvaux. 2008a. Parameters affecting ethyl ester production by *Saccharomyces cerevisiae* during fermentation. Applied and Environmental Microbiology. **74**:454-461.

Saerens, S. M. G., P. J. Verbelen, N. Vanbeneden, J. M. Thevelein, and F. R. Delvaux. 2008b. Monitoring the influence of high-gravity brewing and fermentation temperature on flavour formation by analysis of gene expression levels in brewing yeast. Applied Microbiology and Biotechnology. **80**:1039-1051.

Saerens, S. M. G., K. J. Verstrepen, S. D. M. Van Laere, A. R. D. Voet, P. Van dijck, F. R. Delvauz, and J. M. Thevelein. 2006. *The Sacchoaromyces cerevisiae EHT1* and *EEB1* genes encode novel enzymes with medium –chain fatty acid ethyl ester synthesis and hydrolysis capacity. The Journal of Biological Chemistry. **281**:4446-4456.

Sakamoto, K. and W. N. Konings. 2003. Beer spoilage and hop resistance. International Journal of Food Microbiology. **89:**105-124.

Santayanarayama, T., H. E. Umbarger, and G. Lindengren. 1968. Biosynthesis of branched-chain amino acids in yeast: regulation of leucine biosynthesis in prototrophic and leucine auxotrophic strains. Journal of Bacteriology. **96**:2018-2024.

Seaton, J. C., and I. C. Cantrell. 1993. The determination of beer colour - collaborative trial. Journal of the Institute of Brewing. **99**:21-23.

Sikorska, E., J. Chmielewski, T. Górecki, I. Khmelinskii, M. Sikorski and D, De Keukeleire. 2007. Discrimination of beer flavours by analysis of volatiles using the mass spectrometer as an electronic nose. Journal of the Institute of Brewing. **113**:110-115.

Silva, F., L. C. Nogueira, C. Goncalves, A. A. Ferreira, I. M.P.L.V.O. Ferreira and N. Teixeira. 2008. Electrophoretic and HPLC methods for comparative study of the protein fractions of malts, worts and beers produced from Scarlett and Prestige barley (*Hordeum vulgare L.*) varieties. Food Chemistry. **106**:820–829.

Simpson, W. J., and H. Taguchi. 1995. The genus Pediococcus, with notes on the genera *Tetratogenococcus* and *Aerococcus,* p. 125- 172. .*In*: The Lactic Acid Bacteria. B. J. B. Wood and W. H. Holzapfel (eds). The Genera of Lactic Acid Bacteria. Blackie Academic and Professional, London,

Simpson, W. J., and P. S. Hughes. 1993. Stabilization of foams by hop-derived bitter acids: Chemical interactions in beer foam. *Cerevisiae* Biotechnology. **19:**39–44.

Slaughter, J. C., and G. McKernan. 1988. The Influence of pantothenate concentration and inoculum size on the fermentation of a defined medium by *Saccharomyces cerevisiae.* Journal of the Institute of Brewing **94**:14–18.

Šmogrovičovà, D., and Z. Dömény. 1999. Beer volatile by-product formation at different fermentation temperature using immobilised yeasts. Process Biochemistry. **34**:785–794.

Solay, A., M. Lecina, X. Gamez, J. J. Cairo, P. Riu, X. Rosell, R. Bragos, and F. Godia. 2005. On-line monitoring of yeast cell growth by impedance spectroscopy. Journal of Biotechnology. **118**:398-405.

Sørensen, S. B., L. M. Bech, M. Muldbjerg, T. Beenfeldt, and K. Breddam. 1993. Barley lipid transfer protein 1 is involved in beer foam formation. Master Brewers Association of the Americas. **30**:136–145.

Soumalainen, H. 1981. Yeast esterases and aroma esters in alcoholic beverages. Journal of the Institute of Brewing. **87**:296–300.

Speers, R. A., M. A. Tung, T. D Durance, and G. G. Stewart. 1992. Biochemical aspects of yeast flocculation and its measurements: a review. Journal of the Institute of Brewing. **98**:293–300.

Stewart, G. G. 2006. Studies on the uptake and metabolism of wort sugars during brewing fermentations. Master Brewers Association of the Americas. **43**:265–269.

Suihko, M. L., A. Vilpola and M. Linko. 1993. Pitching rate in high gravity brewing. Journal of the Institute of Brewing. **99**:341-346.

Sumby, K. M., P. R. Grbin and V. Jiranek. 2010. Microbial modulation of aromatic esters in wine: Current knowledge and future prospects. Food Chemistry. **121**:1–16.

Suomalainen, H. 1981. Yeast esterases and aroma esters in alcoholic beverages. Journal of the Institute of Brewing. **87:**296–300.

Tankeviciute, A., R. Kazlauskas and V. Vickackaite. 2001. Headspace extraction of alcohols into a single drop. Analyst. **126**:1674–1677.

Teunissen, A. W. R. H., and H. Y. Steensma. 1995. Review: the dominant flocculation genes of *Saccharomyces cerevisiae*. Yeast. **11**:1001–1013.

Thevelein, J. M., and J. H. de Winde. 1999. Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast *Saccharomyces cerevisiae*. Molecular Microbiology. **33**:904–918.

Thompson, W., J. Shovers, W. E. Sandine, and P. R. Elliker. 1970. Enzymatic removal of diacetyl from beer. Applied Microbiology. **19**:883-889.

Titica, M., S. Landaud, I. C. Trelea, E. Latrille, G. Corrieu, and A. Cheruy. 2000. Modelling of the kinetics of higher alcohol and ester production based on CO_2 emission with a view to control of beer flavor by temperature and top pressure. Journal of the American Society of Brewing Chemists. **58**:167-174.

Torrea, D., P. Fraile, T. Garde, and C. Ancín. 2003. Production of volatile compounds in the fermentation of chardonnay musts inoculated with two strains of *Saccharomyces cerevisiae* with different nitrogen demands. Food Control. **14**:565–571.

Tosun, A., and A. Ergun. 2007. Use of experimental design method to investigate metal ion effects in yeast fermentations. Journal of Chemical Technology and Biotechnology. **82**:11–15.

Trinh, T. T. T., W. Y. Woon, B. Yu, P. Curran, and S. Q. Liu. 2010. Effect of L-isoleucine and L-phenylalanine addition on aroma compound formation during longan juice fermentation by a co-culture of *Saccharomyces cerevisiae* and *Williopsis saturnus.* South African Journal of Enology and Viticulture. **31:**116-124.

Van Bergen, B., and J. D. Sheppard. 2004. The effect of acid washing and fermentation gravity on mechanical shear resistance in brewing yeast. Journal of the American Society of Brewing Chemists. **62**:87–91.

Van Nierop, S. N. E., D. E. Evans, D. E., B. C. Axcell, I. C. Cantrell, and M. Rautenbach. 2004. Impact of different wort boiling temperatures on the beer foam stabilizing properties of lipid transfer protein 1. Journal of Agricultural and Food Chemistry. **52**:3120-3129.

Vanderhaegen, B., F. Delvaux, L. Daenen, H. Verachtert and F. R. Dalvaux. 2007. Aging characteristics of different beer types. Food Chemistry. **103:**404-412.

Vanderhaegen, B., H. Neven, H. Verachtert and G. Derdelinckx. 2006. The chemistry of beer aging – a critical review. Food Chemistry. **95**:357–381.

Venkateshwar, M. K. Chaitanya, M. Altaf, E. J. Mahammad, B. Hameeda, and G. Reddy. 2010. Influence of micronutrients on yeast growth and β-D-fructofuranosidase production. Indian Journal of Microbiology. **50**:325–331.

Verbelen, P. J., T. M. L. Dekoninck, S. M. G. Saerens, S. E. Van Mulders, J. M. Thevelein, and F. R. Delvaux. 2009. Impact of pitching rate on yeast fermentation performance and beer flavour. Applied Microbiology and Biotechnology. **82**:155–167.

Verbelen, P. J., S. Van Mulders, D. Saison, S. Van Laere, F. Delvaux and F. R. Delvaux. 2008. Characteristics of high cell density fermentations with different lager yeast strains. Journal of the Institute of Brewing. **114**:127–133.

Verstrepen, K. J., G. Derdelinckx, J. Dufour, J. Winderrikx, J. M. Thevelein, I. S. Pretorius and F. R. Delvaux. 2003a. Flavour-active esters: adding fruitiness to beer. Journal of Bioscience and Bioengineering. **96**:110-118.

Verstrepen, K. J., G. Derdelinckxa, J. Dufour, J. Winderickx, I. S. Pretorius, J. M. Thevelein and F. R. Delvaux. 2003b. The *Saccharomyces cerevisiae* alcohol acetyl transferase gene *ATF1* is a target of the cAMP/PKA and FGM nutrient-signalling pathways. FEMS Yeast Research. **4**:285-296.

Verstrepen, K. J., S. D. M. Van Laere, B. M. P. Vanderhaegen, G. Derdelinckx, J. Dufour, I. S. Pretorius, J. Winderickx, J. M. Thevelein and F. R. Delvaux. 2003c. Expression levels of the yeast alcohol acetyltransferase genes *ATF1, Lg-ATF1*, and *ATF2* control the formation of a broad range of volatile esters. Applied and Environmental Microbiology. **69:**5228-523.

Verstrepen, K. J., G. Derdelinckx, H. Verachtert, and F. R. Delvaux. 2003d. Yeast flocculation, what brewers should know. Appied Microbiology and Biotechnology. **61**:197–206.

Verstrepen, K. J., S. D. M. Van Laere, J. Vercammen, G. Derdelinckx, J. Dufour, I. S. Pretorius, J. Winderickx, J. M. Thevelein and F. R. Delvaux. 2004. The *Saccharomyces cerevisiae* alcohol acetyltransferase Atf1p is localized in lipid particles. Yeast. **21**:367–377.

Villanueva, K. D., E. Goossens, and C. A. Masschelein. 1990. Subthreshold vicinal diketone levels in lager brewing yeast fermentations by means of ILV5 gene amplification. American Society of Brewing Chemists. **48**:111-114.

Watari, J., M. Sato, M. Ogawa, and K. Shinotsuka. 2000. Genetic and physiological instability of brewing yeast, p. 148-158. *In*: Yeast physiology: a new era of opportunity. Fachverlag Hans Carl, Ntirnberg.

APPENDIX A: List of reagents and media used

a) Dinitrosalicyclic acid (DNS) method

i. DNS reagent

Dinitrosalicyclic acid	1 g
Crystalline phenol (Merck)	200 mg
Sodium sulphite (Merck)	50 mg
1% NaOH	100 ml

ii. 40% Rochelle salt

Potassium sodium tartrate (Merck)	40 g
Distilled water (bring up)	100 ml

b) Ninhyrin Assay

i. Ninhydrin reagent (8% w/v)

Ninhydrin	8 g
Dissolve in acetone	100 ml

c) Brown sugar solution (1g/ml)

Brown sugar (Huletts)	500 g
Distilled water	500 ml

d) Malt extract agar

Malt extract agar (Merck)	25 g
Distilled water	500 ml

e) Diethylpyrocarbonate (DEPC) treated water (0.1%)

DEPC	1 ml
Distilled water	1000 ml

f) 75% ethanol

Absolute ethanol	75 ml
DEPC treated water	25 ml

APPENDIX B: Numerical data – Fermentation performance, post fermentation analysis and ester concentrations and stability for ale and lager beer

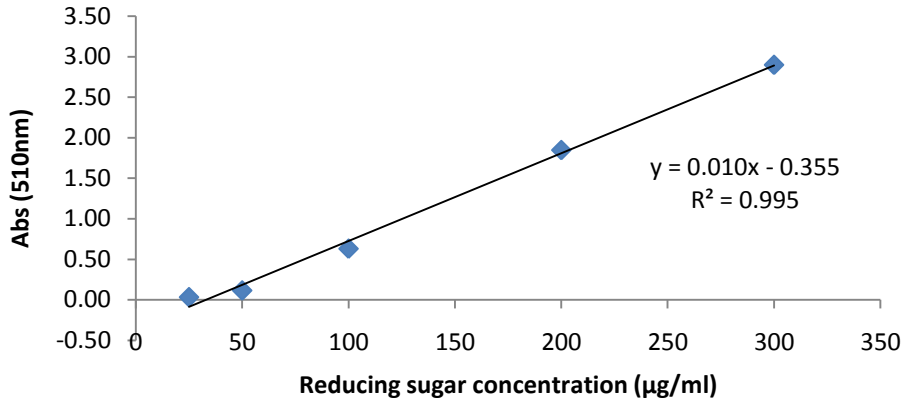


Figure B1: Standard curve used for the determination of reducing sugar content (DNS method) of the samples (used for Figure 2.1a, Figure 2.2a, Figure 2.3a, Figure 2.4a, Figure 3.1a, Figure 3.2a, Figure 3.3a and Figure 3.4a)

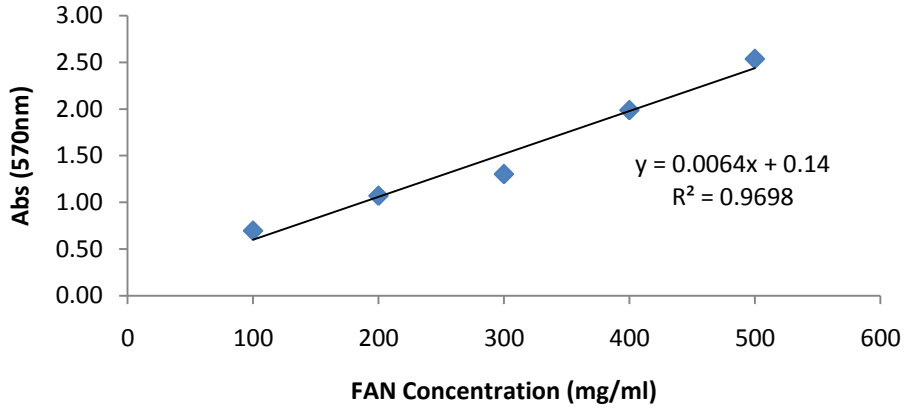


Figure B2: Standard curve used for the determination of FAN (Ninhyrin method) of the samples (used for Figure 2.1b, Figure 2.2b, Figure 2.3b, Figure 2.4b, Figure 3.1b, Figure 3.2b, Figure 3.3b and Figure 3.4b)

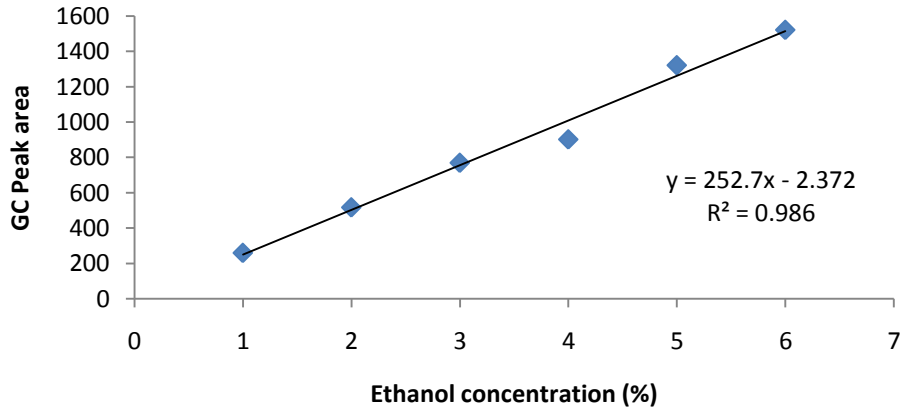


Figure B3: Standard curve used for the determination of ethanol concentration of the samples (used for Figure 2.1a, Figure 2.2a, Figure 2.3a, Figure 2.4a, Figure 3.1a, Figure 3.2a, Figure 3.3a and Figure 3.4a)

Table B1: The absorbance and concentrations (mg/ml) of FAN obtained through the Ninhydrin method under various concentrations of Zinc sulphate by an ale brewing strain (Used for Figure 3.1b) (Dilution factor = 10)

Conc.	Day		1	2	3	4	Average	SD
0.00	1	Absorbance	1.677	1.679	1.683	1.688		
		Concentration	2401	2405	2411	2418	2408.75	7.41
	2	Absorbance	1.654	1.650	1.647	1.649		
		Concentration	2365	2359	2355	2358	2359.25	4.19
	3	Absorbance	1.487	1.487	1.490	1.488		
		Concentration	2104	2104	2109	2107	2106.00	2.45
	4	Absorbance	1.385	1.384	1.381	1.378		
		Concentration	1945	1944	1939	1935	1940.75	4.65
	5	Absorbance	1.263	1.261	1.259	1.252		
		Concentration	1755	1751	1748	1737	1747.75	7.72
	6	Absorbance	1.196	1.195	1.198	1.199		
		Concentration	1650	1648	1653	1654	1651.25	2.75
0.03	1	Absorbance	1.648	1.644	1.654	1.654		
		Concentration	2356	2350	2365	2366	2359.25	7.63
	2	Absorbance	1.581	1.583	1.581	1.579		
		Concentration	2251	2254	2251	2248	2251.00	2.45
	3	Absorbance	1.385	1.382	1.380	1.381		
		Concentration	1946	1940	1938	1939	1940.75	3.59
	4	Absorbance	1.135	1.133	1.135	1.133		
		Concentration	1554	1551	1554	1551	1553.50	1.73
	5	Absorbance	1.173	1.174	1.166	1.165		
		Concentration	1614	1615	1603	1601	1608.25	7.27
	6	Absorbance	1.129	1.126	1.135	1.136		
		Concentration	1545	1541	1555	1556	1549.25	7.41
0.06	1	Absorbance	1.613	1.613	1.616	1.617		
		Concentration	2301	2301	2306	2308	2304.00	3.56
	2	Absorbance	1.519	1.517	1.514	1.513		
		Concentration	2154	2152	2147	2145	2149.50	4.20
	3	Absorbance	1.314	1.317	1.315	1.312		
		Concentration	1834	1839	1836	1832	1835.25	2.99
	4	Absorbance	1.041	1.039	1.045	1.048		
		Concentration	1408	1404	1414	1419	1411.25	6.60
	5	Absorbance	1.067	1.065	1.062	1.063		
		Concentration	1448	1445	1441	1442	1444.00	3.16
	6	Absorbance	1.116	1.111	1.110	1.111		
		Concentration	1525	1517	1515	1517	1518.50	4.43
0.12	1	Absorbance	1.583	1.581	1.586	1.583		
		Concentration	2255	2251	2259	2254	2254.75	3.30
	2	Absorbance	1.393	1.397	1.396	1.394		
		Concentration	1958	1964	1962	1960	1961.00	2.58
	3	Absorbance	1.120	1.122	1.127	1.129		
		Concentration	1531	1534	1542	1546	1538.25	6.95
	4	Absorbance	1.005	1.007	1.008	1.007		
		Concentration	1352	1354	1356	1354	1354.00	1.63
	5	Absorbance	0.964	0.963	0.962	0.956		
		Concentration	1287	1286	1284	1275	1283.00	5.48
	6	Absorbance	0.950	0.927	0.936	0.944		
		Concentration	1265	1229	1244	1256	1248.50	15.59

Table B2: The absorbance and concentrations (mg/ml) of FAN obtained through the Ninhydrin method under various concentrations of L-Leucine by an ale brewing strain (Used for Figure 3.2b) (Dilution factor = 10)

Conc.	Day		1	2	3	4	Average	SD
0.00	1	Absorbance	1.616	1.608	1.608	1.606		
		Concentration	2306	2294	2294	2291	2296.25	6.65
	2	Absorbance	1.586	1.558	1.557	1.571		
		Concentration	2259	2215	2214	2236	2231.00	21.24
	3	Absorbance	1.520	1.513	1.519	1.515		
		Concentration	2156	2145	2155	2149	2151.25	5.19
	4	Absorbance	1.385	1.391	1.391	1.397		
		Concentration	1945	1954	1955	1964	1954.50	7.77
	5	Absorbance	1.195	1.192	1.180	1.183		
		Concentration	1648	1644	1625	1629	1636.50	11.21
	6	Absorbance	1.161	1.158	1.163	1.108		
		Concentration	1595	1590	1599	1512	1574.00	41.50
0.25	1	Absorbance	1.713	1.716	1.733	1.732		
		Concentration	2458	2462	2489	2488	2474.25	16.54
	2	Absorbance	1.654	1.647	1.649	1.647		
		Concentration	2365	2354	2358	2355	2358.00	4.97
	3	Absorbance	1.391	1.396	1.399	1.348		
		Concentration	1955	1962	1967	1888	1943.00	37.00
	4	Absorbance	1.193	1.227	1.199	1.190		
		Concentration	1645	1699	1655	1641	1660.00	26.66
	5	Absorbance	1.130	1.131	1.099	1.099		
		Concentration	1547	1548	1499	1498	1523.00	28.30
	6	Absorbance	0.981	0.924	0.970	0.965		
		Concentration	1314	1225	1297	1289	1281.25	38.92
0.50	1	Absorbance	1.925	1.924	1.839	1.858		
		Concentration	2789	2788	2655	2684	2729.00	69.72
	2	Absorbance	1.648	1.647	1.656	1.642		
		Concentration	2357	2355	2369	2347	2357.00	9.09
	3	Absorbance	1.321	1.322	1.330	1.291		
		Concentration	1845	1847	1859	1799	1837.50	26.40
	4	Absorbance	1.192	1.263	1.263	1.256		
		Concentration	1644	1754	1754	1744	1724.00	53.54
	5	Absorbance	1.131	1.131	1.128	1.142		
		Concentration	1548	1548	1544	1566	1551.50	9.85
	6	Absorbance	1.045	1.005	1.046	1.035		
		Concentration	1414	1351	1415	1399	1394.75	30.07
0.75	1	Absorbance	2.067	2.052	1.995	2.050		
		Concentration	3011	2988	2899	2984	2970.50	49.13
	2	Absorbance	1.962	1.896	1.878	1.884		
		Concentration	2847	2744	2715	2725	2757.75	60.70
	3	Absorbance	1.784	1.781	1.752	1.749		
		Concentration	2569	2564	2518	2514	2541.25	29.27
	4	Absorbance	1.517	1.525	1.494	1.496		
		Concentration	2151	2164	2115	2119	2137.25	24.03
	5	Absorbance	1.327	1.293	1.355	1.348		
		Concentration	1854	1802	1899	1887	1860.50	43.39
	6	Absorbance	1.103	1.092	1.093	1.101		
		Concentration	1505	1488	1489	1501	1495.75	8.54

Table B3: The absorbance and concentrations (mg/ml) of FAN obtained through the Ninhydrin method under various fermentation temperatures by a lager brewing strain (Used for Figure 3.3b) (Dilution factor = 10)

Temp.	Day		1	2	3	4	Average	SD
14 °C	1	Absorbance	1.549	1.551	1.544	1.544		
		Concentration	2201	2205	2194	2193	2198.25	5.74
	2	Absorbance	1.434	1.474	1.355	1.410		
		Concentration	2022	2085	1898	1985	1997.50	78.13
	3	Absorbance	1.307	1.307	1.307	1.327		
		Concentration	1824	1824	1823	1855	1831.50	15.67
	4	Absorbance	1.284	1.264	1.263	1.263		
		Concentration	1788	1756	1754	1754	1763.00	16.69
	5	Absorbance	1.199	1.197	1.179	1.197		
		Concentration	1654	1652	1624	1652	1645.50	14.36
	6	Absorbance	1.179	1.205	1.154	1.116		
		Concentration	1624	1664	1584	1525	1599.25	59.30
18 °C	1	Absorbance	1.485	1.517	1.487	1.519		
		Concentration	2102	2151	2105	2154	2128.00	28.34
	2	Absorbance	1.389	1.391	1.385	1.412		
		Concentration	1952	1954	1945	1988	1959.75	19.22
	3	Absorbance	1.179	1.248	1.257	1.242		
		Concentration	1624	1732	1745	1722	1705.75	55.31
	4	Absorbance	1.178	1.199	1.197	1.221		
		Concentration	1622	1654	1652	1689	1654.25	27.40
	5	Absorbance	1.157	1.128	1.154	1.154		
		Concentration	1589	1544	1584	1584	1575.25	20.97
	6	Absorbance	1.152	1.145	1.131	1.131		
		Concentration	1581	1570	1548	1548	1561.75	16.50
RT	1	Absorbance	1.427	1.430	1.430	1.432		
		Concentration	2011	2015	2015	2019	2015.00	3.27
	2	Absorbance	1.391	1.355	1.408	1.372		
		Concentration	1954	1899	1982	1925	1940.00	35.90
	3	Absorbance	1.179	1.163	1.177	1.180		
		Concentration	1624	1599	1620	1625	1617.00	12.19
	4	Absorbance	0.988	0.989	0.987	0.987		
		Concentration	1325	1327	1324	1324	1325.00	1.41
	5	Absorbance	0.943	0.937	0.937	0.939		
		Concentration	1254	1245	1245	1249	1248.25	4.27
	6	Absorbance	0.860	0.859	0.857	0.860		
		Concentration	1125	1124	1121	1125	1123.75	1.89

Table B4: The absorbance and concentrations (mg/ml) of FAN obtained through the Ninhydrin method under various fermentation pH by al ale brewing strain (Used for Figure 3.4b) (Dilution factor = 10)

pH	Day		1	2	3	4	Average	SD
3	1	Absorbance	1.554	1.551	1.551	1.547		
		Concentration	2209	2205	2204	2199	2204.25	4.11
	2	Absorbance	1.519	1.519	1.519	1.522		
		Concentration	2154	2154	2155	2159	2155.50	2.38
	3	Absorbance	1.427	1.462	1.460	1.443		
		Concentration	2011	2065	2063	2036	2043.75	25.53
	4	Absorbance	1.412	1.413	1.410	1.398		
		Concentration	1988	1989	1985	1965	1981.75	11.30
	5	Absorbance	1.327	1.328	1.322	1.307		
		Concentration	1854	1856	1847	1823	1845.00	15.17
	6	Absorbance	1.263	1.264	1.263	1.259		
		Concentration	1754	1756	1754	1748	1753.00	3.46
5	1	Absorbance	1.520	1.513	1.497	1.515		
		Concentration	2156	2145	2121	2148	2142.50	15.07
	2	Absorbance	1.421	1.425	1.421	1.393		
		Concentration	2001	2008	2001	1958	1992.00	22.91
	3	Absorbance	1.372	1.371	1.379	1.375		
		Concentration	1925	1924	1936	1930	1928.75	5.50
	4	Absorbance	1.201	1.163	1.199	1.186		
		Concentration	1658	1599	1654	1635	1636.50	26.94
	5	Absorbance	1.069	1.042	1.050	1.073		
		Concentration	1452	1409	1422	1458	1435.25	23.54
	6	Absorbance	1.016	1.098	1.093	1.086		
		Concentration	1369	1497	1489	1478	1458.25	60.01
7	1	Absorbance	1.519	1.507	1.533	1.508		
		Concentration	2154	2136	2177	2137	2151.00	19.20
	2	Absorbance	1.519	1.487	1.488	1.522		
		Concentration	2154	2105	2107	2159	2131.25	29.24
	3	Absorbance	1.412	1.410	1.429	1.431		
		Concentration	1988	1985	2014	2017	2001.00	16.83
	4	Absorbance	1.393	1.370	1.354	1.357		
		Concentration	1958	1922	1897	1901	1919.50	27.91
	5	Absorbance	1.186	1.188	1.225	1.195		
		Concentration	1635	1637	1695	1648	1653.75	28.09
	6	Absorbance	1.154	1.130	1.140	1.130		
		Concentration	1585	1547	1563	1547	1560.50	17.99

Table B5: The absorbance and concentrations (mg/ml) of FAN obtained through the Ninhydrin method under various concentrations of Zinc sulphate by a lager brewing strain (Used for Figure 2.1b) (Dilution factor = 10)

Conc.	Day		1	2	3	4	Average	SD
0.00	1	Absorbance	1.648	1.649	1.656	1.657		
		Concentration	2356	2358	2369	2370	2363.25	7.27
	2	Absorbance	1.612	1.615	1.622	1.621		
		Concentration	2300	2305	2315	2314	2308.50	7.23
	3	Absorbance	1.412	1.410	1.414	1.415		
		Concentration	1988	1985	1990	1992	1988.75	2.99
	4	Absorbance	1.185	1.187	1.200	1.196		
		Concentration	1633	1636	1656	1650	1643.75	11.03
	5	Absorbance	0.941	0.940	0.950	0.953		
		Concentration	1252	1250	1265	1270	1259.25	9.78
	6	Absorbance	0.896	0.895	0.894	0.892		
		Concentration	1181	1180	1178	1175	1178.50	2.65
0.03	1	Absorbance	1.657	1.660	1.643	1.644		
		Concentration	2370	2375	2349	2350	2361.00	13.44
	2	Absorbance	1.615	1.616	1.583	1.586		
		Concentration	2304	2306	2255	2260	2281.25	27.51
	3	Absorbance	1.348	1.349	1.337	1.339		
		Concentration	1888	1889	1870	1874	1880.25	9.67
	4	Absorbance	1.074	1.076	1.088	1.088		
		Concentration	1459	1462	1482	1482	1471.25	12.47
	5	Absorbance	0.942	0.943	0.921	0.902		
		Concentration	1253	1255	1220	1190	1229.50	30.84
	6	Absorbance	0.881	0.880	0.848	0.850		
		Concentration	1158	1157	1106	1110	1132.75	28.63
0.06	1	Absorbance	1.645	1.647	1.650	1.650		
		Concentration	2352	2354	2360	2359	2356.25	3.86
	2	Absorbance	1.605	1.607	1.590	1.592		
		Concentration	2289	2292	2265	2268	2278.50	13.96
	3	Absorbance	1.257	1.259	1.263	1.262		
		Concentration	1745	1748	1755	1753	1750.25	4.57
	4	Absorbance	1.010	1.008	0.985	0.986		
		Concentration	1359	1357	1320	1322	1339.50	21.39
	5	Absorbance	0.877	0.878	0.870	0.873		
		Concentration	1152	1153	1140	1145	1147.50	6.14
	6	Absorbance	0.824	0.825	0.836	0.828		
		Concentration	1068	1071	1088	1075	1075.50	8.81
0.12	1	Absorbance	1.641	1.645	1.645	1.645		
		Concentration	2345	2351	2351	2352	2349.75	3.20
	2	Absorbance	1.549	1.551	1.551	1.554		
		Concentration	2201	2205	2204	2209	2204.75	3.30
	3	Absorbance	1.133	1.135	1.138	1.139		
		Concentration	1552	1555	1560	1561	1557.00	4.24
	4	Absorbance	0.988	0.989	0.991	0.991		
		Concentration	1325	1327	1329	1330	1327.75	2.22
	5	Absorbance	0.898	0.898	0.902	0.898		
		Concentration	1184	1185	1190	1185	1186.00	2.71
	6	Absorbance	0.784	0.786	0.786	0.782		
		Concentration	1006	1009	1010	1003	1007.00	3.16

Table B6: The absorbance and concentrations (mg/ml) of FAN obtained through the Ninhydrin method under various concentrations of L-Leucine by a lager brewing strain (Used for Figure 2.2b) (Dilution factor = 10)

Conc.	Day		1	2	3	4	Average	SD
0.00	1	Absorbance	1.654	1.655	1.650	1.647		
		Concentration	2366	2367	2360	2354	2361.75	6.02
	2	Absorbance	1.606	1.600	1.613	1.617		
		Concentration	2290	2281	2301	2308	2295.00	11.92
	3	Absorbance	1.515	1.521	1.513	1.512		
		Concentration	2148	2158	2146	2143	2148.75	6.50
	4	Absorbance	1.386	1.392	1.392	1.384		
		Concentration	1947	1956	1957	1943	1950.75	6.85
	5	Absorbance	1.193	1.192	1.202	1.199		
		Concentration	1645	1644	1659	1655	1650.75	7.41
	6	Absorbance	0.969	0.966	0.939	0.944		
		Concentration	1295	1290	1249	1257	1272.75	23.13
0.25	1	Absorbance	1.785	1.787	1.783	1.778		
		Concentration	2571	2573	2567	2559	2567.50	6.19
	2	Absorbance	1.712	1.709	1.677	1.686		
		Concentration	2456	2451	2401	2416	2431.00	26.77
	3	Absorbance	1.321	1.318	1.302	1.300		
		Concentration	1845	1840	1816	1812	1828.25	16.66
	4	Absorbance	1.199	1.200	1.186	1.185		
		Concentration	1654	1657	1634	1633	1644.50	12.77
	5	Absorbance	1.156	1.152	1.143	1.140		
		Concentration	1588	1581	1567	1562	1574.50	12.07
	6	Absorbance	1.056	1.058	1.040	1.042		
		Concentration	1431	1434	1407	1409	1420.25	14.22
0.50	1	Absorbance	1.941	1.938	1.942	1.947		
		Concentration	2814	2809	2815	2823	2815.25	5.80
	2	Absorbance	1.791	1.787	1.789	1.784		
		Concentration	2580	2574	2576	2569	2574.75	4.57
	3	Absorbance	1.385	1.389	1.387	1.384		
		Concentration	1945	1951	1949	1944	1947.25	3.30
	4	Absorbance	1.291	1.295	1.298	1.288		
		Concentration	1799	1805	1810	1794	1802.00	6.98
	5	Absorbance	1.263	1.263	1.257	1.256		
		Concentration	1754	1755	1745	1744	1749.50	5.80
	6	Absorbance	1.173	1.165	1.171	1.176		
		Concentration	1614	1601	1611	1619	1611.25	7.59
0.75	1	Absorbance	2.138	2.137	2.133	2.130		
		Concentration	3122	3120	3114	3110	3116.50	5.51
	2	Absorbance	1.991	1.992	1.988	1.984		
		Concentration	2892	2893	2887	2882	2888.50	5.07
	3	Absorbance	1.903	1.903	1.899	1.897		
		Concentration	2754	2755	2749	2745	2750.75	4.65
	4	Absorbance	1.677	1.680	1.686	1.688		
		Concentration	2401	2406	2415	2419	2410.25	8.22
	5	Absorbance	1.488	1.487	1.491	1.494		
		Concentration	2106	2105	2111	2116	2109.50	5.07
	6	Absorbance	1.382	1.385	1.387	1.383		
		Concentration	1940	1945	1948	1942	1943.75	3.50

Table B7: The absorbance and concentrations (mg/ml) of FAN obtained through the Ninhydrin method under various fermentation temperatures by a lager brewing strain (Used for Figure 2.3b) (Dilution factor = 10)

Temp.	Day		1	2	3	4	Average	SD
14 °C	1	Absorbance	1.549	1.551	1.544	1.544		
		Concentration	2201	2205	2194	2193	2198.25	5.74
	2	Absorbance	1.484	1.480	1.483	1.487		
		Concentration	2100	2094	2099	2104	2099.25	4.11
	3	Absorbance	1.321	1.325	1.327	1.326		
		Concentration	1845	1851	1854	1853	1850.75	4.03
	4	Absorbance	1.179	1.180	1.184	1.189		
		Concentration	1624	1625	1632	1639	1630.00	6.98
	5	Absorbance	0.943	0.939	0.939	0.943		
		Concentration	1255	1248	1249	1254	1251.50	3.51
	6	Absorbance	0.889	0.889	0.885	0.885		
		Concentration	1171	1170	1164	1164	1167.25	3.77
18 °C	1	Absorbance	1.484	1.491	1.485	1.487		
		Concentration	2100	2111	2102	2105	2104.50	4.80
	2	Absorbance	1.421	1.426	1.414	1.417		
		Concentration	2001	2009	1991	1995	1999.00	7.83
	3	Absorbance	1.257	1.263	1.259	1.259		
		Concentration	1745	1755	1749	1748	1749.25	4.19
	4	Absorbance	0.986	0.989	1.001	1.003		
		Concentration	1322	1326	1346	1349	1335.75	13.72
	5	Absorbance	0.807	0.809	0.813	0.813		
		Concentration	1042	1045	1051	1051	1047.25	4.50
	6	Absorbance	0.772	0.770	0.768	0.767		
		Concentration	987	984	982	980	983.25	2.99
RT	1	Absorbance	1.452	1.455	1.453	1.458		
		Concentration	2050	2054	2051	2059	2053.50	4.04
	2	Absorbance	1.382	1.382	1.385	1.386		
		Concentration	1940	1940	1946	1947	1943.25	3.77
	3	Absorbance	1.199	1.199	1.199	1.195		
		Concentration	1654	1654	1655	1648	1652.75	3.20
	4	Absorbance	0.925	0.923	0.923	0.921		
		Concentration	1227	1224	1224	1220	1223.75	2.87
	5	Absorbance	0.781	0.784	0.783	0.781		
		Concentration	1001	1006	1004	1002	1003.25	2.22
	6	Absorbance	0.699	0.697	0.678	0.681		
		Concentration	874	870	841	846	857.75	16.66

Table B8: The absorbance and concentrations (mg/ml) of FAN obtained through the Ninhydrin method under various fermentation pH by a lager brewing strain (Used for Figure 3.4b) (Dilution factor = 10)

pH	Day		1	2	3	4	Average	SD
3	1	Absorbance	1.632	1.633	1.631	1.630		
		Concentration	2331	2333	2329	2328	2330.25	2.22
	2	Absorbance	1.612	1.613	1.613	1.610		
		Concentration	2300	2301	2302	2297	2300.00	2.16
	3	Absorbance	1.492	1.491	1.488	1.485		
		Concentration	2112	2111	2107	2101	2107.75	4.99
	4	Absorbance	1.427	1.426	1.424	1.426		
		Concentration	2011	2010	2007	2009	2009.25	1.71
	5	Absorbance	1.401	1.405	1.400	1.400		
		Concentration	1971	1976	1968	1969	1971.00	3.56
	6	Absorbance	1.339	1.343	1.329	1.328		
		Concentration	1874	1879	1858	1857	1867.00	11.17
5	1	Absorbance	1.570	1.567	1.565	1.562		
		Concentration	2235	2230	2227	2222	2228.50	5.45
	2	Absorbance	1.547	1.549	1.550	1.546		
		Concentration	2199	2201	2203	2197	2200.00	2.58
	3	Absorbance	1.406	1.407	1.412	1.412		
		Concentration	1978	1979	1988	1987	1983.00	5.23
	4	Absorbance	1.197	1.199	1.196	1.195		
		Concentration	1652	1655	1650	1648	1651.25	2.99
	5	Absorbance	0.988	0.986	1.003	1.005		
		Concentration	1325	1322	1349	1351	1336.75	15.37
	6	Absorbance	0.875	0.876	0.872	0.875		
		Concentration	1148	1150	1144	1149	1147.75	2.63
7	1	Absorbance	1.626	1.631	1.626	1.629		
		Concentration	2322	2329	2322	2326	2324.75	3.40
	2	Absorbance	1.600	1.600	1.600	1.604		
		Concentration	2281	2281	2282	2287	2282.75	2.87
	3	Absorbance	1.408	1.408	1.410	1.407		
		Concentration	1981	1982	1985	1980	1982.00	2.16
	4	Absorbance	1.384	1.387	1.385	1.384		
		Concentration	1944	1948	1946	1943	1945.25	2.22
	5	Absorbance	1.213	1.207	1.227	1.221		
		Concentration	1677	1667	1698	1689	1682.75	13.57
	6	Absorbance	1.130	1.128	1.129	1.127		
		Concentration	1547	1544	1546	1542	1544.75	2.22

Table B9: The absorbance and concentrations (µg/ml) of Reducing Sugars obtained through the DNS method under various concentrations of Zinc sulphate by al ale brewing strain (Used for Figure 3.1a) (Dilution factor = 10)

Conc.	Day		1	2	3	4	Average	SD
0.00	1	Absorbance	2.360	2.365	2.392	2.389		
		Concentration	2514	2519	2544	2541	2529.50	15.20
	2	Absorbance	2.145	2.151	2.152	2.148		
		Concentration	2315	2321	2322	2318	2319.00	3.16
	3	Absorbance	1.856	1.853	1.844	1.838		
		Concentration	2048	2045	2037	2031	2040.25	7.72
	4	Absorbance	1.647	1.640	1.627	1.621		
		Concentration	1854	1848	1836	1830	1842.00	10.95
	5	Absorbance	1.565	1.565	1.544	1.537		
		Concentration	1778	1778	1759	1752	1766.75	13.30
	6	Absorbance	1.400	1.402	1.401	1.411		
		Concentration	1625	1627	1626	1636	1628.50	5.07
0.03	1	Absorbance	2.879	2.882	2.892	2.891		
		Concentration	2536	2539	2548	2547	2542.50	5.92
	2	Absorbance	2.694	2.701	2.689	2.688		
		Concentration	2365	2371	2360	2359	2363.75	5.50
	3	Absorbance	2.466	2.472	2.466	2.462		
		Concentration	2154	2159	2154	2150	2154.25	3.69
	4	Absorbance	2.108	2.115	2.100	2.096		
		Concentration	1822	1829	1815	1811	1819.25	7.93
	5	Absorbance	2.007	2.000	1.982	1.985		
		Concentration	1729	1722	1706	1708	1716.25	11.09
	6	Absorbance	1.870	1.869	1.862	1.867		
		Concentration	1602	1601	1594	1599	1599.00	3.56
0.06	1	Absorbance	2.796	2.790	2.780	2.778		
		Concentration	2459	2454	2444	2443	2450.00	7.79
	2	Absorbance	2.574	2.571	2.571	2.568		
		Concentration	2254	2251	2251	2248	2251.00	2.45
	3	Absorbance	2.310	2.304	2.297	2.291		
		Concentration	2009	2004	1997	1992	2000.50	7.51
	4	Absorbance	1.959	1.957	1.948	1.947		
		Concentration	1684	1682	1674	1673	1678.25	5.56
	5	Absorbance	1.867	1.866	1.841	1.840		
		Concentration	1599	1598	1575	1574	1586.50	13.87
	6	Absorbance	1.614	1.614	1.612	1.607		
		Concentration	1365	1365	1363	1358	1362.75	3.30
0.12	1	Absorbance	2.787	2.791	2.788	2.787		
		Concentration	2451	2455	2452	2451	2452.25	1.89
	2	Absorbance	2.520	2.517	2.522	2.520		
		Concentration	2204	2201	2206	2204	2203.75	2.06
	3	Absorbance	2.248	2.248	2.252	2.259		
		Concentration	1952	1952	1956	1962	1955.50	4.73
	4	Absorbance	1.771	1.772	1.787	1.789		
		Concentration	1510	1511	1525	1527	1518.25	9.00
	5	Absorbance	1.495	1.496	1.490	1.494		
		Concentration	1255	1256	1250	1254	1253.75	2.63
	6	Absorbance	1.406	1.409	1.423	1.392		
		Concentration	1172	1175	1188	1159	1173.50	11.90

Table B10: The absorbance and concentrations (µg/ml) of Reducing Sugars obtained through the DNS method under various concentrations of L-leucine by an ale brewing strain (Used for Figure 3.2a) (Dilution factor = 10)

Conc.	Day		1	2	3	4	Average	SD
0.00	1	Absorbance	2.253	2.257	2.273	2.279		
		Concentration	2415	2419	2434	2439	2426.75	11.56
	2	Absorbance	2.040	2.072	2.095	2.072		
		Concentration	2218	2248	2269	2248	2245.75	20.98
	3	Absorbance	1.972	1.918	1.910	1.900		
		Concentration	2155	2105	2098	2088	2111.50	29.83
	4	Absorbance	1.709	1.705	1.699	1.748		
		Concentration	1912	1908	1902	1948	1917.50	20.74
	5	Absorbance	1.637	1.648	1.684	1.639		
		Concentration	1845	1855	1888	1847	1858.75	19.97
	6	Absorbance	1.398	1.388	1.384	1.388		
		Concentration	1624	1614	1611	1614	1615.75	5.68
0.25	1	Absorbance	2.248	2.247	2.245	2.246		
		Concentration	2411	2410	2408	2409	2409.50	1.29
	2	Absorbance	1.964	2.019	2.080	2.048		
		Concentration	2148	2199	2255	2225	2206.75	45.36
	3	Absorbance	1.748	1.755	1.755	1.756		
		Concentration	1948	1954	1954	1955	1952.75	3.20
	4	Absorbance	1.587	1.586	1.659	1.636		
		Concentration	1799	1798	1865	1844	1826.50	33.45
	5	Absorbance	1.540	1.529	1.533	1.540		
		Concentration	1755	1745	1749	1755	1751.00	4.90
	6	Absorbance	1.290	1.360	1.261	1.263		
		Concentration	1524	1588	1497	1499	1527.00	42.48
0.50	1	Absorbance	2.257	2.256	2.269	2.246		
		Concentration	2419	2418	2430	2409	2419.00	8.60
	2	Absorbance	2.091	2.116	2.080	2.079		
		Concentration	2265	2288	2255	2254	2265.50	15.80
	3	Absorbance	1.529	1.551	1.551	1.540		
		Concentration	1745	1765	1765	1755	1757.50	9.57
	4	Absorbance	1.287	1.420	1.421	1.371		
		Concentration	1521	1644	1645	1599	1602.25	58.26
	5	Absorbance	1.076	1.076	1.123	1.074		
		Concentration	1325	1325	1369	1324	1335.75	22.17
	6	Absorbance	0.858	0.858	0.853	0.883		
		Concentration	1124	1124	1119	1147	1128.50	12.56
0.75	1	Absorbance	2.200	2.199	2.144	2.149		
		Concentration	2366	2365	2314	2319	2341.00	28.37
	2	Absorbance	1.650	1.695	1.684	1.636		
		Concentration	1857	1899	1888	1844	1872.00	25.78
	3	Absorbance	1.371	1.290	1.316	1.312		
		Concentration	1599	1524	1548	1544	1553.75	31.94
	4	Absorbance	1.111	1.099	1.123	1.072		
		Concentration	1358	1347	1369	1322	1349.00	20.12
	5	Absorbance	0.752	0.752	0.763	0.750		
		Concentration	1025	1025	1036	1024	1027.50	5.69
	6	Absorbance	0.482	0.484	0.476	0.491		
		Concentration	775	777	770	784	776.50	5.80

Table B11: The absorbance and concentrations (µg/ml) of Reducing Sugars obtained through the DNS method under various fermentation temperatures by an ale brewing strain (Used for Figure 3.3a) (Dilution factor = 10)

Temp.	Day		1	2	3	4	Average	SD
14 °C	1	Absorbance	2.022	2.022	2.019	2.017		
		Concentration	2201	2201	2199	2197	2199.50	1.91
	2	Absorbance	2.012	1.963	1.970	1.951		
		Concentration	2192	2147	2153	2136	2157.00	24.37
	3	Absorbance	1.816	1.830	1.827	1.884		
		Concentration	2011	2024	2021	2074	2032.50	28.22
	4	Absorbance	1.665	1.637	1.645	1.660		
		Concentration	1871	1845	1852	1866	1858.50	12.07
	5	Absorbance	1.431	1.472	1.421	1.429		
		Concentration	1654	1692	1645	1652	1660.75	21.19
	6	Absorbance	1.368	1.314	1.316	1.316		
		Concentration	1596	1546	1548	1548	1559.50	24.35
18 °C	1	Absorbance	1.972	1.972	1.971	1.965		
		Concentration	2155	2155	2154	2149	2153.25	2.87
	2	Absorbance	1.874	1.876	1.846	1.856		
		Concentration	2064	2066	2038	2048	2054.00	13.37
	3	Absorbance	1.647	1.577	1.637	1.645		
		Concentration	1854	1789	1845	1852	1835.00	30.91
	4	Absorbance	1.504	1.529	1.524	1.505		
		Concentration	1722	1745	1740	1723	1732.50	11.73
	5	Absorbance	1.312	1.371	1.316	1.316		
		Concentration	1544	1599	1548	1548	1559.75	26.23
	6	Absorbance	1.218	1.316	1.219	1.205		
		Concentration	1457	1548	1458	1445	1477.00	47.70
RT	1	Absorbance	1.816	1.821	1.824	1.825		
		Concentration	2011	2015	2018	2019	2015.75	3.59
	2	Absorbance	1.788	1.788	1.757	1.788		
		Concentration	1985	1985	1956	1985	1977.75	14.50
	3	Absorbance	1.529	1.537	1.526	1.517		
		Concentration	1745	1752	1742	1734	1743.25	7.46
	4	Absorbance	1.179	1.191	1.191	1.181		
		Concentration	1421	1432	1432	1423	1427.00	5.83
	5	Absorbance	1.108	1.072	1.097	1.077		
		Concentration	1355	1322	1345	1326	1337.00	15.64
	6	Absorbance	0.884	0.892	0.860	0.835		
		Concentration	1148	1155	1125	1102	1132.50	24.03

Table B12: The absorbance and concentrations (µg/ml) of Reducing Sugars obtained through the DNS method under various fermentation pH by an ale brewing strain (Used for Figure 3.4a) (Dilution factor = 10)

pH	Day		1	2	3	4	Average	SD
14 °C	1	Absorbance	2.188	2.145	2.149	2.148		
		Concentration	2355	2315	2319	2318	2 326.75	18.91
	2	Absorbance	2.111	2.108	2.108	2.111		
		Concentration	2284	2281	2281	2284	2 282.50	1.73
	3	Absorbance	1.975	1.971	2.092	2.078		
		Concentration	2158	2154	2266	2253	2 207.75	60.01
	4	Absorbance	1.916	1.921	1.924	1.918		
		Concentration	2103	2108	2111	2105	2 106.75	3.50
	5	Absorbance	1.790	1.759	1.755	1.754		
		Concentration	1987	1958	1954	1953	1 963.00	16.15
	6	Absorbance	1.637	1.674	1.637	1.695		
		Concentration	1845	1879	1845	1899	1 867.00	26.68
18 °C	1	Absorbance	2.102	2.097	2.076	2.078		
		Concentration	2275	2271	2251	2253	2 262.50	12.26
	2	Absorbance	1.972	1.987	2.093	2.089		
		Concentration	2155	2169	2267	2263	2 213.50	59.76
	3	Absorbance	1.748	1.740	1.759	1.640		
		Concentration	1948	1940	1958	1848	1 923.50	50.87
	4	Absorbance	1.529	1.536	1.515	1.532		
		Concentration	1745	1751	1732	1748	1 744.00	8.37
	5	Absorbance	1.395	1.403	1.414	1.475		
		Concentration	1621	1628	1638	1695	1 645.50	33.73
	6	Absorbance	1.217	1.202	1.259	1.209		
		Concentration	1456	1442	1495	1449	1 460.50	23.70
RT	1	Absorbance	2.130	2.138	2.133	2.136		
		Concentration	2301	2309	2304	2307	2 305.25	3.50
	2	Absorbance	2.105	2.126	2.119	2.116		
		Concentration	2278	2298	2291	2288	2 288.75	8.30
	3	Absorbance	1.792	1.787	1.756	1.788		
		Concentration	1988	1984	1955	1985	1 978.00	15.43
	4	Absorbance	1.623	1.648	1.641	1.594		
		Concentration	1832	1855	1849	1805	1 835.25	22.40
	5	Absorbance	1.590	1.591	1.587	1.586		
		Concentration	1801	1802	1799	1798	1 800.00	1.83
	6	Absorbance	1.539	1.536	1.523	1.513		
		Concentration	1754	1751	1739	1730	1 743.50	11.09

Table B13: The absorbance and concentrations (µg/ml) of Reducing Sugars obtained through the DNS method under various concentrations of Zinc sulphate by a lager brewing strain (Used for Figure 2.1a) (Dilution factor = 10)

Conc.	Day		1	2	3	4	Average	SD
0.00	1	Absorbance	2.238	2.240	2.248	2.258		
		Concentration	2401	2403	2411	2420	2408.75	8.66
	2	Absorbance	2.035	2.033	2.046	2.041		
		Concentration	2213	2212	2224	2219	2217.00	5.60
	3	Absorbance	1.964	1.969	1.965	1.963		
		Concentration	2148	2152	2149	2147	2149.00	2.16
	4	Absorbance	1.802	1.799	1.817	1.816		
		Concentration	1998	1995	2012	2011	2004.00	8.76
	5	Absorbance	1.634	1.641	1.632	1.627		
		Concentration	1842	1849	1840	1836	1841.75	5.44
	6	Absorbance	1.574	1.572	1.586	1.587		
		Concentration	1787	1785	1798	1799	1792.25	7.27
0.03	1	Absorbance	2.249	2.253	2.258	2.275		
		Concentration	2412	2415	2420	2436	2420.75	10.69
	2	Absorbance	2.058	2.053	2.066	2.073		
		Concentration	2235	2230	2242	2249	2239.00	8.29
	3	Absorbance	1.788	1.794	1.776	1.778		
		Concentration	1985	1990	1974	1975	1981.00	7.79
	4	Absorbance	1.700	1.706	1.713	1.729		
		Concentration	1903	1909	1915	1930	1914.25	11.59
	5	Absorbance	1.610	1.617	1.590	1.577		
		Concentration	1820	1826	1801	1789	1809.00	17.07
	6	Absorbance	1.562	1.567	1.496	1.502		
		Concentration	1775	1780	1714	1720	1747.25	35.07
0.06	1	Absorbance	2.247	2.253	2.271	2.264		
		Concentration	2410	2415	2432	2425	2420.50	9.88
	2	Absorbance	2.005	2.004	1.994	1.988		
		Concentration	2186	2185	2175	2170	2179.00	7.79
	3	Absorbance	1.647	1.649	1.627	1.632		
		Concentration	1854	1856	1836	1840	1846.50	9.98
	4	Absorbance	1.613	1.617	1.599	1.611		
		Concentration	1823	1826	1810	1821	1820.00	6.98
	5	Absorbance	1.540	1.538	1.517	1.512		
		Concentration	1755	1753	1734	1729	1742.75	13.18
	6	Absorbance	1.435	1.436	1.370	1.356		
		Concentration	1658	1659	1598	1585	1625.00	39.05
0.12	1	Absorbance	2.235	2.248	2.220	2.219		
		Concentration	2399	2411	2385	2384	2394.75	12.82
	2	Absorbance	1.811	1.815	1.790	1.795		
		Concentration	2006	2010	1987	1991	1998.50	11.21
	3	Absorbance	1.429	1.427	1.395	1.406		
		Concentration	1652	1650	1621	1631	1638.50	15.02
	4	Absorbance	1.275	1.271	1.249	1.248		
		Concentration	1510	1506	1486	1485	1496.75	13.10
	5	Absorbance	1.192	1.179	1.145	1.140		
		Concentration	1433	1421	1389	1385	1407.00	23.66
	6	Absorbance	1.063	1.071	0.983	0.982		
		Concentration	1313	1321	1239	1238	1277.75	45.44

Table B14: The absorbance and concentrations (µg/ml) of Reducing Sugars obtained through the DNS method under various concentrations of L-leucine by a lager brewing strain (Used for Figure 2.2a) (Dilution factor = 10)

Conc.	Day		1	2	3	4	Average	SD
0.00	1	Absorbance	2.253	2.292	2.293	2.259		
		Concentration	2415	2451	2452	2421	2434.75	19.50
	2	Absorbance	2.076	2.078	2.091	2.090		
		Concentration	2251	2253	2265	2264	2258.25	7.27
	3	Absorbance	1.961	1.965	1.968	1.973		
		Concentration	2145	2149	2151	2156	2150.25	4.57
	4	Absorbance	1.636	1.641	1.636	1.637		
		Concentration	1844	1849	1844	1845	1845.50	2.38
	5	Absorbance	1.634	1.637	1.631	1.627		
		Concentration	1842	1845	1839	1836	1840.50	3.87
	6	Absorbance	1.482	1.487	1.486	1.481		
		Concentration	1701	1706	1705	1700	1703.00	2.94
0.25	1	Absorbance	2.249	2.253	2.256	2.255		
		Concentration	2412	2415	2418	2417	2415.50	2.65
	2	Absorbance	1.940	1.944	1.942	1.936		
		Concentration	2125	2129	2127	2122	2125.75	2.99
	3	Absorbance	1.745	1.751	1.752	1.746		
		Concentration	1945	1950	1951	1946	1948.00	2.94
	4	Absorbance	1.667	1.671	1.671	1.665		
		Concentration	1873	1876	1876	1871	1874.00	2.45
	5	Absorbance	1.535	1.536	1.541	1.544		
		Concentration	1750	1751	1756	1759	1754.00	4.24
	6	Absorbance	1.454	1.456	1.456	1.447		
		Concentration	1675	1677	1677	1669	1674.50	3.79
0.50	1	Absorbance	2.247	2.248	2.251	2.246		
		Concentration	2410	2411	2413	2409	2410.75	1.71
	2	Absorbance	2.221	2.224	2.216	2.217		
		Concentration	2386	2388	2381	2382	2384.25	3.30
	3	Absorbance	1.690	1.691	1.695	1.686		
		Concentration	1894	1895	1899	1890	1894.50	3.70
	4	Absorbance	1.601	1.605	1.604	1.600		
		Concentration	1812	1815	1814	1811	1813.00	1.83
	5	Absorbance	1.349	1.348	1.349	1.347		
		Concentration	1578	1577	1578	1576	1577.25	0.96
	6	Absorbance	0.992	0.997	1.004	0.995		
		Concentration	1248	1252	1259	1250	1252.25	4.79
0.75	1	Absorbance	2.188	2.189	2.194	2.193		
		Concentration	2355	2356	2361	2360	2358.00	2.94
	2	Absorbance	1.698	1.699	1.693	1.689		
		Concentration	1901	1902	1897	1893	1898.25	4.11
	3	Absorbance	1.321	1.328	1.321	1.322		
		Concentration	1552	1559	1552	1553	1554.00	3.37
	4	Absorbance	1.167	1.165	1.163	1.168		
		Concentration	1410	1408	1406	1411	1408.75	2.22
	5	Absorbance	1.107	1.107	1.109	1.103		
		Concentration	1354	1354	1356	1350	1353.50	2.52
	6	Absorbance	0.838	0.842	0.735	0.736		
		Concentration	1105	1109	1010	1011	1058.75	55.74

Table B15: The absorbance and concentrations (µg/ml) of Reducing Sugars obtained through the DNS method under various fermentation temperatures by a lager brewing strain (Used for Figure 2.3a) (Dilution factor = 10)

Temp.	Day		1	2	3	4	Average	SD
14 °C	1	Absorbance	2.025	2.022	2.022	2.030		
		Concentration	2204	2201	2201	2209	2203.75	3.77
	2	Absorbance	1.971	1.968	2.014	1.971		
		Concentration	2154	2151	2194	2154	2163.25	20.55
	3	Absorbance	1.817	1.821	1.903	1.900		
		Concentration	2012	2015	2091	2088	2051.50	43.91
	4	Absorbance	1.668	1.637	1.637	1.658		
		Concentration	1874	1845	1845	1864	1857.00	14.45
	5	Absorbance	1.431	1.428	1.398	1.394		
		Concentration	1654	1651	1624	1620	1637.25	17.73
	6	Absorbance	1.288	1.290	1.295	1.355		
		Concentration	1522	1524	1528	1584	1539.50	29.77
18 °C	1	Absorbance	1.973	1.971	1.971	1.964		
		Concentration	2156	2154	2154	2148	2153.00	3.46
	2	Absorbance	1.879	1.874	1.880	1.881		
		Concentration	2069	2064	2070	2071	2068.50	3.11
	3	Absorbance	1.684	1.679	1.679	1.685		
		Concentration	1888	1884	1884	1889	1886.25	2.63
	4	Absorbance	1.529	1.529	1.535	1.538		
		Concentration	1745	1745	1750	1753	1748.25	3.95
	5	Absorbance	1.313	1.314	1.311	1.322		
		Concentration	1545	1546	1543	1553	1546.75	4.35
	6	Absorbance	1.071	1.076	1.097	1.098		
		Concentration	1321	1325	1345	1346	1334.25	13.10
RT	1	Absorbance	1.817	1.821	1.821	1.817		
		Concentration	2012	2015	2015	2012	2013.50	1.73
	2	Absorbance	1.787	1.786	1.748	1.746		
		Concentration	1984	1983	1948	1946	1965.25	21.09
	3	Absorbance	1.495	1.529	1.529	1.501		
		Concentration	1713	1745	1745	1719	1730.50	16.92
	4	Absorbance	1.205	1.205	1.206	1.259		
		Concentration	1445	1445	1446	1495	1457.75	24.84
	5	Absorbance	1.065	1.069	1.098	1.074		
		Concentration	1315	1319	1346	1324	1326.00	13.83
	6	Absorbance	0.855	0.854	0.861	0.838		
		Concentration	1121	1120	1126	1105	1118.00	9.06

Table B16: The absorbance and concentrations (µg/ml) of Reducing Sugars obtained through the DNS method under various fermentation pH by a lager brewing strain (Used for Figure 2.4a) (Dilution factor = 10)

pH	Day		1	2	3	4	Average	SD
14 °C	1	Absorbance	2.129	2.130	2.138	2.134		
		Concentration	2300	2301	2309	2305	2303.75	4.11
	2	Absorbance	2.037	2.041	2.042	2.040		
		Concentration	2215	2219	2220	2218	2218.00	2.16
	3	Absorbance	1.960	1.961	1.956	1.957		
		Concentration	2144	2145	2140	2141	2142.50	2.38
	4	Absorbance	1.927	1.924	1.923	1.929		
		Concentration	2113	2111	2110	2115	2112.25	2.22
	5	Absorbance	1.809	1.810	1.815	1.816		
		Concentration	2004	2005	2010	2011	2007.50	3.51
	6	Absorbance	1.650	1.650	1.647	1.644		
		Concentration	1857	1857	1854	1851	1854.75	2.87
18 °C	1	Absorbance	2.126	2.119	2.126	2.116		
		Concentration	2298	2291	2298	2288	2293.75	5.06
	2	Absorbance	1.999	1.999	2.003	1.998		
		Concentration	2180	2180	2184	2179	2180.75	2.22
	3	Absorbance	1.787	1.783	1.785	1.782		
		Concentration	1984	1980	1982	1979	1981.25	2.22
	4	Absorbance	1.637	1.637	1.643	1.644		
		Concentration	1845	1845	1850	1851	1847.75	3.20
	5	Absorbance	1.591	1.591	1.593	1.593		
		Concentration	1802	1802	1804	1804	1803.00	1.15
	6	Absorbance	1.394	1.398	1.403	1.396		
		Concentration	1620	1624	1628	1622	1623.50	3.42
RT	1	Absorbance	2.139	2.140	2.152	2.144		
		Concentration	2310	2311	2322	2314	2314.25	5.44
	2	Absorbance	2.108	2.107	2.106	2.108		
		Concentration	2281	2280	2279	2281	2280.25	0.96
	3	Absorbance	1.783	1.783	1.784	1.788		
		Concentration	1980	1980	1981	1985	1981.50	2.38
	4	Absorbance	1.637	1.636	1.632	1.637		
		Concentration	1845	1844	1840	1845	1843.50	2.38
	5	Absorbance	1.587	1.589	1.586	1.577		
		Concentration	1799	1800	1798	1789	1796.50	5.07
	6	Absorbance	1.567	1.569	1.571	1.576		
		Concentration	1780	1782	1784	1788	1783.50	3.42

Table B17: Ethanol profiles produced under various concentrations of Zinc sulphate by an ale brewing strain (Used for Figure 3.1a)

Conc.	Day	1	2	3	4	Average	SD
0.00	1	61.84	62.00	60.30	60.05	61.05	1.01
	2	221.03	222.55	218.50	222.80	221.22	1.97
	3	332.48	314.54	342.59	326.67	329.07	11.71
	4	751.74	751.74	647.12	678.46	707.27	52.93
	5	911.21	912.22	894.28	877.60	898.83	16.37
	6	1012.30	1004.46	1012.30	1011.29	1010.09	3.78
0.03	1	78.75	80.77	73.19	68.64	75.34	5.50
	2	246.30	246.30	249.08	250.09	247.95	1.94
	3	401.47	400.46	397.18	393.64	398.19	3.54
	4	795.97	794.96	787.12	787.12	791.29	4.83
	5	956.45	935.47	944.06	944.06	945.01	8.63
	6	1088.22	1089.13	1088.22	1087.20	1088.19	0.78
0.06	1	136.12	136.37	135.87	135.11	135.87	0.55
	2	281.94	253.89	281.94	281.94	274.92	14.03
	3	641.56	640.04	640.80	645.60	642.00	2.48
	4	920.31	848.03	910.96	921.07	900.09	35.01
	5	1008.00	1008.76	979.19	1007.24	1000.80	14.42
	6	1172.02	1166.46	1174.04	1183.90	1174.10	7.27
0.12	1	195.00	196.77	194.50	193.23	194.88	1.47
	2	332.73	332.48	332.48	329.70	331.85	1.44
	3	875.83	869.76	871.53	873.81	872.73	2.65
	4	1063.85	1062.84	1063.60	1075.48	1066.44	6.04
	5	1137.90	1173.03	1138.00	1140.17	1147.28	17.20
	6	1263.75	1267.39	1266.79	1261.48	1264.85	2.76

Table B18: Ethanol profiles produced under various concentrations of L-leucine by an ale brewing strain (Used for Figure 3.2a)

Conc.	Day	1	2	3	4	Average	SD
0.00	1	102.63	102.63	102.51	102.59	102.59	0.06
	2	278.65	304.68	282.06	288.47	288.47	11.55
	3	582.42	584.95	584.29	583.89	583.89	1.07
	4	839.69	809.87	809.87	819.81	819.81	14.06
	5	921.32	910.96	912.73	915.00	915.00	4.53
	6	1009.01	1012.30	1012.30	1008.76	1010.59	1.97
0.25	1	135.11	147.74	127.53	136.79	136.79	8.34
	2	365.59	364.70	364.58	364.96	364.96	0.45
	3	759.58	766.91	759.58	762.02	762.02	3.45
	4	870.90	871.53	869.89	870.78	870.78	0.68
	5	936.37	936.48	936.86	936.57	936.57	0.21
	6	1040.10	1037.57	1062.84	1046.84	1046.84	11.36
0.50	1	152.90	152.92	152.90	152.91	152.91	0.01
	2	398.95	395.41	388.84	394.40	394.40	4.19
	3	792.43	792.18	806.08	796.90	796.90	6.49
	4	792.31	885.69	885.69	854.56	854.56	44.02
	5	978.46	960.77	987.91	975.71	975.71	11.25
	6	1120.21	1121.12	1120.97	1120.77	1120.77	0.40
0.75	1	228.97	228.97	237.23	223.84	229.75	5.54
	2	557.15	559.95	557.53	558.21	558.21	1.24
	3	986.02	987.96	1011.67	995.21	995.21	11.66
	4	1120.21	1087.99	1090.51	1099.57	1099.57	14.63
	5	1164.14	1147.88	1183.92	1165.32	1165.32	14.74
	6	1291.93	1292.77	1292.56	1292.69	1292.49	0.38

Table B19: Ethanol profiles produced under various fermentation temperatures by an ale brewing strain (Used for Figure 3.3a)

Temp.	Day	1	2	3	4	Average	SD
14 °C	1	127.88	127.88	128.11	127.96	127.96	0.11
	2	281.94	293.94	314.79	296.89	296.89	13.57
	3	542.92	533.09	542.11	539.37	539.37	4.46
	4	784.60	769.43	757.05	770.36	770.36	11.26
	5	895.09	893.72	893.47	894.09	894.09	0.71
	6	993.62	987.03	1006.26	995.64	995.64	7.98
18 °C	1	152.29	153.18	157.14	154.20	154.20	2.11
	2	362.81	362.13	380.63	368.52	368.52	8.57
	3	557.50	558.16	557.40	557.69	557.69	0.34
	4	784.62	734.08	755.48	758.06	758.06	20.71
	5	911.21	924.86	911.21	915.76	915.76	6.43
	6	1014.03	1040.05	1012.50	1022.19	1022.19	12.64
RT	1	169.86	178.32	178.47	196.77	180.86	11.35
	2	456.31	481.08	456.06	464.49	464.49	11.74
	3	701.71	544.87	696.65	647.74	647.74	72.77
	4	911.31	917.78	953.19	927.43	927.43	18.41
	5	1047.63	1047.43	1047.68	1047.58	1047.58	0.11
	6	1214.47	1214.50	1164.13	1197.70	1197.70	23.74

Table B20: Ethanol profiles produced under fermentation pH by an ale brewing strain (Used for Figure 3.4a)

pH.	Day	1	2	3	4	Average	SD
14 °C	1	72.18	51.96	53.20	59.11	59.11	9.25
	2	87.27	76.98	79.26	81.17	81.17	4.41
	3	205.14	210.95	232.91	216.33	216.33	11.96
	4	307.08	304.81	278.78	296.89	296.89	12.84
	5	481.69	480.58	475.52	479.26	479.26	2.68
	6	562.95	582.42	582.93	576.10	576.10	9.30
18 °C	1	127.78	149.01	127.53	134.77	134.77	10.07
	2	281.94	281.43	253.89	272.42	272.42	13.11
	3	567.76	557.15	542.64	555.85	555.85	10.30
	4	810.25	810.12	831.35	817.24	817.24	9.98
	5	911.21	922.86	957.71	930.59	930.59	19.76
	6	1021.52	1031.53	1022.43	1025.16	1025.16	4.52
RT	1	77.23	90.88	77.74	81.95	81.95	6.32
	2	155.36	157.47	154.67	155.83	155.83	1.19
	3	256.79	264.25	256.79	259.28	259.28	3.51
	4	382.87	376.45	379.99	379.77	379.77	2.63
	5	479.92	498.29	455.81	478.01	478.01	17.40
	6	542.24	557.40	594.55	564.73	564.73	21.98

Table B21: Ethanol profiles produced under various concentrations of Zinc sulphate by a lager brewing strain (Used for Figure 2.1a)

Conc.	Day	1	2	3	4	Average	SD
0.00	1	132.10	133.21	132.66	132.93	132.73	0.47
	2	307.31	326.90	317.10	322.00	318.33	8.36
	3	557.17	591.90	574.54	583.22	576.71	14.83
	4	822.96	895.14	859.05	877.09	863.56	30.82
	5	912.83	921.17	917.00	919.08	917.52	3.56
	6	1024.83	1024.20	1024.52	1024.36	1024.48	0.27
0.03	1	154.67	162.44	158.55	160.49	159.04	3.32
	2	364.32	383.38	373.85	378.62	375.04	8.14
	3	758.82	787.50	773.16	780.33	774.95	12.25
	4	888.72	837.01	862.86	849.94	859.63	22.08
	5	996.91	1013.94	1005.42	1009.68	1006.49	7.27
	6	1067.21	1082.25	1074.73	1078.49	1075.67	6.42
0.06	1	162.64	154.67	158.66	156.66	158.16	3.41
	2	339.84	355.23	347.53	351.38	348.49	6.57
	3	740.90	718.26	729.58	723.92	728.17	9.67
	4	887.45	922.18	904.82	913.50	906.99	14.83
	5	971.10	949.22	970.93	996.98	972.06	19.54
	6	1218.47	1044.75	1131.61	1088.18	1120.75	74.17
0.12	1	195.91	197.81	196.86	186.81	194.35	5.08
	2	565.01	595.08	580.05	522.88	565.75	31.11
	3	1004.49	971.64	952.51	1047.43	994.01	41.58
	4	1062.84	1065.62	1064.51	1152.05	1086.26	43.88
	5	1160.14	1140.28	1140.38	1256.45	1174.31	55.55
	6	1193.50	1215.06	1204.28	1292.69	1226.38	45.07

Table B22: Ethanol profiles produced under various concentrations of L-leucine by a lager brewing strain (Used for Figure 2.2a)

Conc.	Day	1	2	3	4	Average	SD
0.00	1	112.16	111.98	111.69	111.95	111.95	0.19
	2	278.75	282.04	281.81	280.87	280.87	1.50
	3	557.15	557.52	554.72	556.46	556.46	1.24
	4	794.81	792.94	794.71	794.15	794.15	0.86
	5	860.01	862.31	795.21	839.18	839.18	31.10
	6	999.54	993.65	961.27	984.82	984.82	16.82
0.25	1	129.60	129.43	130.52	129.85	129.85	0.48
	2	339.05	339.08	330.17	336.10	336.10	4.19
	3	759.07	795.21	786.49	780.26	780.26	15.40
	4	885.33	885.28	860.82	877.14	877.14	11.54
	5	918.56	919.70	919.07	919.11	919.11	0.47
	6	998.70	962.63	1006.37	989.23	989.23	19.07
0.50	1	131.49	131.96	129.59	131.02	131.02	1.03
	2	351.21	337.89	349.31	346.14	346.14	5.88
	3	740.93	764.99	733.88	746.60	746.60	13.32
	4	884.93	886.04	883.03	884.67	884.67	1.24
	5	961.00	968.40	961.35	963.58	963.58	3.41
	6	1089.63	1088.11	1088.27	1088.67	1088.67	0.68
0.75	1	236.35	235.97	238.75	237.02	237.02	1.23
	2	591.19	582.07	585.35	586.20	586.20	3.77
	3	1004.49	1003.78	931.97	980.08	980.08	34.02
	4	1131.08	1130.40	1116.30	1125.93	1125.93	6.81
	5	1185.08	1168.01	1165.56	1172.88	1172.88	8.68
	6	1157.59	1157.41	1166.57	1160.52	1160.52	4.28

Table B23: Ethanol profiles produced under various fermentation temperatures by a lager brewing strain (Used for Figure 2.3a)

Temp.	Day	1	2	3	4	Average	SD
14 °C	1	127.60	127.74	127.88	127.74	127.74	0.11
	2	314.54	315.65	313.93	314.71	314.71	0.71
	3	516.71	544.92	542.57	534.73	534.73	12.78
	4	769.43	797.23	784.85	783.84	783.84	11.37
	5	885.99	886.04	885.23	885.75	885.75	0.37
	6	994.66	995.37	986.42	992.15	992.15	4.06
18 °C	1	152.34	153.15	153.26	152.92	152.92	0.41
	2	367.89	359.54	359.41	362.28	362.28	3.97
	3	562.61	557.50	576.28	565.46	565.46	7.93
	4	715.61	709.08	709.29	711.33	711.33	3.03
	5	910.48	911.31	911.46	911.08	911.08	0.43
	6	1040.20	1047.50	1048.13	1045.28	1045.28	3.60
RT	1	169.78	169.81	161.39	166.99	166.99	3.96
	2	456.36	463.97	466.32	462.22	462.22	4.25
	3	693.62	684.09	700.54	692.75	692.75	6.74
	4	919.07	917.86	912.12	916.35	916.35	3.03
	5	1022.28	1023.39	1024.14	1023.27	1023.27	0.76
	6	1221.17	1233.71	1214.58	1223.15	1223.15	7.94

Table B24: Ethanol profiles produced under fermentation pH by a lager brewing strain (Used for Figure 2.4a)

pH	Day	1	2	3	4	Average	SD
3	1	61.84	61.94	61.21	61.67	61.67	0.33
	2	89.91	84.84	86.49	87.08	87.08	2.11
	3	236.83	236.58	235.46	236.29	236.29	0.59
	4	304.45	304.85	301.25	303.52	303.52	1.61
	5	480.58	480.94	481.69	481.07	481.07	0.46
	6	544.87	544.61	544.79	544.76	544.76	0.11
5	1	136.14	134.40	134.43	134.99	134.99	0.82
	2	281.71	282.15	278.75	280.87	280.87	1.51
	3	567.26	569.18	569.16	568.53	568.53	0.90
	4	821.09	810.27	810.24	813.87	813.87	5.11
	5	911.06	911.36	910.59	911.00	911.00	0.32
	6	1008.89	1021.51	1012.45	1014.28	1014.28	5.32
7	1	77.26	77.40	77.35	77.34	77.34	0.06
	2	153.15	163.51	153.08	156.58	156.58	4.90
	3	264.15	263.38	254.24	260.59	260.59	4.50
	4	382.17	377.34	380.61	380.04	380.04	2.01
	5	481.94	483.23	481.74	482.30	482.30	0.66
	6	562.59	565.89	578.54	569.01	569.01	6.87

Table B25: Yeast cell density throughout the fermentation period under different concentrations of Zinc sulphate by an ale brewing strain (Used for Figure 3.1b)

Sample	Day	1	2	3	4	Average
0.00	1	2.35×10^{6}	2.35×10^{6}	2.36×10^{6}	2.34×10^{6}	2.35×10^{6}
	2	3.85×10^{7}	3.85×10^{7}	3.86×10^{7}	3.84×10^{7}	3.85×10^{7}
	3	5.5×10^{10}	5.51×10^{10}	5.5×10^{10}	5.41×10^{10}	5.48×10^{10}
	4	2.68×10^{11}	2.69×10^{11}	2.67×10^{11}	2.72×10^{11}	2.69×10^{11}
	5	2.1×10^{9}	2.13×10^{9}	2.14×10^{9}	2.19×10^{9}	2.14×10^{9}
	6	4.57×10^{7}	4.59×10^{7}	4.55×10^{7}	4.61×10^{7}	4.6×10^{7}
0.03	1	2.46×10^{6}	2.46×10^{6}	2.41×10^{6}	2.47×10^{6}	2.45×10^{6}
	2	3.7×10^{7}	3.71×10^{7}	3.68×10^{7}	3.67×10^{7}	3.96×10^{7}
	3	6.52×10^{10}	6.51×10^{10}	6.52×10^{10}	6.53×10^{10}	6.52×10^{10}
	4	3.2×10^{11}	3.12×10^{11}	3.13×10^{11}	3.03×10^{11}	3.12×10^{11}
	5	3.54×10^{9}	3.54×10^{9}	3.56×10^{9}	3.56×10^{9}	3.55×10^{9}
	6	4.22×10^{7}	4.25×10^{7}	4.23×10^{7}	4.22×10^{7}	4.2×10^{7}
0.06	1	2.56×10^{6}	2.56×10^{6}	2.54×10^{6}	2.46×10^{6}	2.53×10^{6}
	2	3.04×10^{8}	3.04×10^{8}	3.02×10^{8}	3.1×10^{8}	3.05×10^{8}
	3	2.55×10^{11}	2.56×10^{11}	2.58×10^{11}	2.47×10^{11}	2.54×10^{11}
	4	2.21×10^{11}	2.2×10^{11}	2.13×10^{11}	2.34×10^{11}	2.22×10^{11}
	5	5.21×10^{9}	5.23×10^{9}	5.24×10^{9}	5.16×10^{9}	5.2×10^{9}
	6	4.23×10^{7}	4.21×10^{7}	4.2×10^{7}	4.28×10^{7}	3.3×10^{7}
0.12	1	3.55×10^{6}	3.56×10^{6}	3.58×10^{6}	3.67×10^{6}	6.59×10^{6}
	2	2.1×10^{9}	2.11×10^{9}	2.13×10^{9}	2.1×10^{9}	2.11×10^{9}
	3	5.42×10^{11}	5.43×10^{11}	5.46×10^{11}	5.37×10^{11}	5.42×10^{11}
	4	5.92×10^{11}	5.97×10^{11}	5.91×10^{11}	5.96×10^{11}	5.94×10^{11}
	5	7.42×10^{9}	7.43×10^{9}	7.44×10^{9}	7.35×10^{9}	7.4×10^{9}
	6	3.52×10^{7}	3.56×10^{7}	3.5×10^{7}	3.46×10^{7}	3.5×10^{7}

Table B26: Yeast cell density throughout the fermentation period under different concentrations of L-leucine by an ale brewing strain (Used for Figure 3.2b)

Sample	Day	1	2	3	4	Average
0.00	1	2.64×10^{6}	2.65×10^{6}	2.65×10^{6}	2.62×10^{6}	2.64×10^{6}
	2	3.6×10^{7}	3.61×10^{7}	3.58×10^{7}	3.53×10^{7}	3.58×10^{7}
	3	2.15×10^{10}	2.15×10^{10}	2.16×10^{10}	2.14×10^{10}	2.15×10^{10}
	4	3.26×10^{10}	3.24×10^{10}	3.24×10^{10}	3.26×10^{10}	3.25×10^{10}
	5	5.1×10^{9}	5.12×10^{9}	5.1×10^{9}	5.12×10^{9}	5.11×10^{9}
	6	2.64×10^{7}	2.65×10^{7}	2.65×10^{7}	2.62×10^{7}	2.64×10^{7}
0.25	1	3.12×10^{6}	3.1×10^{6}	3.12×10^{6}	3.1×10^{6}	3.11×10^{6}
	2	4.25×10^{7}	4.26×10^{7}	4.23×10^{7}	4.1×10^{7}	4.21×10^{7}
	3	5.69×10^{10}	5.68×10^{10}	5.62×10^{10}	5.77×10^{10}	5.69×10^{10}
	4	3.54×10^{11}	3.54×10^{11}	3.56×10^{11}	3.6×10^{11}	3.56×10^{11}
	5	5.21×10^{9}	5.21×10^{9}	5.23×10^{9}	5.15×10^{9}	5.2×10^{9}
	6	2.74×10^{7}	2.74×10^{7}	2.76×10^{7}	2.6×10^{7}	2.71×10^{7}
0.50	1	2.45×10^{7}	2.44×10^{7}	2.45×10^{7}	2.46×10^{7}	2.5×10^{7}
	2	3.96×10^{8}	3.96×10^{8}	3.95×10^{8}	3.93×10^{8}	3.95×10^{8}
	3	5.21×10^{11}	5.23×10^{11}	5.23×10^{11}	5.17×10^{11}	5.21×10^{11}
	4	4.99×10^{10}	4.98×10^{10}	4.98×10^{10}	5.01×10^{10}	4.99×10^{10}
	5	4.51×10^{9}	4.52×10^{9}	4.55×10^{9}	4.46×10^{9}	4.51×10^{9}
	6	1.33×10^{7}	1.34×10^{7}	1.36×10^{7}	1.25×10^{7}	1.32×10^{7}
0.75	1	3.34×10^{7}	3.34×10^{7}	3.35×10^{7}	3.29×10^{7}	3.3×10^{7}
	2	3.51×10^{9}	3.55×10^{9}	3.54×10^{9}	3.44×10^{9}	3.51×10^{9}
	3	5.35×10^{11}	5.36×10^{11}	5.34×10^{11}	5.31×10^{11}	5.34×10^{11}
	4	5.1×10^{10}	5.1×10^{10}	5.11×10^{10}	5.17×10^{10}	5.12×10^{10}
	5	4.09×10^{9}	4.08×10^{9}	4.1×10^{9}	4.13×10^{9}	4.1×10^{9}
	6	1.52×10^{7}	1.48×10^{7}	1.5×10^{7}	1.46×10^{7}	1.49×10^{7}

Table B27: Yeast cell density throughout the fermentation period under different fermentation temperatures by an ale brewing strain (Used for Figure 3.3b)

Sample	Day	1	2	3	4	Average
14 °C	1	2.7×10^{6}	2.71×10^{6}	2.68×10^{6}	2.67×10^{6}	2.69×10^{6}
	2	2.63×10^{7}	2.63×10^{7}	2.61×10^{7}	2.57×10^{7}	2.61×10^{7}
	3	1.51×10^{10}	1.5×10^{10}	1.5×10^{10}	1.53×10^{10}	1.51×10^{10}
	4	2.54×10^{11}	2.55×10^{11}	2.53×10^{11}	2.54×10^{11}	2.54×10^{11}
	5	4.75×10^{9}	4.78×10^{9}	4.78×10^{9}	4.69×10^{9}	4.8×10^{9}
	6	2.61×10^{7}	2.65×10^{7}	2.63×10^{7}	2.55×10^{7}	2.61×10^{7}
18°C	1	2.5×10^{6}	2.51×10^{6}	2.51×10^{6}	2.52×10^{6}	2.61×10^{6}
	2	2.78×10^{7}	2.78×10^{7}	2.89×10^{7}	2.67×10^{7}	2.51×10^{7}
	3	2.65×10^{11}	2.63×10^{11}	2.63×10^{11}	2.65×10^{11}	2.78×10^{11}
	4	2.48×10^{10}	2.45×10^{10}	2.43×10^{10}	2.44×10^{10}	2.45×10^{10}
	5	1.23×10^{10}	1.25×10^{10}	1.26×10^{10}	1.1×10^{10}	1.2×10^{10}
	6	2.78×10^{7}	2.74×10^{7}	2.47×10^{7}	3.13×10^{7}	2.78×10^{7}
RT	1	1.25×10^{7}	1.23×10^{7}	1.26×10^{7}	1.26×10^{7}	1.25×10^{7}
	2	4.13×10^{8}	4.13×10^{8}	4.15×10^{8}	4.07×10^{8}	4.12×10^{8}
	3	3.96×10^{11}	3.96×10^{11}	3.95×10^{11}	3.93×10^{11}	3.95×10^{11}
	4	3.98×10^{10}	3.95×10^{10}	3.94×10^{10}	4.01×10^{10}	3.97×10^{10}
	5	3.45×10^{9}	3.48×10^{9}	3.41×10^{9}	3.46×10^{9}	3.5×10^{9}
	6	1.82×10^{7}	1.82×10^{7}	1.82×10^{7}	1.78×10^{7}	1.81×10^{7}

Table B28: Yeast cell density throughout the fermentation period under different fermentation pH by an ale brewing strain (Used for Figure 3.4b)

Sample	Day	1	2	3	4	Average
3	1	2.52×10^{6}	2.5×10^{6}	2.5×10^{6}	2.56×10^{6}	2.52×10^{6}
	2	3.25×10^{6}	3.24×10^{6}	3.26×10^{6}	3.25×10^{6}	3.25×10^{6}
	3	7.13×10^{9}	7.13×10^{9}	7.14×10^{9}	7.08×10^{9}	7.12×10^{9}
	4	2.22×10^{9}	2.22×10^{9}	2.21×10^{9}	2.23×10^{9}	2.22×10^{9}
	5	3.54×10^{7}	3.55×10^{7}	3.51×10^{7}	3.56×10^{7}	3.54×10^{7}
	6	1.56×10^{5}	1.54×10^{5}	1.54×10^{5}	1.6×10^{5}	1.56×10^{5}
5	1	3.25×10^{6}	3.24×10^{6}	3.21×10^{6}	3.3×10^{6}	3.25×10^{6}
	2	4.15×10^{7}	4.15×10^{7}	4.12×10^{7}	4.18×10^{7}	4.2×10^{7}
	3	1.21×10^{10}	1.2×10^{10}	1.2×10^{10}	1.23×10^{10}	1.21×10^{10}
	4	3.45×10^{11}	3.46×10^{11}	3.42×10^{11}	3.59×10^{11}	3.48×10^{11}
	5	3.24×10^{9}	3.26×10^{9}	3.22×10^{9}	3.12×10^{9}	3.21×10^{9}
	6	1.12×10^{6}	1.1×10^{6}	1.1×10^{6}	1.16×10^{6}	1.12×10^{6}
7	1	1.5×10^{6}	1.53×10^{6}	1.52×10^{6}	1.53×10^{6}	1.52×10^{6}
	2	3.25×10^{7}	3.24×10^{7}	3.2×10^{7}	3.31×10^{7}	3.3×10^{7}
	3	1.22×10^{9}	1.23×10^{9}	1.25×10^{9}	1.18×10^{9}	1.22×10^{9}
	4	2.54×10^{10}	2.56×10^{10}	2.58×10^{10}	2.48×10^{10}	2.54×10^{10}
	5	5.2×10^{9}	5.22×10^{9}	5.23×10^{9}	4.79×10^{9}	5.11×10^{9}
	6	1.08×10^{6}	1.1×10^{6}	1.11×10^{6}	1.07×10^{6}	1.09×10^{6}

Table B29: Yeast cell density throughout the fermentation period under different concentrations of Zinc sulphate by a lager brewing strain (Used for Figure 2.1b)

Sample	Day	1	2	3	4	Average
0.00	1	8.52×10^{6}	8.49×10^{6}	8.52×10^{6}	8.51×10^{6}	8.51×10^{6}
	2	4.85×10^{7}	4.85×10^{7}	4.86×10^{7}	4.86×10^{7}	4.85×10^{7}
	3	8.41×10^{10}	8.4×10^{10}	8.41×10^{10}	8.42×10^{10}	8.41×10^{10}
	4	3.21×10^{11}	3.2×10^{11}	3.2×10^{11}	3.23×10^{11}	3.21×10^{11}
	5	5.6×10^{9}	5.59×10^{9}	5.59×10^{9}	5.54×10^{9}	5.58×10^{9}
	6	2.58×10^{7}	2.59×10^{7}	2.59×10^{7}	2.56×10^{7}	2.58×10^{7}
0.03	1	8.63×10^{6}	8.64×10^{6}	8.63×10^{6}	8.58×10^{6}	8.62×10^{6}
	2	4.56×10^{7}	4.55×10^{7}	4.55×10^{7}	4.58×10^{7}	4.56×10^{7}
	3	9.65×10^{11}	9.66×10^{11}	9.65×10^{11}	9.64×10^{11}	9.65×10^{11}
	4	4.95×10^{11}	4.96×10^{11}	4.95×10^{11}	4.94×10^{11}	4.95×10^{11}
	5	6.25×10^{9}	6.23×10^{9}	6.25×10^{9}	6.27×10^{9}	6.25×10^{9}
	6	2.68×10^{7}	2.67×10^{7}	2.67×10^{7}	2.7×10^{7}	2.68×10^{7}
0.06	1	2.5×10^{7}	2.51×10^{7}	2.53×10^{7}	2.58×10^{7}	2.63×10^{7}
	2	3.62×10^{8}	3.67×10^{8}	3.66×10^{8}	3.65×10^{8}	3.65×10^{8}
	3	4.54×10^{11}	4.55×10^{11}	4.56×10^{11}	4.51×10^{11}	4.54×10^{11}
	4	4.62×10^{10}	4.62×10^{10}	4.62×10^{10}	4.62×10^{10}	4.62×10^{10}
	5	7.89×10^{9}	7.89×10^{9}	7.89×10^{9}	7.89×10^{9}	7.89×10^{9}
	6	1.95×10^{7}	1.95×10^{7}	1.97×10^{7}	1.93×10^{7}	1.95×10^{7}
0.12	1	6.6×10^{7}	6.59×10^{7}	6.58×10^{7}	6.59×10^{7}	6.59×10^{7}
	2	2.9×10^{9}	2.92×10^{9}	2.92×10^{9}	2.9×10^{9}	2.91×10^{9}
	3	8.97×10^{11}	8.99×10^{11}	8.97×10^{11}	8.95×10^{11}	8.97×10^{11}
	4	4.97×10^{10}	4.97×10^{10}	4.95×10^{10}	4.91×10^{10}	4.95×10^{10}
	5	9.25×10^{9}	9.26×10^{9}	9.26×10^{9}	9.23×10^{9}	9.25×10^{9}
	6	1.34×10^{7}	1.35×10^{7}	1.3×10^{7}	1.29×10^{7}	1.32×10^{7}

Table B30: Yeast cell density throughout the fermentation period under different concentrations of L-leucine by a lager brewing strain (Used for Figure 2.2b)

Sample	Day	1	2	3	4	Average
0.00	1	3.15×10^{6}	3.15×10^{6}	3.12×10^{6}	3.14×10^{6}	3.14×10^{6}
	2	4.89×10^{7}	4.85×10^{7}	4.85×10^{7}	4.93×10^{7}	4.88×10^{7}
	3	4.42×10^{10}	4.45×10^{10}	4.43×10^{10}	4.46×10^{10}	4.44×10^{10}
	4	3.23×10^{10}	3.24×10^{10}	3.25×10^{10}	3.2×10^{10}	3.23×10^{10}
	5	5.6×10^{9}	5.61×10^{9}	5.6×10^{9}	5.55×10^{9}	5.59×10^{9}
	6	2.7×10^{7}	2.69×10^{7}	2.68×10^{7}	2.73×10^{7}	2.7×10^{7}
0.25	1	3.53×10^{6}	3.52×10^{6}	3.51×10^{6}	3.52×10^{6}	3.52×10^{6}
	2	4.51×10^{7}	4.51×10^{7}	4.52×10^{7}	4.66×10^{7}	4.55×10^{7}
	3	5.2×10^{10}	5.21×10^{10}	5.23×10^{10}	$5.\times10^{10}2$	5.21×10^{10}
	4	4.96×10^{11}	4.95×10^{11}	4.95×10^{11}	4.94×10^{11}	4.95×10^{11}
	5	5.26×10^{9}	5.23×10^{9}	5.2×10^{9}	5.31×10^{9}	5.25×10^{9}
	6	2.7×10^{7}	2.71×10^{7}	2.75×10^{7}	2.64×10^{7}	2.7×10^{7}
0.50	1	2.64×10^{7}	2.65×10^{7}	2.65×10^{7}	2.58×10^{7}	2.63×10^{7}
	2	3.64×10^{8}	3.61×10^{8}	3.62×10^{8}	3.73×10^{8}	3.65×10^{8}
	3	4.59×10^{11}	4.57×10^{11}	4.53×10^{11}	4.47×10^{11}	4.54×10^{11}
	4	4.94×10^{10}	4.95×10^{10}	4.96×10^{10}	5.03×10^{10}	4.97×10^{10}
	5	4.9×10^{9}	4.91×10^{9}	4.89×10^{9}	4.86×10^{9}	4.89×10^{9}
	6	1.3×10^{7}	1.29×10^{7}	1.25×10^{7}	1.36×10^{7}	1.3×10^{7}
0.75	1	3.46×10^{7}	3.41×10^{7}	3.42×10^{7}	3.51×10^{7}	3.45×10^{7}
	2	3.32×10^{9}	3.34×10^{9}	3.32×10^{9}	3.34×10^{9}	3.33×10^{9}
	3	4.01×10^{11}	4.01×10^{11}	3.99×10^{11}	3.87×10^{11}	3.97×10^{11}
	4	5×10^{10}	4.98×10^{10}	4.97×10^{10}	4.97×10^{10}	4.98×10^{10}
	5	4.26×10^{9}	4.23×10^{9}	4.22×10^{9}	4.29×10^{9}	4.25×10^{9}
	6	1.5×10^{7}	1.49×10^{7}	1.48×10^{7}	1.53×10^{7}	1.5×10^{7}

Table B31: Yeast cell density throughout the fermentation period under different fermentation temperatures by a lager brewing strain (Used for Figure 2.3b)

Sample	Day	1	2	3	4	Average
14°C	1	4.6×10^{6}	4.61×10^{6}	4.59×10^{6}	4.56×10^{6}	4.59×10^{6}
	2	5.01×10^{7}	5.02×10^{7}	4.99×10^{7}	4.94×10^{7}	4.99×10^{7}
	3	2.64×10^{10}	2.65×10^{10}	2.66×10^{10}	2.65×10^{10}	2.65×10^{10}
	4	3.2×10^{11}	3.21×10^{11}	3.22×10^{11}	3.17×10^{11}	3.2×10^{11}
	5	6.01×10^{9}	5.99×10^{9}	5.99×10^{9}	5.93×10^{9}	5.98×10^{9}
	6	2.5×10^{7}	2.49×10^{7}	2.47×10^{7}	2.58×10^{7}	2.51×10^{7}
18°C	1	2.91×10^{6}	2.91×10^{6}	2.94×10^{6}	2.84×10^{6}	2.9×10^{6}
	2	2.95×10^{7}	2.94×10^{7}	2.95×10^{7}	2.96×10^{7}	2.95×10^{7}
	3	2.53×10^{11}	2.52×10^{11}	2.54×10^{11}	2.45×10^{11}	2.51×10^{11}
	4	4.52×10^{10}	4.56×10^{10}	4.57×10^{10}	4.39×10^{10}	4.51×10^{10}
	5	1.55×10^{10}	1.56×10^{10}	1.58×10^{10}	1.47×10^{10}	1.54×10^{10}
	6	2.51×10^{7}	2.48×10^{7}	2.48×10^{7}	2.73×10^{7}	2.55×10^{7}
RT	1	2.28×10^{7}	2.24×10^{7}	2.2×10^{7}	2.12×10^{7}	2.21×10^{7}
	2	3.49×10^{8}	3.39×10^{8}	3.38×10^{8}	3.54×10^{8}	3.45×10^{8}
	3	4.5×10^{11}	4.51×10^{11}	4.56×10^{11}	4.51×10^{11}	4.52×10^{11}
	4	3.51×10^{10}	3.56×10^{10}	3.52×10^{10}	3.49×10^{10}	3.52×10^{10}
	5	4.6×10^{9}	4.6×10^{9}	4.58×10^{9}	4.58×10^{9}	4.59×10^{9}
	6	1.92×10^{7}	1.93×10^{7}	1.98×10^{7}	1.97×10^{7}	1.95×10^{7}

Table B32: Yeast cell density throughout the fermentation period under different fermentation pH by a lager brewing strain (Used for Figure 2.4b)

Sample	Day	1	2	3	4	Average
3	1	8.53×10^{5}	8.55×10^{5}	8.52×10^{5}	8.44×10^{5}	8.51×10^{5}
	2	4.83×10^{6}	4.82×10^{6}	4.86×10^{6}	4.89×10^{6}	4.85×10^{6}
	3	8.43×10^{9}	8.41×10^{9}	8.4×10^{9}	8.4×10^{9}	8.41×10^{9}
	4	3.21×10^{9}	3.2×10^{9}	3.2×10^{9}	3.23×10^{9}	3.21×10^{9}
	5	5.58×10^{7}	5.6×10^{7}	5.61×10^{7}	5.53×10^{7}	5.58×10^{7}
	6	2.57×10^{5}	2.59×10^{5}	2.56×10^{5}	2.6×10^{5}	2.58×10^{5}
5	1	5.41×10^{6}	5.43×10^{6}	5.44×10^{6}	5.4×10^{6}	5.42×10^{6}
	2	3.2×10^{7}	3.21×10^{7}	3.21×10^{7}	3.22×10^{7}	3.21×10^{7}
	3	4.1×10^{10}	4.12×10^{10}	4.09×10^{10}	4.09×10^{10}	4.1×10^{10}
	4	3.94×10^{11}	3.98×10^{11}	3.99×10^{11}	3.89×10^{11}	3.95×10^{11}
	5	2.84×10^{9}	2.85×10^{9}	2.86×10^{9}	2.85×10^{9}	2.85×10^{9}
	6	2.3×10^{6}	2.29×10^{6}	2.28×10^{6}	2.33×10^{6}	2.3×10^{6}
7	1	2.56×10^{5}	2.51×10^{5}	2.5×10^{5}	2.55×10^{5}	2.53×10^{5}
	2	3.59×10^{7}	3.64×10^{7}	3.61×10^{7}	3.76×10^{7}	3.65×10^{7}
	3	4.54×10^{9}	4.42×10^{9}	4.43×10^{9}	4.77×10^{9}	4.54×10^{9}
	4	4.62×10^{10}	4.63×10^{10}	4.66×10^{10}	4.57×10^{10}	4.62×10^{10}
	5	7.84×10^{9}	7.82×10^{9}	7.81×10^{9}	8.09×10^{9}	7.89×10^{9}
	6	1.95×10^{7}	1.94×10^{7}	1.98×10^{7}	1.93×10^{7}	1.95×10^{7}

Table B33: Colour profiles produced under various nutritional and fermentation conditions in an ale beer (Used for Table 3.1)

Sample	1	2	3	4	Average	Standard deviation
Zinc sulphate (g/l)						
0.00	0.654	0.651	0.659	0.657	0.655	0.00
0.03	0.652	0.651	0.655	0.655	0.653	0.00
0.06	0.649	0.659	0.659	0.651	0.655	0.01
0.12	0.648	0.643	0.649	0.649	0.647	0.00
L-leucine (g/l)						
0.00	0.669	0.647	0.649	0.649	0.654	0.01
0.25	0.664	0.664	0.664	0.669	0.665	0.00
0.50	0.674	0.671	0.675	0.674	0.674	0.00
0.75	0.679	0.682	0.682	0.689	0.683	0.00
Temperature (°C)						
14	0.661	0.665	0.662	0.654	0.661	0.00
18	0.659	0.655	0.671	0.677	0.666	0.01
RT (22.5)	0.615	0.326	0.635	0.334	0.478	0.17
pH						
3	0.658	0.659	0.648	0.647	0.653	0.01
5	0.651	0.649	0.642	0.648	0.648	0.00
7	0.649	0.644	0.629	0.629	0.638	0.01
Commercial beer	0.861	0.863	0.863	0.859	0.862	0.00

Table B34: Spent yeast density produced under various nutritional and fermentation conditions by an ale brewing strain (Used for Figure 3.5)

Sample	1	2	3	4	Average	Standard deviation
Zinc sulphate (g/l)						
0.00	2.124	2.129	2.122	2.219	2.149	0.05
0.03	2.235	2.291	2.265	2.255	2.262	0.02
0.06	2.481	2.482	2.899	2.724	2.647	0.20
0.12	2.849	2.841	2.847	2.855	2.848	0.01
L-leucine (g/l)						
0.00	2.187	2.185	2.176	2.177	2.181	0.01
0.25	2.365	2.325	2.359	2.365	2.354	0.02
0.50	2.487	2.484	2.445	2.511	2.482	0.03
0.75	2.511	2.547	2.598	2.551	2.552	0.04
Temperature (°C)						
14	2.201	2.204	2.215	2.219	2.210	0.01
18	2.441	2.454	2.441	2.441	2.444	0.01
RT (22.5)	2.712	2.766	2.755	2.552	2.696	0.10
pH						
3	1.988	1.983	1.982	1.986	1.985	0.00
5	2.128	2.215	2.154	2.144	2.160	0.04
7	1.954	1.945	1.995	2.124	2.005	0.08

Table B35: Foam head stability rating produced under various nutritional and fermentation conditions in an ale beer (Used for Table 3.1)

Sample	1	2	3	Average	Standard deviation
Zinc sulphate (g/l)					
0.00	3	2	2	2.33	0.58
0.03	3	2	2	2.33	0.58
0.06	2	2	2	2.00	0.00
0.12	2	2	2	2.00	0.00
L-leucine (g/l)					
0.00	3	2	2	2.33	0.58
0.25	3	2	2	2.33	0.58
0.50	3	2	2	2.33	0.58
0.75	3	3	2	2.67	0.58
Temperature (°C)					
14	3	2	2	2.33	0.58
18	3	2	2	2.33	0.58
RT (22.5)	2	2	1	1.67	0.58
pH					
3	2	1	1	1.33	0.58
5	3	2	2	2.33	0.58
7	2	2	1	1.67	0.58
Commercial beer	5	5	5	5.00	0.00

Table B36: Colour profiles produced under various nutritional and fermentation conditions in a lager beer (Used for Table 2.1)

Sample	1	2	3	4	Average	Standard deviation
Zinc sulphate (g/l)						
0.00	0.608	0.609	0.612	0.615	0.611	0.00
0.03	0.654	0.634	0.685	0.681	0.664	0.02
0.06	0.625	0.641	0.659	0.655	0.645	0.02
0.12	0.645	0.621	0.698	0.694		0.04
L-leucine (g/l)						
0.00	0.615	0.619	0.624	0.621	0.620	0.00
0.25	0.65	0.647	0.612	0.624	0.633	0.02
0.50	0.634	0.639	0.641	0.649	0.641	0.01
0.75	0.618	0.622	0.645	0.643	0.632	0.01
Temperature (°C)						
14	0.681	0.685	0.681	0.679	0.682	0.00
18	0.673	0.675	0.674	0.677	0.675	0.00
RT (22.5)	0.649	0.658	0.683	0.688	0.670	0.02
pH						
3	0.682	0.681	0.621	0.629	0.653	0.03
5	0.614	0.619	0.645	0.648	0.632	0.02
7	0.674	0.673	0.651	0.655	0.663	0.01
Commercial beer	0.861	0.863	0.863	0.859	0.862	0.00

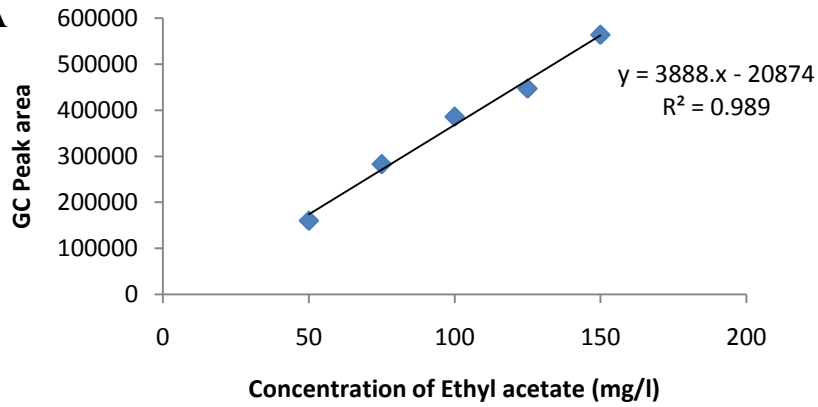
Table B37: Spent yeast density produced under various nutritional and fermentation conditions by a lager brewing strain (Used for Figure 2.5)

Sample	1	2	3	4	Average	Standard deviation
Zinc sulphate (g/l)						
0.00	2.451	2.549	2.514	2.455	2.492	0.05
0.03	2.715	2.791	2.722	2.744	2.743	0.03
0.06	2.788	2.854	2.891	2.892	2.856	0.05
0.12	3.142	3.111	3.654	3.523	3.358	0.27
L-leucine (g/l)						
0.00	2.586	2.543	2.584	2.302	2.504	0.14
0.25	2.675	2.781	2.649	2.644	2.687	0.06
0.50	2.719	2.842	2.015	2.162	2.435	0.41
0.75	2.914	2.945	2.965	2.811	2.909	0.07
Temperature (°C)						
14	2.492	2.52	2.503	2.469	2.496	0.02
18	2.943	2.971	3.005	3.022	2.985	0.04
RT (22.5)	3.145	3.154	3.158	3.102	3.140	0.03
pH						
3	2.314	2.149	2.245	2.019	2.182	0.13
5	2.518	2.516	2.489	2.473	2.499	0.02
7	2.458	2.126	2.014	2.51	2.277	0.24

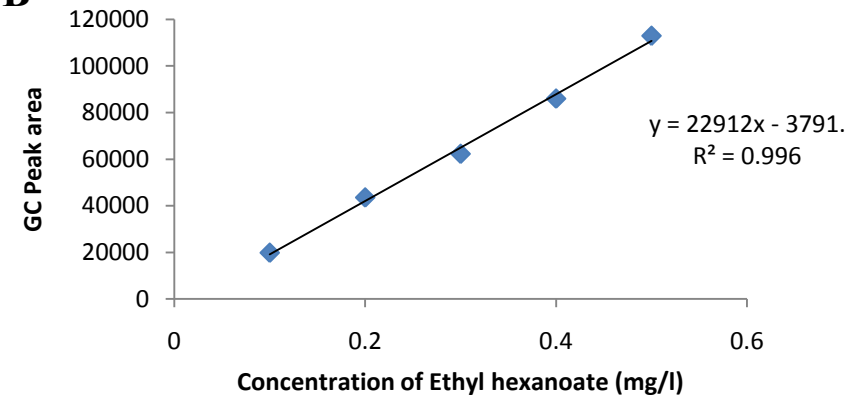
Table B38: Foam head stability rating produced under various nutritional and fermentation conditions in a lager beer (Used for Table 3.2)

Sample	1	2	3	Average	Standard deviation
Zinc sulphate (g/l)					
0.00	2	2	2	2.00	0.00
0.03	2	2	2	2.00	0.00
0.06	2	2	3	2.33	0.58
0.12	1	2	2	1.67	0.58
L-leucine (g/l)					
0.00	2	2	2	2.00	0.00
0.25	3	2	2	2.33	0.58
0.50	3	2	2	2.33	0.58
0.75	2	3	3	2.67	0.58
Temperature (°C)					
14	2	2	2	2.00	0.00
18	2	2	2	2.00	0.00
RT (22.5)	2	2	2	2.00	0.00
pH					
3	2	2	2	2.00	0.00
5	2	2	3	2.33	0.58
7	1	2	2	1.67	0.58
Commercial beer	5	5	5	5.00	0.00

A


600000
500000
400000
300000
200000
100000
0
GC Peak area
y = 3888.x - 20874
R² = 0.989
0
50
100
150
200
Concentration of Ethyl acetate (mg/l)

B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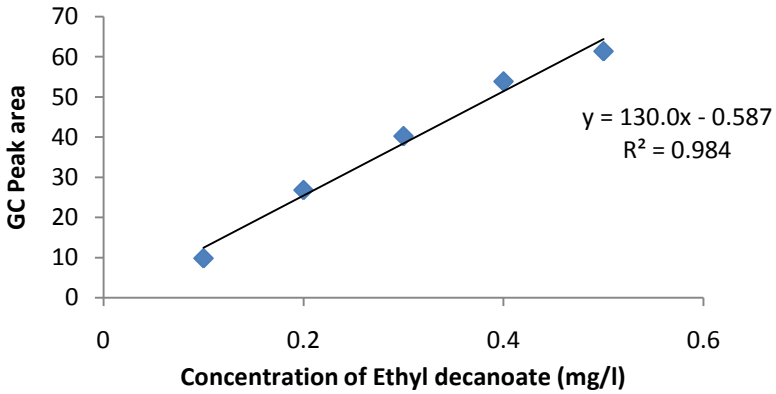
100000
80000
60000
40000
20000
0
GC Peak area
y = 22912x - 3791.
R² = 0.996
0
0.2
0.4
0.6
Concentration of Ethyl hexanoate (mg/l)

C


70
60
50
40
30
20
10
0
GC Peak area
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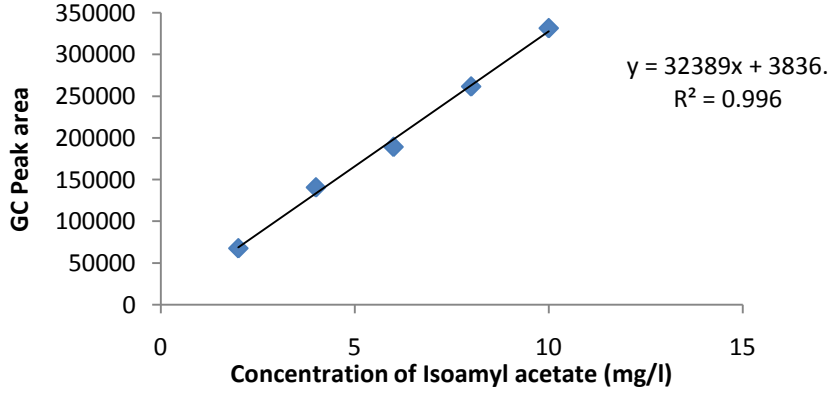
R² = 0.984
0
0.2
0.4
0.6
Concentration of Ethyl decanoate (mg/l)

D


350000
300000
250000
200000
150000
100000
50000
0
GC Peak area
y = 32389x + 3836.
R² = 0.996
0
5
10
15
Concentration of Isoamyl acetate (mg/l)

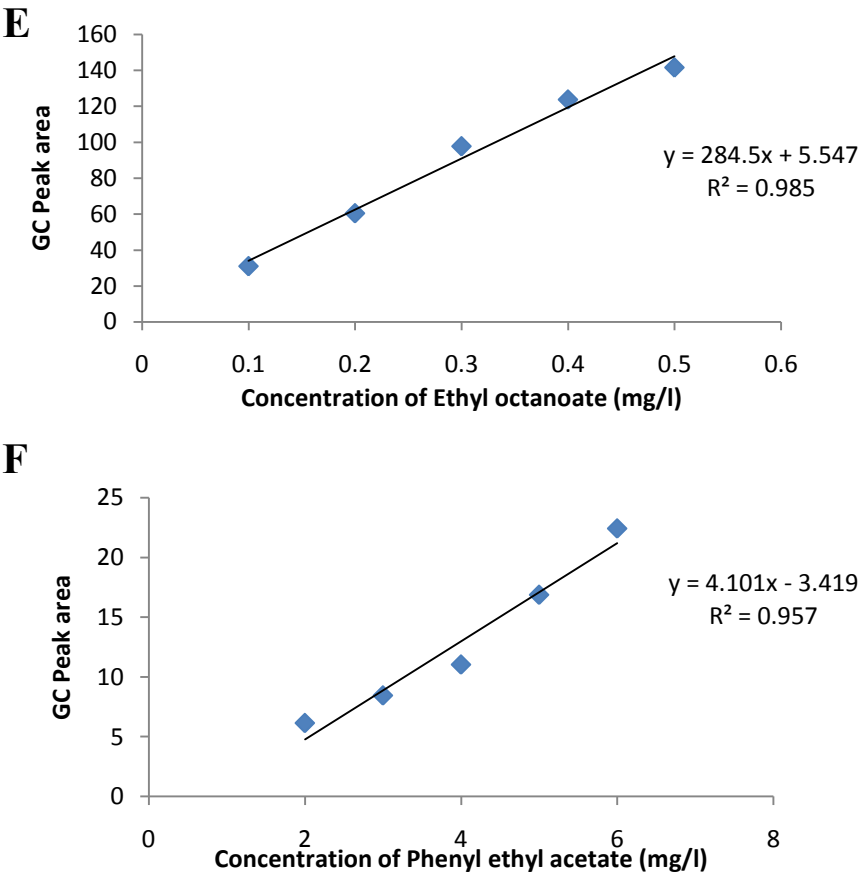


Figure B4: Standard curve used for the determination of a. Ethyl acetate, b. Ethyl hexanoate c. Ethyl decanoate d Isoamyl acetate. e. Ethyl octanoate and f. Phenyl ethyl acetate of the samples

Table B39: Gas chromatographic peak area values for esters concentrations in ale beer under various concentrations of Zinc sulphate (Used for Table 3.2 and Figure 3.6 a)

Sample	Ester					
	Ethyl acetate	**Isoamyl acetate**	**Phenyl ethyl acetate**	**Ethyl decanoate**	**Ethyl hexanoate**	**Ethyl octanoate**
0.00	113329.31	87989.50	5.25	253.77	54727.48	51.02
	109207.29	86020.25	5.16	253.89	52436.22	50.42
	107111.92	87536.05	5.10	249.35	57041.65	50.59
	122738.65	85365.99	5.11	260.77	54720.84	50.88
Average	113096.79	86727.95	5.16	254.45	54731.55	50.73
SD	6927.39	1238.32	0.07	4.72	1880.18	0.27
0.03	120431.29	131481.45	7.06	326.51	62380.29	53.47
	116053.03	131711.41	7.23	337.41	62520.06	51.79
	115815.96	130717.07	6.49	305.75	62222.19	52.50
	129456.82	130704.11	6.89	318.51	61188.83	55.66
Average	120439.27	131153.51	6.92	322.05	62077.84	53.36
SD	6375.22	520.01	0.32	13.34	605.03	1.68
0.06	133069.63	157823.42	6.50	456.57	57270.78	55.46
	133229.24	158558.65	6.71	467.45	63915.43	54.64
	132883.57	157729.49	6.25	456.53	61388.17	55.50
	132372.46	156654.18	6.09	485.05	46043.61	52.39
Average	132888.73	157691.44	6.39	466.40	57154.50	54.50
SD	372.04	784.61	0.28	13.46	7897.25	1.46
0.12	137431.33	208340.55	8.94	635.66	64579.90	58.51
	137152.42	208664.44	9.29	625.41	63690.89	58.06
	136777.90	210309.80	8.41	643.46	63663.39	57.40
	137896.49	202970.45	8.75	601.11	61555.44	56.54
Average	137314.54	207571.31	8.85	626.41	63372.40	57.63
SD	471.37	3186.11	0.37	18.42	1283.94	0.85

Table B40: Gas chromatographic peak area values for esters concentrations in ale beer under various concentrations of L-leucine (Used for Table 3.2 and Figure 3.6 b)

Sample	Ester					
	Ethyl acetate	Isoamyl acetate	Phenyl ethyl acetate	Ethyl decanoate	Ethyl hexanoate	Ethyl octanoate
0.00	101593.84	41235.98	14.81	329.29	55575.25	50.77
	101045.59	41239.22	14.62	333.20	54429.62	50.74
	101962.84	41018.97	14.62	326.43	55414.86	49.43
	101216.68	40840.83	15.15	325.00	56812.53	51.11
Average	101454.74	41083.75	14.80	328.48	55558.06	50.51
SD	408.90	191.96	0.25	3.61	977.73	0.74
0.25	105682.39	199180.94	17.72	301.04	56354.28	50.14
	105483.70	199138.83	17.74	301.95	56354.28	50.20
	105733.33	198623.85	17.91	300.65	58026.89	50.17
	105795.15	198325.87	17.44	295.94	57043.94	49.26
Average	101454.74	41083.75	14.80	328.48	55558.06	50.51
SD	408.90	191.96	0.25	3.61	977.73	0.74
0.50	106044.39	226313.20	19.52	547.82	54567.09	53.01
	106117.10	216765.25	19.52	553.42	55529.42	52.90
	106100.38	217111.49	19.56	545.93	53810.98	52.84
	105898.19	217578.86	19.41	541.04	53719.33	52.38
Average	106040.01	219442.20	19.51	547.05	54406.70	52.78
SD	99.53	4592.78	0.06	5.12	839.36	0.28
0.75	120858.42	266543.58	24.44	674.79	59539.13	53.90
	122026.47	268033.47	24.40	612.74	59332.91	53.98
	126043.08	267009.98	24.22	652.54	59653.69	53.67
	129958.99	264451.25	24.65	643.57	59312.29	53.01
Average	124721.74	266509.57	24.43	645.91	59459.51	53.64
SD	4137.81	1506.71	0.18	25.71	165.07	0.44

Table B41: Gas chromatographic peak area values for esters concentrations in ale beer under various fermentation temperatures (Used for Table 3.2 and Figure 3.6c)

Sample	Ester					
	Ethyl acetate	Isoamyl acetate	Phenyl ethyl acetate	Ethyl decanoate	Ethyl hexanoate	Ethyl octanoate
14 °C	90502.47	108938.71	12.39	152.19	55575.25	166.87
	90210.84	109262.60	12.23	152.32	54590.00	166.73
	90308.05	107950.84	12.23	151.89	55048.26	164.59
	90930.18	108938.71	12.63	151.76	57018.74	168.29
Average	90487.88	108772.71	12.37	152.04	55558.06	166.62
SD	318.81	568.79	0.19	0.26	1053.71	1.53
18 °C	98104.09	177162.89	20.69	299.25	54383.79	167.58
	97933.01	177732.94	20.83	300.03	55735.63	167.21
	98115.76	176178.27	20.43	285.72	53810.98	167.18
	98119.64	177396.10	20.66	300.42	52757.00	167.87
Average	98068.12	177117.55	20.65	296.35	54171.85	167.46
SD	90.32	668.48	0.16	7.11	1241.29	0.33
RT	118766.52	219741.47	18.50	561.54	49228.46	149.00
	118902.61	216917.15	18.41	562.11	50488.65	149.14
	115702.54	214850.74	18.38	567.21	50144.96	150.42
	121962.70	214996.49	18.64	554.57	46708.07	147.29
Average	118833.59	216626.46	18.48	561.36	49142.53	148.96
SD	2556.30	2280.19	0.11	5.19	1707.91	1.29

Table B42: Gas chromatographic peak area values for esters concentrations in ale beer under various fermentation pH (Used for Table 3.2 and Figure 3.6d)

Sample	Ester					
	Ethyl acetate	Isoamyl acetate	Phenyl ethyl acetate	Ethyl decanoate	Ethyl hexanoate	Ethyl octanoate
3	78359.30	46991.50	11.34	330.40	32204.39	90.21
	78398.19	48060.34	11.51	332.27	32502.26	84.37
	78430.85	46787.45	11.30	327.73	32410.61	89.72
	78234.88	45819.02	11.06	328.86	31585.75	95.98
Average	78355.80	46914.58	11.30	329.82	32175.75	90.07
SD	85.76	919.26	0.19	1.97	412.59	4.75
5	98368.50	80455.82	16.80	315.65	31929.44	130.59
	98500.70	80267.96	16.81	317.00	31814.88	130.39
	98436.54	80238.81	16.84	313.16	31700.32	130.42
	98051.99	80138.41	16.77	316.22	31471.19	130.53
Average	98339.43	80275.25	16.81	315.51	31728.96	130.48
SD	199.09	132.55	0.03	1.66	195.65	0.09
7	109271.29	124712.15	19.79	336.07	35664.20	131.92
	109185.75	124948.59	19.61	336.85	35824.59	132.41
	109286.84	131497.64	19.64	336.37	35618.37	131.18
	109536.08	123905.66	19.98	332.08	33441.68	132.10
Average	109319.99	126266.01	19.75	335.34	35137.21	131.90
SD	150.76	3516.22	0.17	2.20	1133.81	0.52

Table B43: Gas chromatographic peak area values for esters concentrations in lager beer under various concentrations of Zinc sulphate (Used for Figure 2.5)

Sample	Ester					
	Ethyl acetate	Isoamyl acetate	Phenyl ethyl acetate	Ethyl decanoate	Ethyl hexanoate	Ethyl octanoate
0.00	117579.40	55439.85	15.41	310.50	53.40	97509.89
	118862.11	51819.08	15.08	300.49	51.41	99821.77
	118749.78	52794.32	15.12	313.53	49.41	101425.65
	119484.32	53866.07	16.03	292.29	53.40	93156.49
Average	118668.90	53479.83	15.41	304.20	51.90	97978.45
SD	794.94	1551.22	0.44	9.70	1.91	3594.06
0.03	118771.94	85236.76	17.17	356.01	53.11	102264.25
	118901.38	82365.47	16.85	344.26	52.54	101175.90
	118622.32	80644.97	16.64	335.26	51.69	99736.99
	118706.62	73191.61	16.56	352.13	56.23	110116.40
Average	118750.56	80359.70	16.81	346.92	53.39	103323.39
SD	117.73	5140.42	0.27	9.18	1.98	4645.45
0.06	118706.66	94117.82	16.99	523.84	73.31	103487.78
	118784.73	92618.54	16.63	517.33	76.16	100946.78
	118746.59	90921.03	16.60	517.29	71.31	101249.22
	118629.75	95003.66	17.79	527.74	67.93	94535.83
Average	118716.93	93165.26	17.00	521.55	72.18	100054.90
SD	66.29	1790.96	0.56	5.15	3.46	3849.96
0.12	118500.11	87555.81	20.14	604.48	75.37	106473.30
	118383.50	125012.72	20.00	599.78	79.00	106908.64
	118616.02	116285.50	19.62	596.64	78.43	104841.92
	118374.79	170038.61	19.31	581.34	78.90	101785.38
Average	118468.60	124723.16	19.77	595.56	77.93	105002.31
SD	113.68	34186.64	0.38	10.01	1.72	2321.80

Table B44: Gas chromatographic peak area values for esters concentrations in lager beer under various concentrations of L-leucine (Used for Figure 2.6)

Sample	Ester					
	Ethyl acetate	Isoamyl acetate	Phenyl ethyl acetate	Ethyl decanoate	Ethyl hexanoate	Ethyl octanoate
0.00	121496.71	39315.63	14.19	261.06	57.10	99808.02
	114455.54	35942.00	14.35	258.45	49.43	96601.17
	118617.03	37893.76	13.93	244.16	48.95	94281.50
	120983.84	37473.67	11.54	327.80	49.13	90196.19
Average	118888.28	37656.27	13.50	272.87	51.15	95221.72
SD	3210.30	1388.28	1.32	37.37	3.97	4044.63
0.25	119966.04	152224.66	18.09	318.81	73.14	98731.13
	120054.69	179756.28	17.19	317.01	66.52	98217.89
	121671.25	188179.69	16.56	266.22	90.50	99730.12
	114845.50	109557.33	14.44	293.41	74.73	99134.39
Average	119134.37	157429.49	16.57	298.86	76.22	98953.38
SD	2964.73	35416.07	1.55	24.65	10.16	639.38
0.50	117601.02	162268.49	20.70	306.99	76.45	107909.92
	124060.77	205990.40	19.47	309.28	67.34	101201.11
	114536.80	206401.09	21.03	335.22	73.63	100717.65
	119511.30	166432.74	21.44	259.77	71.34	117906.68
Average	118927.47	185273.18	20.66	302.81	72.19	106933.84
SD	7811.44	25383.98	4.91	53.30	1.86	2882.28
0.75	119193.28	169807.68	27.53	396.18	77.87	111731.74
	114977.81	229951.78	24.76	415.95	78.44	110203.47
	115204.66	193381.69	22.05	331.40	75.90	106450.38
	131594.08	209041.12	16.03	302.78	80.42	113140.86
Average	120242.46	200545.57	22.59	361.58	78.16	110381.61
SD	7811.44	25383.98	4.91	53.30	1.86	2882.28

Table B45: Gas chromatographic peak area values for esters concentrations in lager beer under various fermentation temperatures (Used for Figure 2.7)

Sample	Ester					
	Ethyl acetate	Isoamyl acetate	Phenyl ethyl acetate	Ethyl decanoate	Ethyl hexanoate	Ethyl octanoate
14 °C	119238.11	42748.87	13.87	290.25	51.78	115844.55
	119000.57	44296.74	13.81	298.22	49.57	102087.82
	120093.53	43398.59	13.04	261.59	49.41	79131.69
	117866.55	44257.87	14.67	307.91	52.40	115047.19
Average	119049.69	43675.52	13.85	289.49	50.79	103027.81
SD	917.85	743.96	0.66	19.95	1.52	17133.23
18 °C	138057.71	80080.11	20.41	508.34	57.45	110865.64
	136901.72	78784.55	20.75	417.64	56.81	112238.11
	135256.97	80570.48	20.42	522.52	55.08	123808.97
	140275.60	112754.78	21.04	619.96	61.03	104406.35
Average	137623.00	88047.48	20.65	517.11	57.59	112829.77
SD	2109.00	16488.75	0.30	82.82	2.50	8076.81
RT	167572.83	182448.78	22.48	588.32	72.54	119868.00
	166674.44	151285.06	22.51	587.85	62.88	116295.93
	166561.29	151109.18	22.51	584.67	79.01	118403.89
	166806.83	187355.71	22.83	619.79	70.64	119682.41
Average	166903.85	168049.68	22.58	595.16	71.26	118562.56
SD	457.14	19562.63	0.17	16.50	6.64	1645.30

Table B46: Gas chromatographic peak area values for esters concentrations in lager beer under various fermentation pH (Used for Figure 2.8)

Sample	Ester					
	Ethyl acetate	**Isoamyl acetate**	**Phenyl ethyl acetate**	**Ethyl decanoate**	**Ethyl hexanoate**	**Ethyl octanoate**
3	121489.32	31417.25	14.83	306.93	52.42	45356.23
	119156.36	32798.64	17.01	301.39	49.79	45379.14
	111694.81	35618.11	13.85	298.74	45.99	35849.79
	117449.33	37598.69	12.00	308.13	52.68	39124.00
Average	117447.45	34358.17	14.42	303.80	50.22	41427.29
SD	4177.32	2779.06	2.09	4.47	3.11	4742.27
5	109328.23	53171.32	15.66	300.50	48.92	111252.86
	117606.03	54366.48	15.13	300.42	50.25	113065.25
	128605.45	50068.46	14.87	285.72	48.20	90677.35
	119096.34	56310.47	15.57	322.41	51.25	72301.44
Average	118659.01	53479.18	15.31	302.26	49.66	96824.23
SD	7901.37	2616.06	0.37	15.13	1.36	19244.98
7	117608.37	63112.16	19.97	361.16	56.74	97299.09
	113751.35	63509.89	19.91	362.32	47.89	101102.58
	125221.50	88702.38	19.48	348.16	49.42	97322.00
	117255.89	56634.36	19.55	372.50	61.50	96057.23
Average	118459.28	67989.70	19.73	361.04	53.89	97945.23
SD	4832.68	14163.55	0.25	9.98	6.38	2186.27

Table B47: Gas chromatographic peak area values for esters concentrations in ale beer stored at 4 °C (Used for Table 3.3)

Storage time (weeks)	Ester					
	Ethyl acetate	Isoamyl acetate	Phenyl ethyl acetate	Ethyl decanoate	Ethyl hexanoate	Ethyl octanoate
0	103108.33	44160.71	14.96	331.30	49.60	56331.36
	105837.92	44484.60	15.04	331.74	49.48	56170.97
	104162.06	44355.04	15.04	326.50	49.34	58118.55
	99227.81	42997.94	14.77	334.94	49.04	54360.88
Average	103084.03	43999.57	14.95	331.12	49.36	56245.44
SD	2805.77	680.89	0.13	3.48	0.24	1535.47
2	102983.91	43396.32	14.82	330.39	48.26	56102.24
	102983.91	43512.93	14.79	331.13	48.35	56125.15
	102953.19	43636.00	14.79	328.66	48.26	56125.15
	102956.30	42884.58	14.75	328.96	48.09	55712.72
Average	102969.33	43357.46	14.79	329.78	48.24	56016.31
SD	16.88	330.09	0.03	1.17	0.11	202.68
4	100734.53	43415.76	14.75	330.73	47.61	56102.24
	100650.93	43350.98	14.80	330.86	46.53	56423.01
	100709.25	43027.09	14.71	330.08	47.86	56033.50
	100761.74	43318.59	14.71	327.73	47.81	55712.72
Average	100714.11	43278.10	14.74	329.85	47.45	56067.87
SD	47.26	172.15	0.04	1.45	0.63	291.33
6	100619.04	43525.88	14.63	326.52	46.10	55781.46
	100596.88	43512.93	14.59	326.55	46.16	55506.51
	101028.09	43389.85	14.62	326.55	46.02	55712.72
	100145.84	42981.75	14.66	324.04	45.56	56102.24
Average	100597.46	43352.60	14.63	325.92	45.96	55775.73
SD	360.50	254.72	0.03	1.25	0.27	247.04
8	96936.44	43172.84	14.81	315.79	44.85	55116.99
	96908.21	43239.56	14.82	315.70	44.11	55185.73
	96614.87	43065.96	14.62	315.95	45.07	55121.58
	97154.80	42620.28	14.91	314.58	45.27	54958.90
Average	96903.58	43024.66	14.79	315.50	44.82	55095.80
SD	221.78	278.91	0.12	0.62	0.51	96.51
10	94025.27	41258.65	14.68	311.88	42.95	54819.13
	94040.82	41239.22	14.69	311.88	43.24	54727.48
	94056.37	41145.29	14.28	310.62	42.97	54434.20
	93819.19	40701.56	15.02	312.08	42.15	54569.38
Average	93985.41	41086.18	14.67	311.61	42.83	54637.55
SD	111.54	261.15	0.30	0.67	0.47	170.35
12	93886.06	40286.98	14.55	307.90	41.56	53513.11
	93885.29	40248.11	14.55	307.90	41.23	53288.57
	93886.06	40274.03	14.54	307.90	41.18	53157.97
	93819.19	39626.25	14.53	307.05	41.63	53077.77
Average	93869.15	40108.84	14.54	307.69	41.40	53259.36
SD	33.31	322.14	0.01	0.42	0.23	190.17

Table B48: Gas chromatographic peak area values for esters concentrations in ale beer stored at Room temperature (Used for Table 3.3)

Storage time (weeks)	Ester					
	Ethyl acetate	Isoamyl acetate	Phenyl ethyl acetate	Ethyl decanoate	Ethyl hexanoate	Ethyl octanoate
0	103090.84	43318.59	14.98	331.39	49.57	56340.53
	102712.12	44274.07	14.98	331.30	49.63	56042.66
	102945.03	44180.14	14.98	331.30	49.60	56594.86
	103590.48	44193.09	14.89	330.50	48.67	55930.39
Average	103084.62	43991.47	14.95	331.12	49.37	56227.11
SD	371.57	450.51	0.04	0.41	0.47	300.09
2	97555.84	43173.16	14.88	324.79	49.15	55804.37
	97534.11	43169.60	14.88	324.03	49.15	55653.15
	97534.46	43169.60	14.88	324.79	48.84	55534.00
	97474.19	42593.08	14.84	324.74	49.20	56125.15
Average	97524.65	43026.36	14.87	324.59	49.09	55779.17
SD	35.14	288.86	0.02	0.38	0.16	255.82
4	96612.54	42545.79	14.63	320.91	48.18	54429.62
	96609.04	42355.05	14.62	313.61	48.24	54436.49
	96610.98	42549.03	14.59	320.85	48.09	54383.79
	96538.66	42067.08	14.66	327.53	47.27	54360.88
Average	96592.81	42379.24	14.63	320.72	47.94	54402.69
SD	36.12	227.01	0.03	5.69	0.45	36.39
6	93633.32	40928.28	14.43	314.39	44.68	54431.91
	93644.21	40840.83	14.54	314.91	44.69	54454.82
	93632.55	40840.83	14.43	314.41	44.94	55689.81
	93632.55	40403.58	14.33	313.10	43.82	53054.86
Average	93635.66	40753.38	14.43	314.20	44.53	54407.85
SD	5.71	236.82	0.09	0.77	0.49	1076.54
8	89523.00	39529.08	14.14	306.58	42.95	53810.98
	89601.93	39305.59	14.18	306.32	42.58	53810.98
	89558.39	39276.44	14.10	305.28	42.95	54040.10
	89374.86	38427.85	14.12	307.40	42.82	53220.75
Average	89514.55	39134.74	14.13	306.40	42.83	53720.70
SD	98.56	484.58	0.04	0.87	0.17	350.37
10	84948.03	37682.91	13.87	302.82	41.24	50076.22
	84946.08	37505.41	13.85	302.68	41.31	50397.00
	84961.64	37514.48	13.90	302.63	41.52	50442.82
	84854.71	37384.28	13.81	303.16	40.61	49388.84
Average	84927.62	37521.77	13.86	302.82	41.17	50076.22
SD	49.09	122.73	0.04	0.24	0.39	486.41
12	81314.80	32630.22	13.16	283.66	35.28	46249.82
	83820.03	32824.56	13.17	282.92	35.46	45677.00
	81197.76	32468.28	13.17	282.44	35.40	45502.87
	78911.05	32698.89	13.09	282.93	34.46	47143.41
Average	81310.91	32655.48	13.15	282.99	35.15	46143.27
SD	2005.64	148.50	0.04	0.50	0.47	739.18

Table B49: Gas chromatographic peak area values for esters concentrations in lager beer stored at 4 °C (Used for Table 2.2)

Storage time (weeks)	Ester					
	Ethyl acetate	Isoamyl acetate	Phenyl ethyl acetate	Ethyl decanoate	Ethyl hexanoate	Ethyl octanoate
0	98191.58	38418.46	13.72	313.67	138.84	34905.79
	98233.96	38214.08	13.70	314.43	134.09	34239.03
	98284.51	38174.25	13.73	319.42	136.08	33228.82
	99123.41	37990.67	13.58	310.46	131.49	31647.62
Average	98458.36	38199.36	13.68	314.50	135.12	33505.31
SD	444.99	175.50	0.07	3.71	3.11	1417.41
2	98306.28	38126.96	13.60	310.84	134.20	33330.32
	98337.00	38108.50	13.58	311.08	132.41	34138.22
	98486.70	38070.93	13.63	302.51	137.27	33121.59
	98236.68	38014.57	13.43	316.30	131.36	33230.88
Average	98341.67	38080.24	13.56	310.18	133.81	33455.25
SD	105.40	49.60	0.09	5.70	2.59	463.22
4	98307.84	37997.08	13.50	308.79	136.42	34000.74
	98361.11	38129.68	13.48	308.31	132.43	33104.86
	98191.23	37861.69	13.54	308.61	133.63	33107.15
	98506.53	37597.40	13.39	307.62	135.35	32680.98
Average	98341.68	37896.46	13.48	308.34	134.46	33223.43
SD	130.81	227.42	0.06	0.51	1.77	555.59
6	97773.59	38149.31	13.45	293.35	136.22	31677.40
	97905.40	37886.96	13.45	292.99	132.24	32474.76
	97759.59	37914.16	13.40	290.10	131.38	31492.04
	97593.75	38154.49	13.45	329.95	131.17	33221.71
Average	97758.08	38026.23	13.43	301.60	132.75	32216.48
SD	127.74	145.55	0.03	18.95	2.36	794.28
8	93110.74	37601.93	13.31	274.19	126.06	30914.42
	95055.28	37595.78	13.29	273.28	125.36	30955.66
	93128.62	37945.26	13.21	275.78	125.89	30669.25
	91077.16	37561.45	13.25	271.85	124.97	30644.05
Average	93092.95	37676.10	13.26	273.78	125.57	30795.84
SD	1624.37	180.32	0.04	1.65	0.50	161.93
10	89841.07	36630.26	13.17	273.93	123.62	30151.43
	90545.63	36629.29	13.16	274.58	123.92	30241.01
	90029.26	36970.67	13.13	275.80	123.24	30099.64
	89979.88	36614.72	13.14	272.37	123.49	30280.19
Average	90098.96	36711.24	13.15	274.17	123.57	30193.07
SD	308.25	173.10	0.02	1.43	0.28	82.37
12	86627.00	36507.18	13.04	270.94	122.30	29939.25
	89114.34	37031.89	13.09	271.11	122.18	29973.62
	90558.46	36733.91	12.99	272.33	122.35	29693.17
	88960.21	36285.97	13.03	266.61	121.86	30306.31
Average	88815.00	36639.74	13.04	270.25	122.18	29978.09
SD	1626.62	319.05	0.04	2.50	0.22	251.95

Table B50: Gas chromatographic peak area values for esters concentrations in lager beer stored at Room temperature (Used for Table 2.2)

Storage time (weeks)	Ester					
	Ethyl acetate	Isoamyl acetate	Phenyl ethyl acetate	Ethyl decanoate	Ethyl hexanoate	Ethyl octanoate
0	98544.25	38164.53	13.71735	311.3571	136.3668	33945.75
	98558.25	38345.91	13.7532	309.1658	136.8676	34177.17
	98473.48	38215.38	13.62016	314.388	135.6297	34218.41
	98447.82	38159.35	13.69562	320.4627	133.9576	34353.6
Average	98505.95	38221.29	13.69658	313.8434	135.7054	34173.73
SD	53.65056	86.83932	0.056212	4.904881	1.271309	169.6409
2	96765.35	38126.96	13.5287	304.4629	131.1136	32580.16
	96908.05	38075.14	13.49741	305.2564	133.0945	32208.98
	96762.63	38091.01	13.55085	298.742	131.9559	31539.93
	96829.12	38042.42	13.44686	299.6499	128.5212	32571
Average	96816.29	38083.88	13.50595	302.0278	131.1713	32225.02
SD	68.45691	35.12676	0.045084	3.306777	1.944294	488.3413
4	96595.04	37851.33	13.30354	300.4695	130.3908	32505.01
	96453.51	37916.43	13.39381	305.6336	130.0322	31998.18
	96173.32	37848.41	13.2782	298.4285	126.3471	31817.17
	96338.8	37914.16	13.22267	297.4516	124.1559	31546.8
Average	96390.17	37882.58	13.29955	300.4958	127.7315	31966.79
SD	178.5572	37.80367	0.071339	3.648693	3.003725	403.9136
6	95763.72	37773.59	13.03717	300.0792	120.2715	31590.34
	95831.38	37805.01	13.08651	300.4695	123.2606	31319.97
	95390.06	37751.25	13.03713	296.9183	125.96	31136.67
	95312.29	37689.38	13.09028	295.2793	123.2771	31090.84
Average	95574.36	37754.81	13.06277	298.1865	123.1923	31284.45
SD	261.1288	48.87556	0.029626	2.506969	2.324034	226.6804
8	89602.32	37307.19	12.9921	274.1998	127.8553	27113.21
	89990.88	37405.66	12.98492	274.1991	125.0096	27248.4
	89219.33	37243.71	13.04148	274.3552	124.3921	26567.9
	89170.33	37353.83	12.97791	276.1965	123.7689	26554.15
Average	89495.72	37327.6	12.9991	274.7377	125.2565	26870.91
SD	382.4566	68.88331	0.028838	0.975333	1.805082	362.1079
10	88781.5	36589.45	12.74471	265.5144	122.1838	25491
	88834.93	36630.26	12.74516	266.2376	119.9841	24986.93
	88793.17	37025.41	12.37101	268.1186	122.2891	25697.22
	88831.66	37330.19	12.41744	263.7921	119.9841	24966.3
Average	88810.32	36893.83	12.56958	265.9157	121.1103	25285.36
SD	26.99175	351.1103	0.20337	1.791365	1.301118	366.4124
12	84487.65	35807.58	11.96503	257.2452	119.0052	22443.63
	84508.26	35646.93	11.79721	255.826	117.2152	21962.46
	84547.14	33389.42	11.70243	141.5169	114.0309	22665.88
	84438.31	33389.74	11.59076	141.3327	114.859	19708.55
Average	84495.34	34558.42	11.76386	198.9802	116.2776	21695.13
SD	45.32131	1351.25	0.158452	66.46181	2.264138	1356.536

APPENDIX C: Numerical data - Gene expression data

Table C1: Gene expression on Day 1 of fermentation by a lager yeast strain (Used for Figure 4.2)

Sample	Target	RQ	Ct	Ct Mean	Ct SD
Zinc	*atf1*	0	0 0 0	0	0
	atf2	0.217707	36.83834 0 0	36.83834	0
	eeb1	0	0 0 0	0	0
	eth1	0	0 0 0	0	0
L-leucine	*atf1*	0.035844	39.3867 0 0	39.3867	0
	atf2	0	0 0 0	0	0
	eeb1	0	0 0 0	0	0
	eth1	0.053747	37.57281 37.05653 36.67609	37.10181	0.450072
Temperature	*atf1*	0.023629	32.70694 33.10904 32.74051	32.85216	0.223092
	atf2	0.021952	30.27115 32.83391 34.16719	32.42408	1.980084
	eeb1	0	0 0 0	0	0
	eth1	0.004136	32.30318 32.35248 32.28065	32.3121	0.036734
pH	*atf1*	0.051816	33.37072 33.41482 34.47022	33.75192	0.622454
	atf2	0.054367	32.72094 33.18196 33.542	33.1483	0.411562
	eeb1	0	0 0 0	0	0
	eth1	0.006663	30.60646 28.93143 29.09764	29.54517	0.922849

Table C2: Gene expression on Day 2 of fermentation by a lager yeast strain (Used for Figure 4.2)

Sample	Target	RQ	Ct	Ct Mean	Ct SD
Zinc	*atf1*	0.011174	34.96846 35.3946 35.51911	35.29406	0.288765
	atf2	0.002469	38.59607 39.13743 0	38.86675	0.382801
	eeb1	0	0 0 0	0	0
	eth1	0.002675	31.25509 34.62466 32.86869	32.91615	1.685287
L-leucine	*atf1*	0.000727	32.19196 32.31683 31.70226	32.07035	0.324827
	atf2	0.003021	32.13242 29.11146 32.98275	31.40888	2.034545
	eeb1	0	0 0 0	0	0
	eth1	0.001465	31.45239 27.37191 28.9875	29.2706	2.054919
Temperature	*atf1*	0.00081	36.44676 35.70847 35.40848	35.85457	0.534337
	atf2	0.001079	37.76512 35.93069 36.80777	36.83453	0.917504
	eeb1	0	0 0 0	0	0
	eth1	0.002577	34.85283 32.54674 35.01393	34.13783	1.380279
pH	*atf1*	0.006979	26.68208 26.66103 26.74941	26.69751	0.046167
	atf2	0.011715	26.48544 28.0508 27.49686	27.34437	0.793743
	eeb1	0	0 0 0	0	0
	eth1	0.01168	26.56917 26.2477 26.91685	26.57791	0.334665

Table C3: Gene expression on Day 3 of fermentation by a lager yeast strain (Used for Figure 4.2)

Sample	Target	RQ	Ct	Ct Mean	Ct SD
Zinc	*atf1*	0	0 0 0	0	0
	atf2	68.94142	0 36.89851 36.51305	36.70578	0.272559
	eeb1	121.0231	35.84863 36.39804 36.55647	36.26771	0.37148
	eth1	0	0 0 0	0	0
L-leucine	*atf1*	0	0 0 0	0	0
	atf2	0.068816	29.91622 29.71015 29.66343	29.76327	0.134508
	eeb1	0.072545	29.48817 29.30941 29.36886	29.38881	0.091034
	eth1	0.055525	33.53792 33.35789 33.25384	33.38322	0.143723
Temperature	*atf1*	0	0 0 0	0	0
	atf2	30.81719	35.29373 35.45601 34.89166	35.2138	0.290543
	eeb1	23.51473	34.12033 35.19191 35.06234	34.79153	0.584872
	eth1	0	0 0 0	0	0
pH	*atf1*	0	0 0 0	0	0
	atf2	0.027866	29.5969 29.79239 29.8306	29.73997	0.125363
	eeb1	0.013411	30.23528 30.16823 30.36366	30.25572	0.099308
	eth1	0.053913	31.45881 32.48858 31.6241	31.85716	0.553034

Table C4: Gene expression on Day 4 of fermentation by a lager yeast strain (Used for Figure 4.2)

Sample	Target	RQ	Ct	Ct Mean	Ct SD
Zinc	*atf1*	0	0 0 0	0	0
	atf2	0.008945	0 38.07547	38.07547	0
	eeb1	0.007907	37.00875 36.00097 36.00443	36.33805	0.580845
	eth1	0	0 0 0	0	0
L-leucine	*atf1*	0	0 0 0	0	0
	atf2	3.649065	31.01338 33.33954	32.20977	1.164511
	eeb1	1.644938	32.2764 32.57042 32.23299	32.65767	0.474367
	eth1	1.395162	33.16961 38.77565 0	38.77565	0
Temperature	*atf1*	0	0 0 0	0	0
	atf2	0.000662	32.56981 33.31758	33.11757	0.480082
	eeb1	0.000526	33.4653 31.49265 31.68995	31.54314	0.129194
	eth1	0.001145	31.44681 36.44704 36.12088	36.29992	0.165407
pH	*atf1*	0	36.33185 0 0 0	0	0
	atf2	0.001012	37.03086 37.50613	37.31684	0.251955
	eeb1	0.001388	37.41352 35.28653 35.84848	35.35577	0.461989
	eth1	0.004647	34.93232 39.49326 0 0	39.49326	0

Table C5: Gene expression on Day 5 of fermentation by a lager yeast strain (Used for Figure 4.2)

Sample	Target	RQ	Ct	Ct Mean	Ct SD
Zinc	*atf1*	0	0 0 0	0	0
	atf2	0.684716	38.61165 34.54122 34.28405	35.81231	2.427711
	eeb1	2.729445	33.01244 33.54769 34.08792	33.54935	0.537745
	eth1	0	0 0 0	0	0
L-leucine	*atf1*	0	0 0 0	0	0
	atf2	461.9213	37.01956 35.65334 36.9524	36.54176	0.770133
	eeb1	2125.185	34.69714 34.46252 34.43162	34.53043	0.145204
	eth1	0	0 0 0	0	0
Temperature	*atf1*	918.5372	37.0373 36.38655 37.44255	36.95546	0.532738
	atf2	1090.127	30.91666 33.24672 30.86787	31.67708	1.359568
	eeb1	1190.917	31.73351 31.73017 31.45071	31.63813	0.162318
	eth1	1134.662	0 36.21521 0	36.21521	0
pH	*atf1*	1.711723	36.12906 36.28379 37.94195	36.78493	1.004986
	atf2	1.766169	31.71023 31.64176 31.77339	31.70846	0.065833
	eeb1	3.25932	30.46967 30.89784 30.958	30.77517	0.266279
	eth1	1.559287	35.96204 36.2906 36.78572	36.34612	0.414637

Table C6: Gene expression on Day 6 of fermentation by a lager yeast strain (Used for Figure 4.2)

Sample	Target	RQ	Ct	Ct Mean	Ct SD
Zinc	*atf1*	0	0 0 0	0	0
	atf2	0.547091	31.60223 31.82792 31.51417	31.64811	0.161828
	eeb1	0	0 0 0	0	0
	eth1	0	0 0 0	0	0
L-leucine	*atf1*	0	0 0 0	0	0
	atf2	1499.957	0 34.3283 35.66564	34.99697	0.945643
	eeb1	357.48	32.154 32.185 32.214	32.1843	0.03001
	eth1	0	0 0 0	0	0
Temperature	*atf1*	0	0 0 0	0	0
	atf2	0.181881	30.58888 30.97937 30.64008	30.73611	0.212222
	eeb1	0	0 0 0	0	0
	eth1	0	0 0 0	0	0
pH	*atf1*	0	0 0 0	0	0
	atf2	2.765815	33.52045 32.61877 32.24588	32.79503	0.655312
	eeb1	0	0 0 0	0	0
	eth1	0	0 0 0	0	0

Table C7: Gene expression on Day 1 of fermentation by an ale yeast strain (Used for Figure 4.1)

Sample	Target	RQ	Ct	Ct Mean	Ct SD
Zinc	*atf1*	0	0 0 0	0	0
	atf2	0	0 0 0	0	0
	eeb1	0	0 0 0	0	0
	eth1	0	0 0 0	0	0
L-leucine	*atf1*	0	0 0 0	0	0
	atf2	133.488	38.056 0 0	38.056	0
	eeb1	0	0 0 0	0	0
	eth1	0	0 0 0	0	0
Temperature	*atf1*	0	0 0 0	0	0
	atf2	27.11	30.2753 27.3504 29.8711	29.1676	1.5863
	eeb1	0	0 0 0	0	0
	eth1	0	0 0 0	0	0
pH	*atf1*	0	0 0 0	0	0
	atf2	0.227	35.8604 33.8462 34.3361	34.6809	1.0504
	eeb1	0	0 0 0	0	0
	eth1	0	0 0 0	0	0

Table C8: Gene expression on Day 2 of fermentation by an ale yeast strain (Used for Figure 4.1)

Sample	Target	RQ	Ct	Ct Mean	Ct SD
Zinc	*atf1*	0.049234	36.24457 0	36.24457	0
	atf2	0.030853	0 30.92659 37.08588	34.00623	4.355275
	eeb1	0.260949	0 37.0067 35.40503	34.80009	2.563181
	eth1	0.045461	31.98856 35.06006 34.48219	34.68883	0.32218
L-leucine	*atf1*	0	34.52425 0 0	0	0
	atf2	0.412687	0 38.49465	38.49465	0
	eeb1	3.316783	0 37.67506	37.67506	0
	eth1	1.286455	0 38.17628 34.642	36.40914	2.499113
Temperature	*atf1*	0.142431	0 36.8702	37.87643	1.423026
	atf2	0.232649	38.88266 36.20402 32.50548	34.25596	1.857169
	eeb1	1.585109	34.05839 0 34.50575	32.29403	3.127845
	eth1	0.390448	30.08231 31.84338 31.80703	31.6831	0.246805
pH	*atf1*	7.027467	31.39888 33.28298 0	35.17611	2.677286
	atf2	0.660443	37.06924 0 34.10518	35.67504	2.220115
	eeb1	3.316033	37.2449 0 30.6782	33.83761	4.468082
	eth1	0.566323	36.99702 31.92672 37.0514 32.28707	33.75506	2.860394

Table C9: Gene expression on Day 3 of fermentation by an ale yeast strain (Used for Figure 4.1)

Sample	Target	RQ	Ct	Ct Mean	Ct SD
Zinc	*atf1*	0.099922	35.4642 35.36776 0	35.41599	0.068193
	atf2	2.516442	33.64848 33.64848 0	33.64848	0
	eeb1	0	0 0 0	0	0
	eth1	3.270094	37.10038 36.86077 36.61404	36.8584	0.243175
L-leucine	*atf1*	0.008632	32.29404 33.24205 36.53952	34.0252	2.228458
	atf2	0.001303	39.90041 39.37881 0	39.63961	0.368826
	eeb1	0.393069	36.37738 36.29762 35.82709	36.16736	0.297372
	eth1	2.244098	32.52024 31.95719 32.11546	32.19763	0.29038
Temperature	*atf1*	0	0 0 0	0	0
	atf2	4.214391	31.2874 31.2874 0	31.2874	0
	eeb1	0.438394	34.58192 34.50512 33.77715	34.28807	0.444124
	eth1	2.487607	29.95376 30.44695 30.58074	30.32715	0.33021
pH	*atf1*	0.011278	0 34.72102 34.72102	34.72102	0
	atf2	0	0 0 0	0	0
	eeb1	0.41264	37.53457 37.09455 36.84552	37.15821	0.348908
	eth1	2.413916	33.63672 33.11731 32.8896	33.21454	0.382934

Table C10: Gene expression on Day 4 of fermentation by an ale yeast strain (Used for Figure 4.1)

Sample	Target	RQ	Ct	Ct Mean	Ct SD
Zinc	*atf1*	0	0 0 0	0	0
	atf2	37.30898	35.14628 39.64658	37.30898	1.255243
	eeb1	0.282885	37.13408 38.47092 38.66814	38.38475	0.334907
	eth1	0.151749	38.01517 35.43407 36.04065	35.73736	0.428919
L-leucine	*atf1*	33.96294	0 0 35.08602	33.96294	1.830919
	atf2	32.30389	32.83985 32.30389 0	32.30389	0
	eeb1	0.684925	0 33.9212 33.83316	34.01961	0.25059
	eth1	0.806585	34.30447 30.11082 0	30.23782	0.1796
Temperature	*atf1*	31.96491	30.36481 34.65836 39.27147	36.96491	1.809109
	atf2	39.1117	34.65836 0 0	39.1117	0
	eeb1	0.778406	39.1117 36.04348 0	35.89388	0.211565
	eth1	0.3328	35.74428 0 33.44752	33.57383	0.178633
pH	*atf1*	0	33.70015 0 0 0	0	0
	atf2	31.43486	32.22153 30.22052 31.86253	31.43486	1.066858
	eeb1	0.576174	32.9505 35.46728 32.9505	34.20889	1.77963
	eth1	0.630324	30.35993 30.70685 30.70685	30.53339	0.245309

Table C11: Gene expression on Day 5 of fermentation by an ale yeast strain (Used for Figure 4.1)

Sample	Target	RQ	Ct	Ct Mean	Ct SD
Zinc	*atf1*	0.004188	31.24776 31.03271 31.0743	31.11826	0.114065
	atf2	0	0 0 0	0	0
	eeb1	0.000903	30.09755 29.77514 30.18007	30.01759	0.213976
	eth1	0.015371	32.71636 32.48173 30.72354	31.97388	1.08916
L-leucine	*atf1*	0.490778	35.14753 35.50116 35.41363	35.35411	0.184172
	atf2	0	0 0 0	0	0
	eeb1	0.491255	31.75982 31.92178 31.83835	31.83998	0.080994
	eth1	0.381089	38.28132 38.06159 38.41169	38.25153	0.176943
Temperature	*atf1*	0.005848	34.8291 33.7178 33.96365	34.17019	0.583728
	atf2	0	0 0 0	0	0
	eeb1	0.000616	29.25021 29.82679 29.44939	29.5088	0.292846
	eth1	0.001517	35.58324 32.53619 34.63954	34.25299	1.559868
pH	*atf1*	0.012846	34.67805 33.39915 34.59531	34.22417	0.715687
	atf2	0	0 0 0	0	0
	eeb1	0.007619	32.32078 32.74937 32.8192	32.62979	0.269872
	eth1	0.013407	37.85963 37.85963 37.85963	37.85963	0

Table C12: Gene expression on Day 6 of fermentation by an ale yeast strain (Used for Figure 4.1)

Sample	Target	RQ	Ct	Ct Mean	Ct SD
Zinc	*atf1*	0.012481	34.26721 35.15979	34.68867	0.448359
	atf2	0	34.639 0 0	0	0
	eeb1	0.00348	0 32.46022 32.17818	32.16916	0.295672
	eth1	0	31.86908 0 0	0	0
L-leucine	*atf1*	0.010456	0 34.0116 33.77592	33.90765	0.120269
	atf2	0	33.93543 0 0	0	0
	eeb1	0.003754	0 29.64103 30.34947	29.82175	
	eth1	0	29.47476 0 0	0	0
Temperature	*atf1*	0.001855	0 35.40071 35.55472	35.45362	0.087585
	atf2	0	35.40543 0 0	0	0
	eeb1	0.001041	0 32.28758 32.236	32.51398	0.437565
	eth1	0	33.01836 0 0	0	0
pH	*atf1*	0.002069	0 36.71092 38.25815	38.1097	1.330774
	atf2	0	39.36002 0 0	0	0
	eeb1	0.006422	0 31.62356 28.99891	29.95048	1.453493
	eth1	0	29.22896 0 0	0	0

Table C13: Relative expression of *EEB1, EHT1, ATF2,* and *ATF1* genes involved in the synthesis of esters in an ale brewing yeast strain under the optimum fermentation conditions and nutritional supplementation (Used for Figure 4.1)

Gene	Sample	Gene expression (fold)					
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
ATF1	Zinc	0.000	0.049	0.100	0.000	0.042	0.012
	L-leucine	0.000	0.000	0.009	0.000	0.491	0.010
	Temp.	0.000	0.142	0.000	0.000	0.006	0.002
	pH	0.000	7.070	0.011	0.000	0.013	0.002
ATF2	Zinc	0.000	0.031	2.516	0.359	0.000	0.000
	L-leucine	133.488	0.413	0.000	55.072	0.000	0.000
	Temp.	27.110	0.233	4.414	37.303	0.000	0.000
	pH	0.227	0.660	0.000	7.733	0.000	0.000
EEB1	Zinc	0.000	0.261	0.000	0.283	0.001	0.003
	L-leucine	0.000	3.317	0.393	0.685	0.491	0.004
	Temp.	0.000	1.585	0.438	0.778	0.006	0.001
	pH	0.000	3.316	0.413	0.576	0.008	0.006
EHT1	Zinc	0.000	0.045	3.270	0.152	0.015	0.000
	L-leucine	0.000	1.286	2.244	0.807	0.381	0.000
	Temp.	0.000	0.390	2.438	0.333	0.002	0.000
	pH	0.000	0.566	2.314	0.630	0.013	0.000

Table C14: Relative expression of *EEB1, EHT1, ATF2,* and *ATF1* genes involved in the synthesis of esters in a lager brewing yeast strain under the optimum fermentation conditions and nutritional supplementation (Used for Figure 4.2)

Gene	Sample	Gene expression (fold)					
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
ATF1	Zinc	0.000	0.011	0.000	0.000	0.000	0.000
	L-leucine	0.036	0.001	0.000	0.000	0.000	0.000
	Temp.	0.024	0.001	0.000	0.000	918.537	0.000
	pH	0.052	0.007	0.000	0.000	1.712	0.000
ATF2	Zinc	0.218	0.002	68.941	0.009	0.685	0.547
	L-leucine	0.000	0.000	0.069	3.649	461.921	1499.957
	Temp.	0.022	0.001	30.817	0.001	1090.127	0.182
	pH	0.054	0.012	0.028	0.001	1.766	2.766
EEB1	Zinc	0.000	0.000	121.023	0.008	2.729	0.071
	L-leucine	0.000	0.000	0.073	1.645	2125.185	357.480
	Temp.	0.000	0.000	23.515	0.001	1190.127	0.022
	pH	0.000	0.000	0.013	0.001	1.766	0.481
EHT1	Zinc	0.000	0.003	0.000	0.000	0.000	0.000
	L-leucine	0.054	0.001	0.056	1.395	0.000	0.000
	Temp.	0.004	0.002	0.000	0.001	1134.662	0.000
	pH	0.088	0.012	0.054	0.004	1.559	0.000