THE IMPACT OF YEAST NUTRITION ON FLAVOUR FORMATION DURING FERMENTATION

By

Normando Mendes Ribeiro Filho

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

Division of Food Science

School of Bioscience

University of Nottingham

ACKNOWLEDGEMENTS

First, I am extremely thankful for universal energy, which created and guides everything.

I would also like to thank all Brazilian people who pay their taxes, which support my scholarship/fees by CAPES/Brazilian education ministry.

I am grateful to at Federal University of Paraiba/Brazil for their support over this last years.

I would also like to acknowledge, and thank Prof Ian Fisk and Dr Chris Powell for the supervision and guidance. I am very thankful.

Thanks are also due to Dr Rob Linforth, Dr Darren Greetham, Dr Ayed Charfedinne, Dr Sarah Nicholls, Melanie Stuart, Ms. Sharon Lim Mui Ting, Ms. Khatija Nawaz Husain, Ms. Stephanie Brindley and the magic Jenny Drury for technical assistance and patient explanations regarding their professional fields.

Indeed, I am grateful to all those members of flavour group and yeast group, past and present, who have assisted me.

I would like to thank Bárbara and Henrique, niece and nephew, because on the top of their innocence they brought me faith

Finally, I would like to thank all my family and friends for their support and total understanding my absence over the last years. People, I love you all!

"The suffering of today is the
enjoyment of tomorrow"

(Cristina Garcia)

ABSTRACT

This thesis generates a knowledge about the role of yeast nutrition on flavour formation during fermentation, specifically related to the influence of essential inorganic elements on yeast-flavour formation. Two yeast nutritional influences, which are total carbohydrates and essential inorganic elements, were considered. Therefore, data were collected to cover five steps, which are divided in 5 result chapters: a characterization of yeast strains (Chapter 3); a more complete understanding of yeast flavour formation (Chapter 4); a new understanding of the role of the essential inorganic element and their effect on yeast-flavour formation during supplemented fermentations (Chapter 5); a creation of a more complete synthetic sweet wort as a tool for fermentation studies (Chapter 6); and finally, a knowledge of the role of inorganic-phosphate, potassium, magnesium as independent variables (input) on flavour formation as dependent variables (output) during alcoholic fermentation using RSM (Chapter 7). All flavour compounds were measured using high-performance liquid chromatography (carbohydrates, ethanol, glycerol and acetic acid) and gas chromatography-mass spectrometry (aromas). Results suggested that alcoholic fermentation can be divided into two domain phases, which are carbohydrates domain and ethanol domain. Carbohydrates phase occurs from the inoculation time to when ethanol concentration became higher than dissolved sugars in wort. During carbohydrate phase, acetic acid was accumulated; in contrast, during ethanol phase, acetic acid was consumed. Acetate esters and fatty acid esters are influenced by carbohydrates domain and ethanol domain; however, most of esters were produced under carbohydrates domain (16h). Afterwards, when singular or a complex mix of eight different essential inorganic elements were added during fermentation, ammonia-nitrogen, inorganic phosphate, potassium and magnesium significantly increased the production of target industrial compounds (ethanol and glycerol) and decreased acetic acid; furthermore increased the formation of higher alcohols and esters. Copper, iron, manganese or a composite mixture of all nutrient supplementations influenced negatively flavour formation. Zinc had less impact on flavour formation. Subsequently, a synthetic sweet wort was developed as a more controllable medium for fermentation studies. To evaluate the interactions among inorganic-phosphate, potassium and magnesium, a RSM was applied. Observed that ethanol is magnesium dependent, yeast growth is potassium/magnesium dependent, and supplementation level of inorganic phosphate, potassium and/or magnesium increase the flavour formation without affect the yeast growth. In conclusion, magnesium is the most important inorganic element for flavour formation (including the formation of ethanol, higher alcohols, acetate esters/fatty acid esters, and off-flavour reduction). Magnesium interacts with phosphorous and potassium as all together acts as important co-factor for fermentation and yeast-flavour formation.

Table of Contents

List of Figures

Fig. 7.7: Principle component analysis (Bio-Plot) to identify the correlation and similarities among all 27 variables including ethanol, glycerol, acetic acid, higher alcohols, fatty acid, acetate esters and fatty acid ethyl esters. Fermentation were conducted in a synthetic sweet wort (SW) using a *Saccharomyces cerevisiae* strain (NCYC2592). All treatments applied by a response surface method: T1 (500, 180 and 116.8 mg/L); T2 (500, 1000 and 160 mg/L); T3 (500, 0 and 0 mg/L); T4 (500, 650 and 32 mg/L); T5 (500, 1000 and 0 mg/L); T6 (500, 0 and 160 mg/L); T7 (500, 1000 and 103.7 mg/L); T8 (500, 515 and 160 mg/L); T9 (500, 0 and 55.2 mg/L); T10 (157.5, 1000 and 160 mg/L); T11 (157.5, 750 and 0 mg/L); T12 (157.5, 250 and 160 mg/L); T13 (157.5, 0 and 21.6 mg/L); T14 (0, 600 and 160 mg/L); T15 (0, 1000 and 84.8 mg/L); T16 (0, 1000 and 0 mg/L); T17 (0, 0 and 0 mg/L); T18 (0, 415 and 24 mg/L); T19 (0, 0 and 160 mg/L); T20 (0, 1000 and 160 mg/L); T21 (0, 0 and 96.8 mg/L); T22 (350, 755 and 132 mg/L); T23 (350, 1000 and 36 mg/L); T24 (350, 300 and 0 mg/L); T25 (350, 0 and 160 mg/L); T26 (250, 500 and 80 mg/L); T27 (250, 500 and 80 mg/L); and T28 (250, 500 and 80 mg/L).

List of Table

List of equations

CHAPTER I:
INTRODUCTION

1. CHAPTER I

1.1. INTRODUCTION

Large brewing companies have been instrumental in increasing the understanding of the brewing process and key outputs, such as the formation of flavours by yeast. Flavour is a very important attribute in the majority of food products, due to its power in defining brands and in driving consumer acceptance. Alcoholic beverages are classed as food products and, as expected, flavour acts as an important attribute. Beer is the most popular alcoholic beverage consumed worldwide (De Keukeleire 2000); traditionally beer is produced using four essential ingredients: malted barley, water, hops and yeast (Briggs *et al.*, 2004). The brewing process involves several physical, chemical and biochemical transformations, which convert wort to beer (Masschelein, 1997).

Brewery yeasts belong to the genus *Saccharomyces*, members of which are important for food, alcoholic beverages, fermented products and pharmaceutical industries. In the brewery, yeast utilise wort as the primary substrate, which supports yeast growth and survival due to its physical and nutritional characteristics. During fermentation, 90-93% of wort sugars are converted into alcohol, glycerol and CO_2, which can then be converted into other end products, including organic acids, esters, aldehydes and higher alcohol (Ingledew *et al.* 2009, Gibson *et al.* 2007). To maintain physiological activities, yeasts have developed sophisticated homeostatic systems, which provide the mechanism of cellular control against environmental stress, which

includes nutritional stress. Environmental stress involves important physical requirements of yeasts such as hydrogen-ionic potential (pH), temperature, oxygen and osmotic stress. These last two physical stresses influence metabolic activities due to their relationship with the Pasteur Effect and the Crabtree effect, respectively. Nutritional stress is related to the metabolic activity of yeast; this thesis focuses on carbon source and essential inorganic elements, due to their impact on flavour formation. Carbon, nitrogen, phosphorus, potassium, magnesium, copper, zinc, iron manganese, hydrogen and oxygen are important for living organisms because they require these multiple chemical elements for growth and maintenance (Canadell *et al.* 2014). In the brewery, the composition of wort compounds are important variables in producing a good quality beer. However, sugars (carbon source), amino acids and essential inorganic elements are important for yeast growth and fermentation control, and as a consequence they can influence the characteristics of the final product in terms of flavour production.

Brewery fermentation is a bio-process, which involves several key metabolic pathways, including glycolysis and routes to flavour formation. These pathways involve multiple independent and dependent variables. Although analysis of fermentation using one-variable-at-a-time analysis has typically been applied for many years, it has several disadvantages (Myers *et al.* 2009, Bezerra *et al.* 2008, Barros Neto *et al.* 2003). Firstly, it increases the necessary number of experiments, experimental time and the consumption of materials/reagents, and as a result it increases the cost of experiments and process. Secondly, it does not include the interactive effects between the input variables, and as a consequence, does not describe the effects of input variables

on the response (Khuri and Mukhopadhyay 2010, Bezerra *et al.* 2008). In order to overcome this problem, process optimization studies have used multivariate methods including Principal Component Analysis (PCA) and response surface methodology (RSM) (Bezerra *et al.* 2008, Bas & Boyacı 2007, Barros Neto *et al.* 2003).

This chapter presents an overview of the brewing process including: Environmental stress (pH, temperature, oxygen and osmotic pressure), metabolic effects (Pasteur Effect and Crabtree effect), and nutritional stress (carbon, nitrogen, phosphorus, potassium, magnesium, copper, zinc, iron manganese, hydrogen and oxygen) and their effects on flavour formation. Furthermore, it introduces the multivariate methods Principal Component Analysis (PCA) to describe the effects of output variables and response surface methodology (RSM) to describe the effects of input variables. All these key concepts are explained for an understanding and contextualization of this thesis. In summary, it provides a better understanding about 'The impact of nutritional stress on flavour formation during yeast fermentation' generating scientific know-how and industrial solutions.

1.2 The brewing process

Beer production involves four classic ingredients; water, malt/barley, hops and yeast. Brewing classically involves five stages to produce the final product, they are in order: malting, wort production, fermentation, maturation and packaging (Stewart 2016, Briggs *et al.* 2004, Boulton and Quain 2001).

The first step is the malting process where occurs the liberation of sugars and all compounds stored in the seed (barley). Malting is divided into steeping, germination and kilning. The steeping process increases the moisture content (the degrees of steeping) to 40-46% and, as a consequence, it activates germination. Germination is responsible for the activation of hydrolysing enzymes which soften cell walls, reduce protein and leave starch intact. After germination, kilning stops germination by drying where generally pale malts are dried at about 80ºC and other malt styles are dried at higher temperatures (up to 105 ºC). As a consequence, this step of process generates flavour and stabilizes the malt (Stewart 2016, Briggs *et al.*, 2004). After malting, the production of wort is the next brewing step. Wort contributes several compounds which are essential for yeast fermentation and the quality of beer production such as carbohydrates, amino acids, minerals, in addition to other characteristics such as colour, sweetness and bitterness (Francis & Newton 2005; Swiegers *et al.* 2005).

1.2.1. Wort

Wort is the complex matrix or medium where yeast growth and beer fermentation occurs. All raw materials such as malt, hops and water contribute to wort composition; however, a very large number of compounds comes from malt (Stewart 2016). Wort contains sugars (fructose, sucrose, glucose, maltose, maltotriose, and non-fermentable dextrins), nitrogenous materials (amino acids, peptides, proteins, ammonium ions, nucleic acids, malt endosperm degradation products), vitamins, essential inorganic elements, organic acids, fatty acids, and

several other trace constituents (Briggs *et al.*, 2004, Boulton and Quain 2001, Bamforth 2001).

Wort is produced from malt and water via a sequence of mechanical/physicochemical processes. Milling, mashing, boiling, filtration and cooling are the sequential stages that are responsible for transporting the nutritional content from malt (solid phase) into water (liquid phase) producing wort. Firstly, milling is a mechanical process that breaks down malt into smaller particles, named grist (Stewart 2016). Second, the grist is mixed with water to form a liquor or mash. Third, to promote the transport of sugars into the liquid phase and raise enzymatic activities, the mixture is held between 63-67°C (Briggs *et al.* 2004). During this process, called boiling, temperature is the most important parameter. Whilst temperatures above 65°C might result in shorter processing times; it will however, reduce enzymatic activity which could result in less fermentable wort (Briggs *et al.* 2004, Muller 1991). Also during boiling, proteins are precipitated and hops are added into wort. Hops are the third essential ingredient of beer production, which influence flavour and have antimicrobial activity (Bamforth 2006, Briggs *et al.* 2004, Boulton & Quain 2001). Finally, wort goes through two physical processes: filtration and cooling, which contributes to the separation of solids (Briggs *et al.* 2004). After cooling, wort is ready for fermentation that is the metabolic process where yeast consumes wort nutrients for growth and as result produces ethanol and flavour compounds.

1.2.2. Fermentation

Fermentation can be defined as the processes by which microrganisms (generally yeast or bacteria) catalyse the conversion of sugars into ethanol and associated products. This process typically takes place under anaerobic conditions, and the microorganisms can comprise a part of the end product. Although fermentations can occur in solid, and semi-solid states, the production of beverages occurs exclusively in liquid systems. Alcoholic beverages are predominantly produced through yeast fermentation, where yeast consumes sugars converting it into ethanol (Walker and Stewart 2016). The yeast *Saccharomyces cerevisiae* dominates alcoholic beverages worldwide because of their capacity to produce and tolerate high levels of ethanol and their profound influence on flavour formation (Walker and Stewart 2016, Schawan 2001).

1.3. Yeast: the key to fermentation

Yeasts are eukaryote unicellular microfungi that are widely found in the environment and are important for industry and medical science (Walker 2009). Yeasts have ellipsoid shape with diameter from 5 to 10μm. Similarly to all yeast cells, *S. cerevisiae* has a cell wall, which is outside the cell membrane and affords the cell structural support and protection. In addition, it prevents an excessive transport/accumulation of water in the cell (Walker and Stewart 2016). Yeast cells contain numerous important organelles (fig. 1.1), which include the nucleus, mitochondria, endoplasmic reticulum (ER), Golgi apparatus, vacuoles, microbodies, and secretory vesicles (Walker and Stewart

2016). All organelles exert important functions, which combine with a complex extracellular and intracellular membrane network (Walker and Stewart 2016, Takahashi *et al.* 2006).

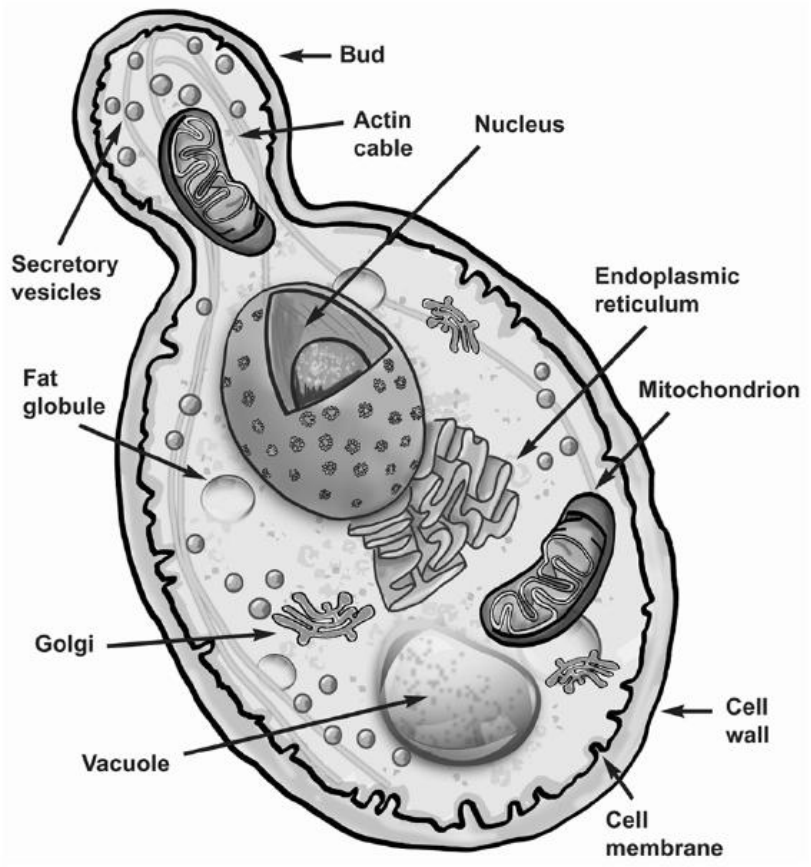

Fig. 1.1: Main features of a typical *Saccharomyces cerevisiae* cell (Reproduced from Walker and Stewart 2016)

Yeast fermentation has been used for production and conservation of food and drink for much of human history (Walker 2009). Most traditionally, yeasts have been used for baking and alcoholic beverage production (He *et al.* 2014, Walker 2009). Nowadays, yeasts are used for many industrial processes due to their potential in producing a range of biotechnological products, including chemicals, industrial enzymes, pharmaceutical compounds, as well as in agriculture and in environmental situations through bioremediation (Walker

2011, Walker 2009). The yeast *S. cerevisiae* is arguably the most important 'cell factory' in biotechnology, through its use in food production and fermentation, as well as the chemical and pharmaceutical industries (Walker 2009).

In the brewery, yeast are generally *Saccharomyces* genus. *Saccharomyces cerevisiae*, *Saccharomyces pastorianus* and *Brettanomyces bruxellesis* are used to produce ale, lager and lambic beer, respectively (Walker and Stewart 2016). Lager yeast are a natural hybrids of *S cerevisiae* and *Saccharomyces eubayanus* (Walker and Stewart 2016). Ale products produced with *S. cerevisiae* are typically fermented at warmer temperatures than lager style beers produces using *S. pastorianus*. Lambic beers are produced by spontaneous fermentation; however, some Belgian-style beers use inoculated *Brettanomyces* ssp (Walker and Stewart 2016, Spitaels *et al.* 2014). Generally brewing research aims to improve the efficiency of sugar conversion to alcohol. Understanding the behaviour of different yeast via the utilization of different nutrients and consequently the production of desirable flavours is important for process control (Walker and Stewart 2016, Boulton and Quain 2001).

1.4. Yeast: Physical Requirements, nutritional intakes and metabolism

1.4.1. Homeostasis in Yeast *Saccharomyces cerevisiae*

To adequately explain yeast fermentation and control, it is necessary to understand yeast homeostasis. Homeostasis is the mechanism of cellular control that maintains the internal equilibrium (Sala 2015) against environmental changes, and as a consequence, it maintains physiological

activities. In the brewery, yeast *Saccharomyces* are exposed to a range of environments that can be aggressive towards the cell. Related to this, several wort nutrients, especially minerals, which are charged ions (Canadell *et al.* 2014) and whose uptake is governed by homeostatic mechanisms.

1.4.2. Environmental and nutritional stress of yeast

As described at section 1.2.1.1., wort is the medium which supports yeast growth and survival, due to its physical and nutritional characteristics. *Saccharomyces* yeast are facultative aerobic microorganisms, whose aim is to reproduce by budding and, as a consequence, they make ethanol and other metabolic compounds (Ingledew 2009). Yeasts undergo constant changes caused by variables present in their environment, which includes nutritional levels, osmotic stress, toxic molecules and temperature. During fermentation, two main stress responses occur, which are heat shock response (HSR) and global (general) stress response (GSR). The heat shock response is activated by sub lethal heat stress (Gibson *et al.* 2007; Morimoto *et al.* 1996). The GSR includes environmental stress factors such as pH, temperature, oxygen (oxidative), osmotic stress, and nutritional limitation (or absence) of carbon sources, amino acids and essential inorganic elements (Gibson *et al.* 2007, Ruis and Schuller 1995). These environmental stress factors affect yeast adaptation/metabolism and, consequently, the characteristics of the end product.

1.4.3. Physical requirements of yeasts

The concentration of hydrogen ions (pH), temperature, oxygen and osmotic stress are the most important physical requirements of yeasts (Fig. 1.2). In alcoholic fermentation, source of osmotic stress involves the yeast inoculation into wort, which is a complex and highly concentrated solution containing high concentrations of sugars (Briggs *et al.*, 2004). Therefore, oxygen and sugar concentration are related to the Pasteur Effect and Crabtree effect, respectively. All physical properties described below are crucial for yeast survival and efficient fermentation.

1.4.3.1. pH

The importance of pH during fermentation control is well known. The pH affects beer taste (sourness), viscosity, flavour stability and nonbiological/biological stability. The pH (and buffering) is among the most relevant control parameter to yeast performance (Bamforth 2003), and generally, initial pH of wort is about 5.20. Recently, pH control has been studied using online probes due to importance of this parameter (Ashraf 2011). Variation on initial wort pH affects the fermentation and wort buffering capacity. During fermentation, wort pH decreases because yeasts consume buffering compounds (including amino acids, small peptides, vitamins and phosphates), and form/secrete organic acids (Ashraf 2011, Boulton and Quain 2001; Briggs *et al.* 2004, Imai and Ohno 1995).

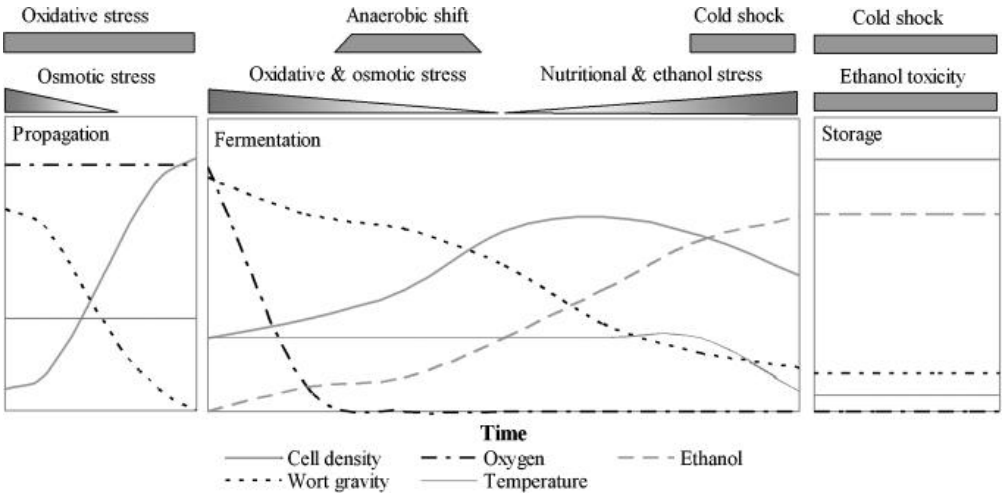

Fig. 1.2: Schematic representation of the sequential nature and temporal of potential stresses by yeast during brewery propagation, fermentation and storage (Reproduced from Gibson *et al.* 2007)

Temperature influences the rate of pH decrease and oxygen intake rate during fermentation, consequently affecting fermentation time and the consumption rate of buffering compounds (including amino acids, small peptides and phosphates). Wort acidity decreases quickly during the first 20 hours of fermentation, and then the rate of pH becomes slower. Although pH decreases faster in fermentations conducted at 20°C and 15°C than those conducted at 10°C, the final pH is not effected (Ashraf 2011).

1.4.3.2. Temperature

Fermentation temperature is a crucial variable (Barker *et al.* 1992; Titica *et al.* 2000) because it regulates yeast growth, fermentation length and affects flavour formation. (Verbelen *et al.* 2009; Verbelen *et al.* 2009).. Ashraf (2011) showed that fermentation of a lager strain (W34/70) at low fermentation temperature produced decreased ethanol, higher alcohols and esters during the

process and influenced in final product. The author studied the effect of temperature on the fermentation process and measured fermentation at 10°C, 15°C and 20°C. His data demonstrated that when the fermentation was conducted at 20°C more ethanol, higher alcohols and ester were formed than in fermentations conducted at 10°C and 15°C. In addition, fermentation at 15°C produced better growth and ethanol, higher alcohols and esters formation than the lowest temperature tested (10°C). In addition, fermentations conducted at 20°C finished quicker than the lower temperatures tested (10°C and 15°C). Landaud *et al.* (2001) and Barker *et al.* (1992) showed that in brewery fermentation higher alcohol formation was reduced by 15-23% when fermentation temperature was reduced from 17°C to 12°C or 16°C to 10°C. These results indicate the flavour formation can decrease up to 23% for approximately each 5°C reduction in the fermentation process temperature. Furthermore, lower temperatures are implicated in lower flavour formation.

The negative effect of low fermentation temperature on flavour formation are reduced when the quantities of dissolved oxygen in the wort is increased; however, there is still a negative effect of up to 8% of flavour reduction (Ashraf 2011, Saerens *et al.* 2008). Fermentation temperature can affect the process control influencing growth rate and as a consequence, yeast metabolic activities and oxygen rate intakes.

1.4.3.3. Oxygen role

In brewing, oxygen has a complex role. However in yeast metabolism the main role of oxygen is to promote fatty acid and ergosterol biosynthesis, which are essential for yeast growth and survival during anaerobic growth and fermentation (Boulton and Quain 2001). Yeast cells can survive under anaerobic condition for long fermentation periods, however they require oxygen for lag (adaptation) and exponential growing phases. The presence of oxygen is important for satisfactory growth and fermentation. Brewery fermentation is conducted in batch or continuous systems, but in both systems, oxygen dissolved in the wort and the headspace of fermenter are generally the only oxygen sources. Therefore, some fermentation systems use oxygen addition and some studies have been conducted to evaluate the influence of oxygen addition in flavour formation (Ashraf 2011). However, high-gravity worts have less available oxygen dissolved (Verbelen *et al.*, 2009, Verbelen *et al.*, 2008), which can cause problems associated with anaerobiosis. On the other hand, highly oxygenated worts can cause lower yeast growth and viability reduction as well (Ashraf 2011). Then, low or high levels of oxygen might affect the favourable lipid synthesis, as a consequence, it may causes sluggish or stuck fermentations and beer flavour changes (Ashraf 2011, Verbelen *et al.*, 2009, Kirsop, 1974).

Brewery yeasts are facultative anaerobes, able to survive in the presence or absence of oxygen. Brewing strains grow quickly in the presence of oxygen and the growth rate is reduced when oxygen is limited or absent. This occurs when exponential phase finishes, the dissolved oxygen is totally consumed (Fig. 1.3.), as a consequence yeasts grows anaerobically and the growth is mostly

influenced by ethanol presence. In addition, when oxygen becomes limiting the sterol content decreases affecting yeast growth (Verbelen *et al.* 2009, Aries and Kirsop 1977).

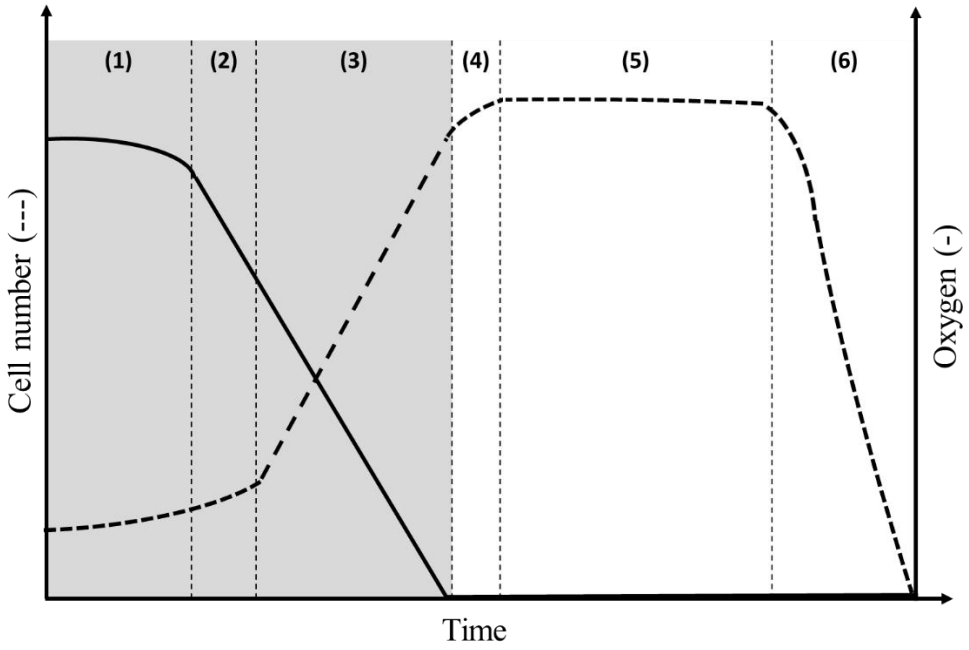

Fig. 1.3: Schematic summarizes the oxygen consumption and yeast growth main during four growth phases including (1) lag phase (adaptation), (2) acceleration, (3) exponential phase, (4) deceleration, (5) stationary phase and (6) decline phase. The exponential phase finishes when dissolved oxygen is fully consumed, as a consequence, deceleration phase begins and yeast grows anaerobically.

During lag and exponential phases, yeast produces sterols and fatty acids. Sterols and unsaturated fatty acids regulate membrane fluidity, permeability and the activity of membrane-bound enzymes; consequently, they control the response to stress (Swan and Watson 1998; Chatterjee *et al.* 2000; Jones *et al.* 2005). Oxygen is essential for suitable quantities of membrane compounds synthetises, which affects yeast growth and health (David & Kirsop, 1973). In addition, oxygen is important for mitochondrial development (O'Connor-Cox *et al.*, 1993) and other cellular activities. Although oxygen is very important

for yeast growth, health and development and additional oxygen increases growth rate, it does not increase the final cell density (Ashraf 2011). Excess of oxygen may be problematic causing yeast degeneration and reduces flavour formation due to the toxic effect of reactive oxygen species (ROS) (Ashraf 2011).

1.4.3.4. Osmotic stress

Osmotic stress is defined as an imbalance between intra- and extracellular concentration, which results in osmotic pressure (Csonka and Hanson 1991). Osmotic pressure is the force developed between two solutes of different concentration separated by a permeable membrane. Osmotic pressure can cause lethal physiological changes (Dihazi *et al.* 2001) and can occur during very low external osmotic potential (e.g. yeast in deionized water) or high external osmotic potential (e.g. yeast in wort). Yeast in deionized water increases the influx of water, reducing the hypo-osmotic stress and cell's dehydration (Klipp *et al.* 2005, Pratt *et al.* 2003).

Osmoregulation is the homeostatic process which occurs in yeast cells to monitor and adjust osmotic pressure, which affects cells' shape, turgor and water content (Klipp *et al.* 2005). In the brewery, efficient osmoregulation is necessary and fermentation can be controlled by dissolved solids such as initial gravity, specific gravity, sugar total, degree of brix and degree of Plato. They are a measurement of the concentration of dissolved solids. Brewing fermentation can be classify by initial gravity as low (<10°P), normal (10-13°P), medium (13.1-16°P), high (16.1-18°P) and very high (>18°P), which

generates different external osmotic potential. Osmotic stress starts when yeast is inoculated into wort (Puligundla *et al.* 2011, Gilson *et al.* 2007) and influences the Pasteur effect and Crabtree effect (Briggs *et al.* 2004).

1.4.3.5. Crabtree effect and Pasteur effect

During fermentation, many types and levels of stress can affect yeast metabolism and drive the final product to have different characteristics/profile. Quantities of sugars and oxygen in solution are the two biggest effects, which occur during yeast fermentation (including all brewing strains). These two effects are called the Pasteur Effect (oxygen effect) and the Crabtree effect (sugars effect) are very important influence on yeast metabolism (Walker and Stewart 2016, Briggs *et al.* 2004, De Deken 1966). Pasteur effect (Fig. 1.4) is relative to oxygen presence, which activates cells' respiration, on the other hand, Crabtree effect (Fig 1.4) is relative to sugar concentration, which enhances the rule of glycolysis pathway and drives carbon units into ethanol production.

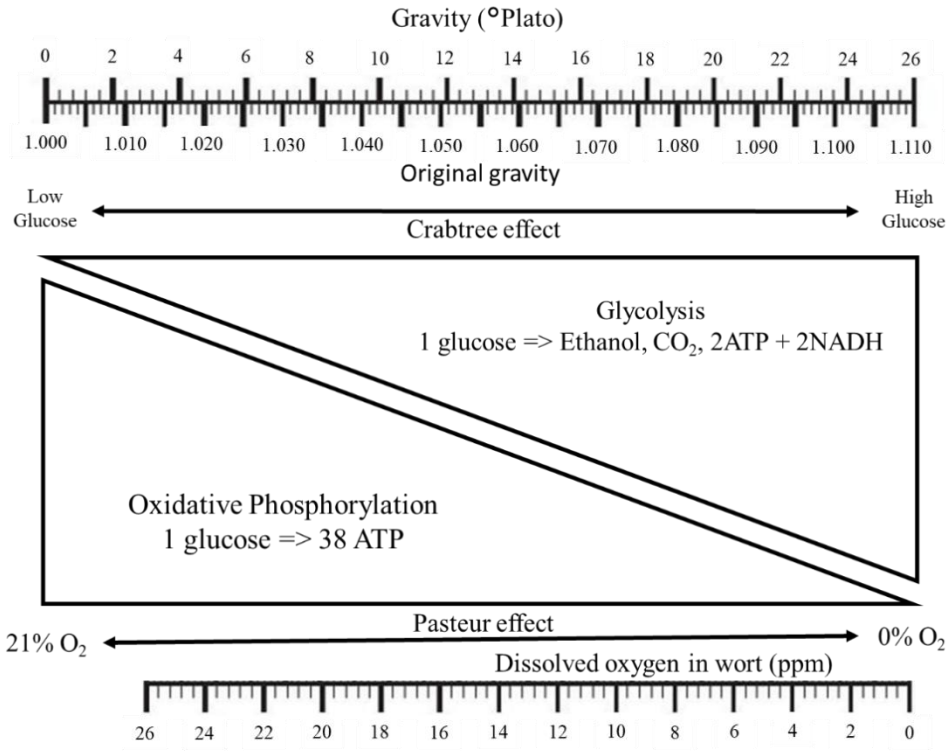

Fig. 1.4: Schematic summarizes the relationship between gravity and dissolved oxygen. In addition, summarizes the relationship between Crabtree effect and Pasteur effect and their effects in glycolysis and oxidative phosphorylation.

Pasteur and Crabtree effects may be a competition between glycolytic and oxidative phosphorylation system for inorganic phosphate and adenine nucleotides (Pfeiffer and Morley 2014, Briggs *et al.* 2004). In the presence of glucose, glycolysis supplies the cellular necessities for ATP and, also, glycerol is produced as a result of the osmotic stress (Crabtree effect) helping to supply ATP necessities (Walker and Stewart 2016). It is well known that glycerol is formed as an osmoprotectant; however, metabolically its role is for redox balance as a primary role and as a consequence has a purpose in NAD regeneration. The Crabtree effect results in a reduction of oxidative phosphorylation and consequently a reduction of ATP transport from mitochondria to cytosol due to the reduction of mitochondrial ATP generation

(Pfeiffer and Morley 2014, Dashko *et al.* 2014, Briggs *et al.* 2004). Then, respiratory rates and oxidative phosphorylation drop down.

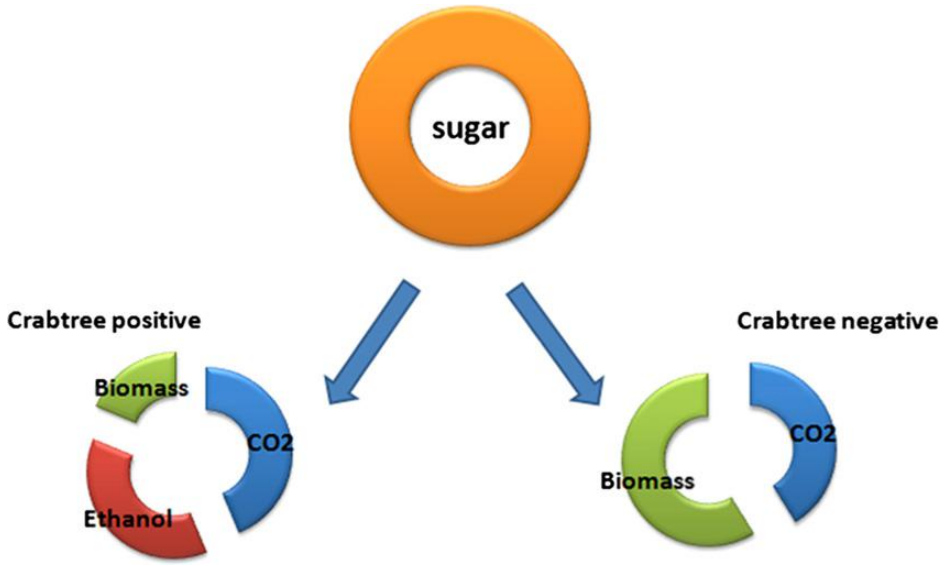

Fig. 1.5: Crabtree effect results in lower biomass production because a fraction of sugar is converted into ethanol. This means that more glucose has to be consumed to achieve the same yield of cells if comparing with Crabtree-negative yeasts. Because only a fraction of sugar is used for the biomass and energy production, this could theoretically result in lower growth rate in Crabtree positive yeasts and these could then simply be out-competed by Crabtree-negative yeasts and other microorganisms. However, ethanol could be used as a tool to slow down and control the proliferation of other competitive microorganisms (Dashko *et al.* 2014).

Pfeiffer and Morley (2014) reported two common yeast behaviour called Crabtree-negative and Crabtree-positive (Fig.1.5); brewery yeasts are Crabtree-positive. Crabtree-positive yeasts, including all brewery strains, produce acetyl-CoA via respiration and fermentation (Dashko *et al.* 2014, Briggs *et al.* 2004), because they are capable of producing biomass, CO_2 and ethanol. Crabtree positive yeast can also produce acetyl-CoA using ethanol as a source of carbon via aldehyde dehydrogenase (Ald) and acetyl-CoA synthetase (Acs) (Pfeiffer and Morley 2014)

1.5. Nutritional intakes

Carbon, nitrogen, phosphorus, potassium, magnesium, copper, zinc, iron manganese, hydrogen and oxygen are required by living organisms for growth and maintenance (Canadell *et al.* 2014). In the brewery, several wort compounds are important variables which contribute to the production of a great beer. The most important are sugars (carbon source), amino acids and essential inorganic elements which fuel yeast growth, fermentation control and as a consequence, influence the quality of the final product (flavour production).

1.5.1. Carbon source

Yeast can assimilate a wide range of carbon sources, including glucose, fructose, sucrose, maltose, galactose, mannose, raffinose and occasionally trehalose. Furthermore, most yeast can metabolise isomaltose and some can use pentose and maltotetraose, but they cannot usually assimilate dextrins (Stewart 2016, Briggs *et al.* 2004, Boulton and Quain 2001). Brewery yeast can use the majority of simple carbohydrates; however, ale and lager strains can be differentiated because of their ability to consume melibiose (disaccharide). Ale strains cannot grow on melibiose, whilst lager have α-D-galactose activity, which hydrolyses mebibiose into galactose and glucose (Briggs *et al.* 2004, Barnett, 1981).

Wort contains sucrose, fructose, glucose, maltose, maltotriose, maltotetraose and dextrins (Stewart 2016, Briggs *et al.* 2004). Basically, all yeast consume sucrose, fructose, glucose and maltose, some might consume maltotriose but

generally brewery strains do not assimilate maltotetraose and dextrins. Glucose is the preferred source of carbon and energy for the yeast *Saccharomyces cerevisiae* (Galdieri *et al.* 2014). During fermentation, sucrose is the first sugar to be fully consumed since it is present in low quantities in the wort and is broken down extracellularly to glucose and fructose by invertase. Sucrose degradation is controlled by six genes which encode invertase (SUC1-5 and SUC7). Monosaccharides (glucose and fructose) are easily transported across membrane by facilitated diffusion. In addition, glucose is an easily fermentable sugar and is consumed more or less simultaneously with fructose (Walker and Stewart 2016).

Maltose and maltotriose consumption are activated by specific permeases. Maltose is the most abundant sugar present in the wort and requires the presence of at least one of five MAL loci (MAL1-4 and MAL6), each one consisting of three genes MAL S (α-glucosidase), MAL T (permease) and MAL R (regulates the other genes). MAL S and MAL T are regulated by the presence of maltose and glucose reduction. In the brewery, maltose uptake starts when 50% of glucose has been consumed by the yeast (Walker and Stewart 2016, Stewart 2016). Generally, maltose starts to be consumed after 20h of fermentation and the activation of the process requires cellular energy from a proton symport system and potassium (K^+) presence (Boulton and Quain 2001, Priest and Stewart 2006). In summary, di- and trisaccharides are converted to hexose monomers that once transported into yeast cell, they are metabolised via phosphorylated intermediates and then go through by the glycolysis pathway.

1.5.2. Essential inorganic elements

Sugars are very important for fermentation and amino acids (nitrogen source) stimulate carbon consumption and increase biomass production (Beltran *et al.* 2005). However, essential inorganic elements influence growth and consequently fermentation control and the quality and consistency of the final product (flavours). Essential inorganic elements are present mainly as charged ions in wort (Barreto *et al.* 2012, Canadell *et al.* 2014, Sala 2015). Charged ions are used to establish chemical gradients used in processes such as ATP synthesis in mitochondria by oxidative phosphorylation or secondary transport (Canadell *et al.* 2014). All yeast have their specific means of accumulating ions that depends on yeast physiology (Eide *et al.* 2005, Canadell *et al.* 2014). Indeed, the yeast environment during alcoholic fermentation involves several physical, chemical and biochemical transformations simultaneously (Silva *et al.* 2006, Deepak 2008, Smart 2003). This group of transformations occurs in wort and produces the appearance and composition of the final product.

1.5.2.1. Nitrogen

Nitrogen availability has a central role in general yeast metabolism because it supports protein and amino acid synthesis (Ljungdahl and Daignan-Fornier 2012). Yeasts *Saccharomyces* are able to assimilate nitrogen from different sources and this is referred to as ‘assailable nitrogen’ or ‘assimilable nitrogen’ (Silva el at. 2006, Priest and Stewart 2006). Yeast assimilable nitrogen (YAN) can be divided into two groups: 1) free amino nitrogen (FAN), and 2) ammonia-nitrogen (NH_3) (Ljungdahl and Daignan-Fornier, 2012, Silva el at.

2006). Yeast growth is nitrogen dependent mainly in amino acids forms because they are key to the synthesis of proteins and other nitrogenous components (Priest and Stewart 2006). Ammonia nitrogen is assimilated during glutamate and glutamine formation. Glutamate is synthetized by NADPH-dependent glutamate dehydrogenase (GDH1) from α-ketoglutarate (fig. 1.6, reaction 1) and glutamine is synthetized by glutamine synthetase (GLN1) from glutamate (Ljungdahl and Daignan-Fornier 2012). Ammonia/amino acid supplementation effects flavour formation due to effects on yeast physiology and consequently fermentation performance (Beltran *et al.* 2005). Although nitrogen is essential for yeast growth, other inorganic elements are also required for growth quality because they are involved in metabolic activities.

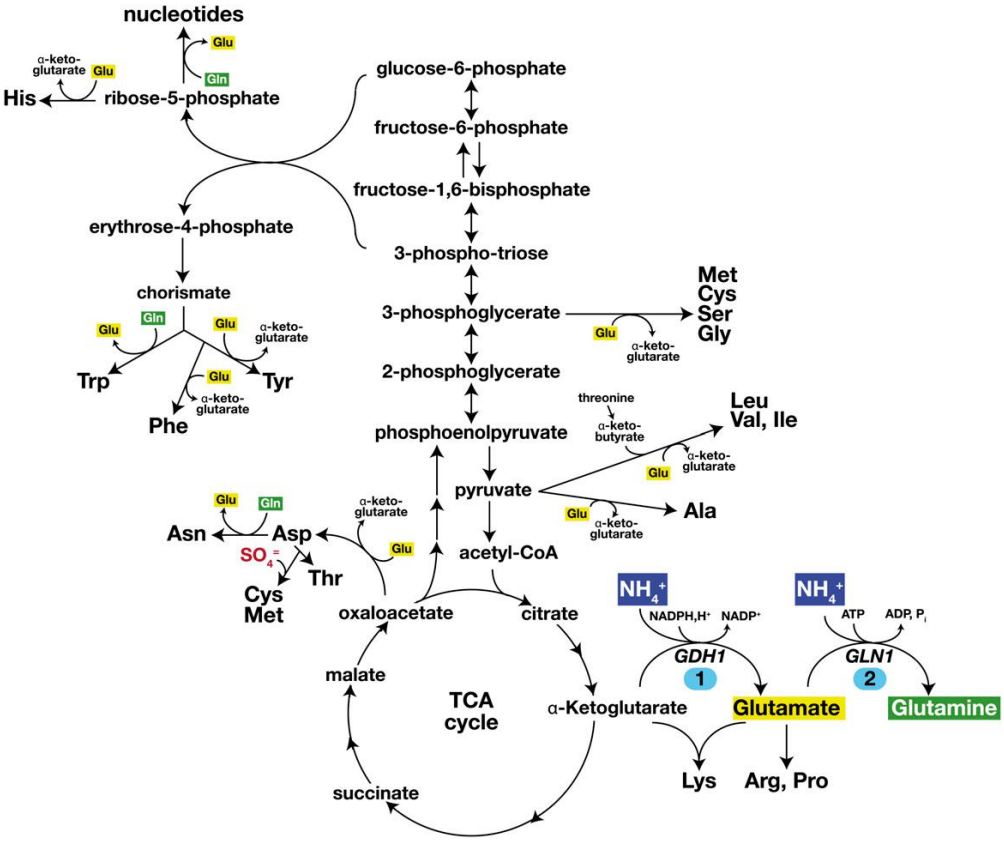

Fig. 1.6: General biosynthesis of amino acids from glucose and ammonia (Reproduced from Ljungdahl and Daignan-Fornier 2012).

1.5.2.2. Phosphorous

Phosphorus is one of the key minerals required for yeast growth. In the brewery, wort contains phosphorus in the form of phosphate salts (Walker and Stewart 2016). Inorganic phosphate (Pi) is the major intracellular anion present in the yeast cell and is involved in cellular biosyntheses such as nucleic acids, nucleoproteins and phospholipids and ATP, as well as several metabolic pathways (Walker and Stewart 2016, Canadell *et al.* 2014). As a function, the yeast vacuole (fig. 1.7) stores phosphates as polyphosphates forms (Walker and Stewart 2016). Pi is transported across the cell membrane and acts as a substrate and enzyme effector (Walker and Stewart 2016, Canadell *et al.* 2014).

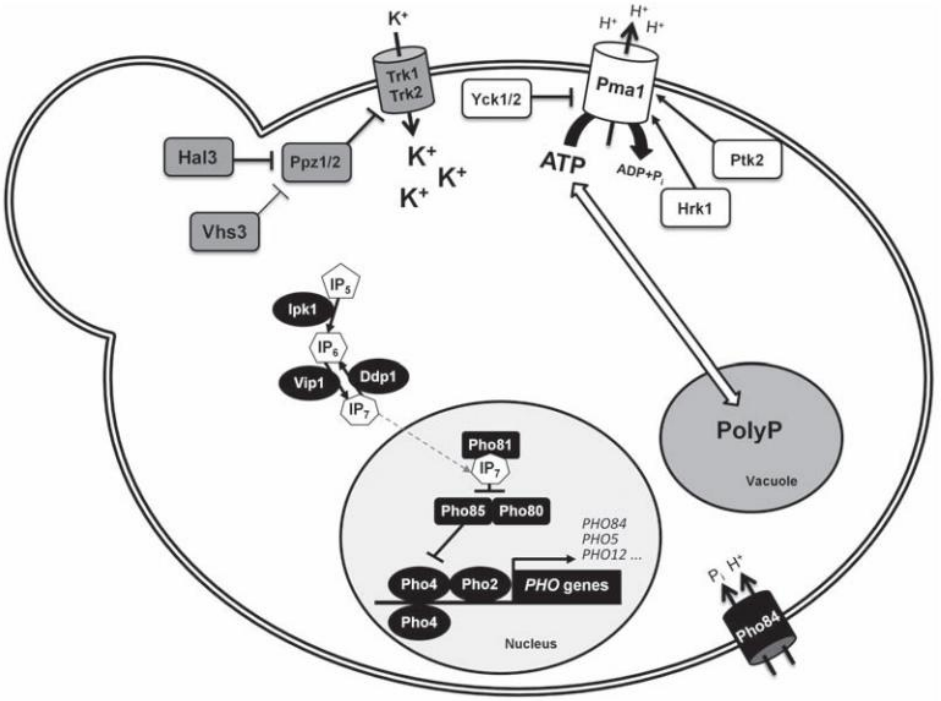

Fig. 1.7: Schematic *S. Cerevisiae* plasma-membrane: schematic description of potassium/phosphorus influx and proton efflux system. In addition, the main elements of PHO signalling pathway and also shows the mechanisms of regulation for transporter activities (reproduced from Canadell *et al.* 2014).

Fig. 1.7 shows the schematic description of potassium/phosphorus influx and proton efflux system in plasma-membrane, plus their respective transporters. Pho84 and Pho89 are responsible for Pi transport through plasma-membrane and have high-affinity for Pi uptake (K_m~10mM) (Persson *et al.* 1999, Sala 2015). Pho84 is activated in acid pH (Pattison-Granberg and Persson 2000), which is valuable for yeast fermentation because wort is acidic (pH~5.2) and becomes more acidic during fermentation. On the other hand, Pho89 increases its transport efficiency in alkaline pH (Zvyagilkaya *et al.* 2008, Canadell *et al.* 2014). High concentration of extracellular Pi (in wort) influences PHO84 and PHO89 activation. Furthermore, phosphate metabolism is altered due to the absence of extracellular potassium or influx perturbation (Canadell *et al.* 2014). Lack of potassium inhibits phosphate intake and, as a result it affects yeast growth rate (Barreto *et al.* 2012, Sala 2015) and consequently, fermentation and flavour production.

1.5.2.3. Potassium

Potassium is a major intracellular monovalent cation and in yeast cell biology is important for the export of H^+, Na^+ and toxic cations like lithium (Canadell *et al.* 2014) as well as for phosphorus uptake (Sala 2015, Barreto *et al.* 2012). The yeast *Saccharomyces cerevisiae* has an intracellular concentration of potassium between 200 to 300mM, but yeast cells can proliferate in media with low extracellular potassium (below 1mM) (Barreto *et al.* 2012, Sala, 2015). However, under low potassium concentrations, important control factors are reduced such as growth, viability and yeast integrity. Yeast plasma-membrane

has a high capacity to transport potassium, which allows the positive influx of potassium ion through membrane (Rodriguez-Navarro, 2000; Arino *et al.* 2010).

Saccharomyces cerevisiae has two main transporters responsible for potassium uptake, Trk1 and Trk2 (Gaber *et al.* 1988, Ko *et al.* 1990, Ko and Gaber 1991). During growth, Trk1 and Trk2 need high potassium concentration (>10mM) (Canadell *et al.* 2014), which is supplied due to wort composition. Trk1 is most physiologically relevant because it regulates the highest amount of potassium that passes through the cell membrane (Sala 2015). Intracellular potassium has many functions such as maintaining yeast volume, intracellular pH, protein synthesis, redox homeostasis, cell cycle progression and enzymatic activation, etc. (Barreto *et al.* 2012, Page and Di Cera, 2006, Merchan *et al.* 2004).

During fermentation, potassium keeps the plasma-membrane stable against the negative influence of macromolecules and is involved in acetaldehyde dehydrogenase (ACDH). The existence of two acetaldehyde dehydrogenase (ACDH) types, which are localized in the mitochondrial and the cytosol (Boubekeur *et al.* 2001). In mitochondria, ACDH is activated by K^+ (K-ACDH) using as coenzymes NAD1 and NADP1 (Boubekeur *et al.* 2001, Jacobson and Bernofsky 1974). In addition, its expression is repressed by the presence of glucose. However, in the cytosol, ACDH is activated by Mg^{2+} (Mg-ACDH) using NADP1 as coenzyme (Dickinson 1996). Potassium and phosphorus have an interactive relationship (fig. 1.7), which are related to yeast phenotype/genotype, the minerals available in the wort and, as a consequence, nutrient uptake (Gonzalez *et al.* 2013). Additionally, the potassium transport system has been reported to be responsible for magnesium uptake under

potassium-deficient conditions, even though this occurs at a relatively slower rate (Udeh and Kgatla 2013).

1.5.2.4. Magnesium

Magnesium is the most abundant divalent cation involved in biological processes (Gibson 2011, Walker 2004; Briggs *et al.* 2004). Although magnesium can be transported into cells via potassium and phosphorus transport systems, two plasma membrane transporters, ALR1 and ALR2, have been reported to be required for magnesium transport (Knoop *et al.*, 2005, Walker 2004). Magnesium has a vital importance for yeast division/growth, metabolic activities, respiro-fermentative metabolism, mitochondrial structure and function, response to environmental stress, fermentation performance, ethanol production, etc. (Walker 2004).

In the brewery, evidence has suggested that magnesium contributes to protection against the effects of heat and ethanol stresses (Gibson 2011), increased ethanol production, maintenance of cellular integrity (including structural stabilization of nucleic acids, polynucleotides, chromosomes, polysaccharides and lipids), while also broadly protecting against cell death (Walker 2000). Magnesium activates over 300 enzymes including many glycolytic enzymes such as pyruvate kinase, hexokinase, phosphofructokinase, phosphoglycerate kinase and enolase (Walker *et al.* 2006, Walker 2004). Conversely, magnesium absence or limitation reduces yeast division/growth (Walker 2004, Walker 2000), and has a negative effect on several physiological/metabolic activities and protein transport through intracellular

organelles membranes (Koop *et al.*, 2005). These may result in sluggish or stuck fermentations and reduction of ethanol production and flavour generation. Although magnesium is a very important cation, several other cations such as heavy metals (including zinc, copper, iron and manganese) are involved in yeast metabolic activity during growth and fermentation (Wietstock *et al.* 2015).

1.5.2.5. Heavy metals

Heavy metals or transition metals are critical for yeast cells due to their necessity in cellular functions, but also in some instances due to their toxicity (De Freitas *et al.* 2003). Heavy metals are essential nutrients for all organisms because they serve as cofactors and perform structural roles within cells (Cyert and Philpott 2013). Yeast, like most eukaryotic organisms, contain proteins which have transition metals as cofactors, including iron, copper, zinc and manganese. These cations are involved in ensuring metabolic activity of yeast during growth and fermentation (Wietstock *et al.* 2015, Cyert and Philpott 2013). However, under certain situations these metals can be toxic and the yeast *Saccharomyces cerevisiae* has developed sophisticated homeostatic mechanisms to avoid such effects (Cyert and Philpott 2013). In the brewery, metal ions have been identified in several process steps (Wietstock *et al.* 2015) and are crucial during fermentation and beer stability, as discussed below.

1.5.2.6. Iron

Iron, copper and manganese promote the staling of beer because metals ions contribute to reactive oxygen species, which destabilize beer flavour (Porter and Bamforth 2016). The yeast *Saccharomyces cerevisiae* obtains iron from the environment by two methods of uptake: by directly assimilating ions, or via breakdown of low molecular weight compounds (De Freitas *et al.* 2003). Iron is captured and stored in yeast vacuoles by the Fet5p/Fth1 complex (Fig. 1.8) and can be used if iron limitation is encountered (Urbanowski & Piper 1999). Iron is found throughout the yeast cell but it is most abundant in the mitochondria due to its role in the electron transport chain (De Freitas *et al.* 2003). Mitochondrial ion metabolism supports iron homeostasis but an excess of iron causes oxidative stress and reduces metabolic and respiration capacity (De Freitas *et al.* 2003). When iron is present at toxic concentrations, detoxification occurs by respiration intensification, which increases copper utilization (Szczypka *et al.* 1997). On the other hand, when cell-iron levels are low, the mitochondrial-iron can be transported to support organelles requirement such as vacuoles (De Freitas *et al.* 2003). Furthermore, iron might be able to substitute copper in some metabolic activities and vice-versa.

1.5.2.7. Copper

It has been described previously that transition metals are important for yeast survival and metabolism (Section 1.5.2.5.). Metal ions such as iron, copper, zinc and manganese are necessary to maintain yeast intracellular functions. Iron and copper participate directly in the vital reaction of electron transfer and

are metabolic enzymatic cofactors of many enzymes (De Freitas *et al.* 2003). Copper acts as an essential cofactor for some enzymes (including cytochrome c oxydase, lactase and Cu,Zn-superoxide dismutase) and is an important divalent cation for yeast metabolism mainly during iron homeostasis (Keller *et al.* 1991, Geller and Winge, 1982).

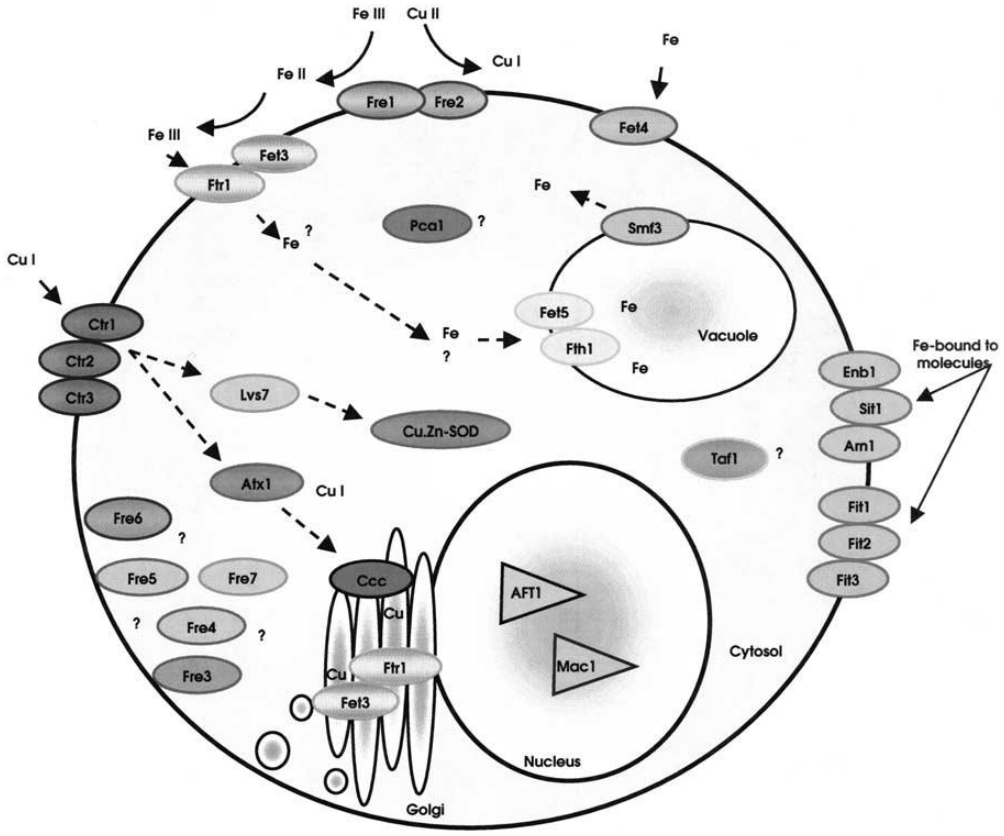

Fig. 1.8: Copper and iron metabolism in yeast (De Freitas *et al.* 2003)

The relationship between iron and copper is shown in fig. 1.8. Copper ions are transported into yeast cell through Ctr1/Ctr2/Ctr3 transporters; however, the Fet3p-Ftr1p complex transports copper ions through the plasma membrane during iron deficiency (Lin *et al.* 1997, O'Halloran & Culotta 2000). Although copper ion is important for yeast growth and fermentation, an excess of copper is toxic and can damage the cell structure (Sturtz *et al.* 2001) due to copper

electron negativity. Yeasts have developed very sophisticated mechanisms to maintain metal homeostasis to avoid toxicity (De Freitas *et al.* 2003), and as a result prevent cell damage.

1.5.2.8. Zinc

Zinc is arguably the most significant heavy metal ion in wort, due to its importance in ethanol production, which converted by ADH (alcohol dehydrogenases) (Section 1.7.2). As with the previously described heavy metal ions, zinc impacts on yeast fermentation activity and growth since they are biologically important as cofactors for enzymatic reaction including alcohol dehydrogenase, alkaline phosphatase, carbonic anhydrase and several carboxypeptidases (Turrens and Boveris 1980, Crapo *et al.* 1992). In brewing, zinc is essential for maintaining yeast growth rate and ethanol production, however, as with other metal ions, an excess or deficiency of zinc can result in sluggish or stuck fermentations (Walker and Stewart 2016, Field *et al.* 2003),

Furthermore, zinc (as well as copper (section 1.5.2.7) and manganese (Section 1.5.2.8)) function to combat superoxide anion toxicity in eukaryotic cells via enzymes called superoxide dismutases (SODs) (Sturtz *et al.* 2001). Two intracellular SODs exist, Cu/Zn SOD and MnSOD which function to convert the superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2) by oxidation and reduction of the metal cofactor (Field *et al.* 2003, Sturtz *et al.* 2001). Cu/ZnSOD (SOD1) is most abundant in the cytosol, but occurs in small amounts in the intermembrane space (IMS) of mitochondria. Conversely,

MnSOD (SOD2) is most abundant in mitochondria, although it can also be found in the cytosol (Field *et al.* 2003).

1.5.2.9. Manganese

Manganese is detected in commercial beer at levels between 0.04-0.51, (Porter and Bamforth 2016, Briggs *et al.* 2004). In the brewery, manganese can be found in all of the raw materials typically used, but hops contain the highest levels of manganese (Porter and Bamforth 2016). Consequently, "sweet wort" and "hopped wort" differ in their manganese composition and as a consequence, hop content may have an influence over yeast activity.

Manganese has several important functions in the yeast *Saccharomyces cerevisiae* metabolism: manganese-dependent enzymes include oxidoreductases (e.g. pyruvate carboxylase), dehydrogenases, transferases and hydrolases (e.g. acetyl-CoA synthesise) (Stehlik-Tomas *et al.* 2004, Crowley *et al.* 2000). Yeast-manganese transport can be stimulated by glucose (Stehlik-Tomas *et al.* 2004, Kihn *et al.* 1998, Blackwell *et al.* 1998). In addition, similar to magnesium (Section 1.5.2.4), manganese uptake is phosphate-dependent. When yeast are actively dividing, uptake of manganese as $MnHPO^4$ occurs through the phosphate transporter Pho84. However, when manganese is in excess, secretion occurs and $MnHPO^4$ passes out of the cell via Pho84; this secretory pathway is dependent on sugar transferases (Cyert and Philpott 2013). When yeast is a lacking PHO84 this exposes a resistance to manganese toxicity (Jensen *et al.* 2003). During yeast growth, manganese ions are present at concentrations between 2-10 μM (Stehlik-Tomas *et al.* 2004). Manganese can

promote production of reactive oxygen species (ROS) in response to oxidative stress (Zufall and Tyrell 2008).

1.6. Yeast metabolism

Nutritional composition and the importance of the yeast environment have already been discussed in this chapter; in this subsection we describe the key yeast metabolic pathways which are relevant to this study.

1.6.1. Glycolysis

The Embden-Myerhof-Parnas pathway, or glycolysis, occurs under aerobic and anaerobic conditions, and is the principal carbohydrate catabolic pathway in yeast. The glycolysis pathway ends with the generation of two pyruvate molecules, however, all of the glycolytic reactions are reversible (except the initial phosphorylation of glucose, the phosphorylation of fructose-6-phosphate to yield fructose 1,6 bisphosphate and the dephosphorylation of phospho-enol-pyruvate to form pyruvate). Glycolysis is constitutively active in yeast (Briggs *et al.* 2004) and during growth, the flow from glucose to pyruvate energy in the form of ATP, and NADH as reducing power (Briggs *et al.* 2004). Fig. 1.9 illustrates the Glycolysis pathway and its genes and enzymes.

Glycolysis is divided into two stages. The first stage, conversion of glucose to fructose 1, 6-bisphosphate consumes a phosphate ion from ATP converting it to ADP, this consumption happens during the conversion of glucose into glucose 6-phosphate; and fructose 6-phosphate to fructose 1, 6-bisphosphate. However, the second stage of glycolysis generates two molecules of ATP,

which are the only energetic source in the complete fermentative pathway and these generations occur during the conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate; and phosphoenolpyruvate to pyruvate (Walker and Stewart 2016, Briggs *et al.* 2004, Walker 2000).

Fermentative metabolism via the glycolysis pathway generates two molecules of ATP, which is significantly less than is theoretically achievable using respiratory (aerobic) metabolism. However, in brewing yeast, aerobic metabolism is repressed due to the presence of glucose in the media, a phenomenon known as the Crabtree effect (Section 1.4.3.5). This causes yeast to exclusively use the fermentative pathway, even in the presence of oxygen (Walker and Stewart 2016, Briggs *et al.* 2004, Walker 2000).

Gene expression and enzyme activity control sugar transport into the cell and carbon flux, which involves phosphorylation of glucose and regulation of the activities of phosphofructokinase and pyruvate kinase (Briggs *et al.* 2004). Therefore, the extracellular glucose concentration is higher than the intracellular concentration of glucose; however, nitrogen starvation affects rates of sugar transport, which brings a progressive decay in rates of glycolytic flux, although it does not directly affect the activities of glycolytic enzymes (Walker 2000).

Glycolysis can occur in the reverse direction, called gluconeogenesis. In this case, the reverse direction of carbon past the irreversible steps of glycolysis is catalysed by three additional enzymes (phospho-enol-pyruvate carboxykinase, fructose 1, 6-bisphosphatase and glucose 6-phosphate phosphatase).

Glycolysis/gluconeogenesis is an amphibolic pathway because it serves both anabolic and catabolic roles, (Briggs *et al.* 2004, Walker 2000).

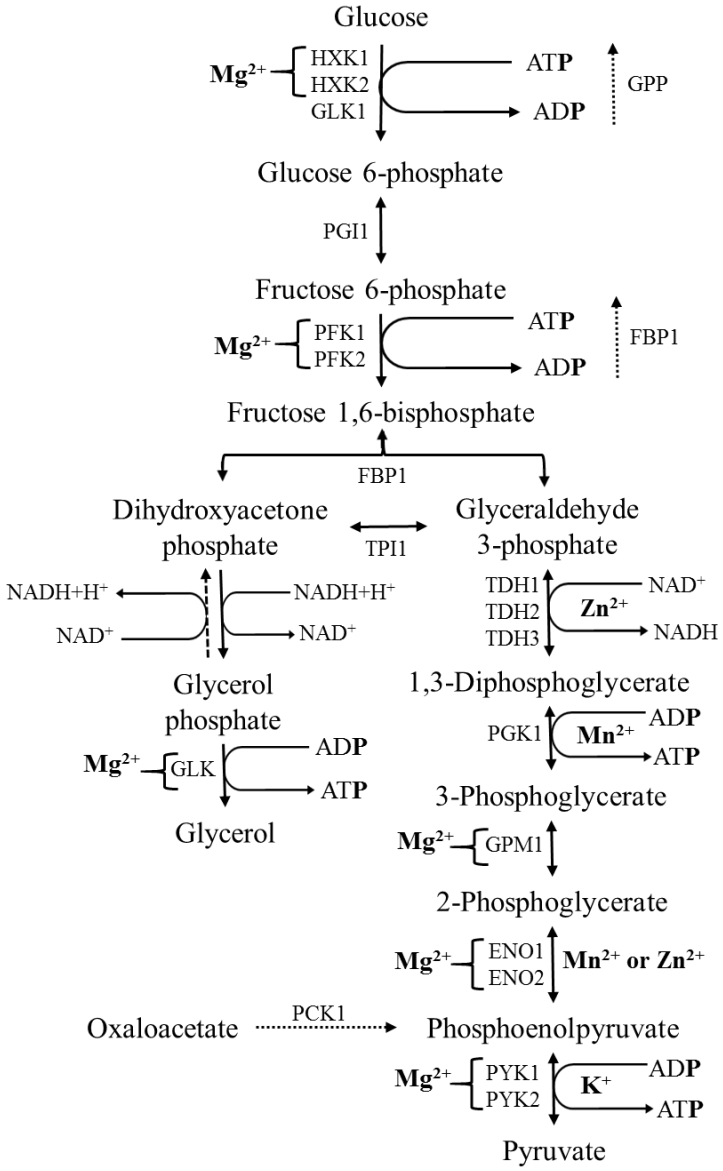

Fig. 1.9: The main glycolytic pathway. The genes and enzymes responsible for each step are: HXK1, HXK2, hexokinases (Magnesium as a cofactor); GLK1, glucokinase; PGI1, phosphoglucose isomerase; PFK1, PFK2, phosphofructokinase; FBP1, fructose 1, 6-bisphosphatase; FBA1, fructose 1, 6-bisphosphate aldolase (Magnesium as a cofactor); TPI1, triose phosphate isomerase; TDH1, TDH2, TDH3, glyceraldehyde 3-phosphate dehydrogenase; PGK1, phosphoglycerate kinase; GPM1, glycerophosphate mutase (Magnesium as a cofactor); ENO1, ENO2', enolase (Magnesium as a cofactor); PYK1, PYK2, pyruvate kinase (Magnesium as a cofactor). Three gluconeogenic enzymes are also shown: GPP, glucose 6-phosphate phosphatase; FBP1; fructose 1, 6-bisphosphatase; PCK1, phosphoenolpyruvate carboxykinase.

All glycolytic reactions end in pyruvate formation. Pyruvate is a very important precursor of various compounds, mainly organic acids such as citrate, malate, acetate, succinate, lactate and 2-oxoglutarate. In addition, 2-oxoglutarate, is an intermediate in the TCA cycle, important in glutamate metabolism (Akram 2014, Boulton and Quain 2001) and consequently, the synthesis of amino acids and higher alcohols generation.

1.6.2. Pyruvate

Pyruvate is a key intermediate in carbohydrate metabolism, which consists of a 3-carbon backbone and exists in two groups: ketone and carboxylate. In *Saccharomyces* yeast, pyruvate is present between fermentative and respiratory metabolism (Fig. 1.10). In anaerobic and oxygen-limited growth conditions, pyruvate is converted to acetaldehyde by pyruvate decarboxylase (Boubekeur *et al.* 2001). Acetaldehyde subsequently serves as an electron acceptor to re-oxidade NADH formed in glycolysis.

Pyruvate formed from glycolysis can be converted in the TCA cycle either to oxaloacetate via pyruvate carboxylase, or to acetyl-CoA via pyruvate dehydrogenase complex. In brewery systems, the majority of pyruvate is converted into acetaldehyde via pyruvate decarboxylase and then acetaldehyde into ethanol via alcohol dehydrogenase (Briggs *et al.* 2004, Boulton and Quain 2001). In the brewery, pyruvate decarboxylase, which converts pyruvate into acetaldehyde, is very active early in fermentation when wort sugar concentration is high due to the Crabtree effect (Walker and Stewart 2016).

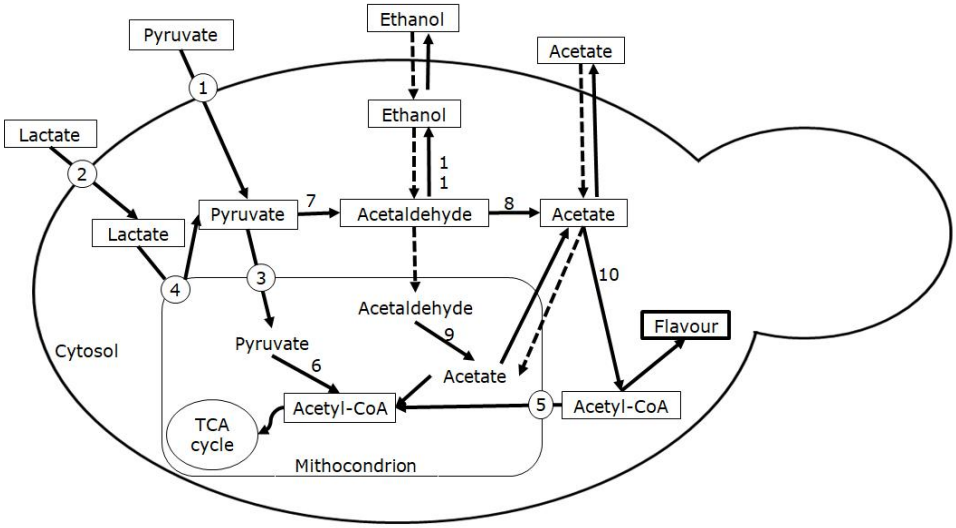

Fig. 1.10: Schematic summary of the various metabolic pathways involved during yeast *Saccharomyces* growth and fermentation considering different types of respiratory substrates: pyruvate, acetaldehyde, acetate and ethanol. Furthermore, acetyl-CoA role as key flavours precursors. Numbered reactions are catalysed by the following enzymes: (1) pyruvate carrier; (2) lactate carrier; (3) mitochondrial pyruvate carrier; (4) D-lactate and L-lactate dehydrogenase; (5) carnitine shuttle; (6) pyruvate dehydrogenase complex; (7) pyruvate decarboxylase; (8) acetaldehyde dehydrogenase-NADP1-dependent (Ald6p); (9) acetaldehyde dehydrogenase-NAD1-dependent (Ald4p); (10) acetyl-CoA synthase (ACS); (11) alcohol dehydrogenase (Adh2p).

During the respiratory growth of *Saccharomyces cerevisiae* on sugars, the pyruvate dehydrogenase complex converts pyruvate into acetyl CoA (Fig. 1.10). Formation of acetyl-CoA can occur via two mechanisms, which are 1) oxidative decarboxylation of pyruvate by the mitochondria, and 2) pyruvate dehydrogenase complex. Otherwise, the pyruvate dehydrogenase complex can be avoided by the intensive action of the enzymes pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl-CoA synthetase. This phenomenon happens when the concentration of sugars comes to be similar to ethanol concentration (mix of sugars-ethanol) or ethanol concentration becomes higher than sugars concentration (Kocharin and Nielsen 2013). Subsequently, two

carbon units of acetyl-CoA are the starting point for the TCA cycle (Jones *et al.*, 1993).

1.6.3. Tricarboxylic Acid (TCA)

In yeasts, eukaryotes cells, tricarboxylic acid (TCA) or Krebs cycle is an essential role reducing NADH and driving aerobic respiration to yield ATP via oxidative reactions. In addition, TCA cycle is an important biosynthetic pathway to build metabolites such as alfa-ketoglutarate, succinyl-CoA and oxaloacetate, which are required for synthesis of glucose and amino acids (Lee *et al.*, 2010). TCA cycle localizes in the mitochondrial matrix (Hellemond *et al.* 2005, Zatta *et al.*, 2000) and involves 8 enzymes (Fig. 1.11). TCA cycle has eight stages (Akram, 2014). These eight-stages can be well visualized in fig. 1.11.

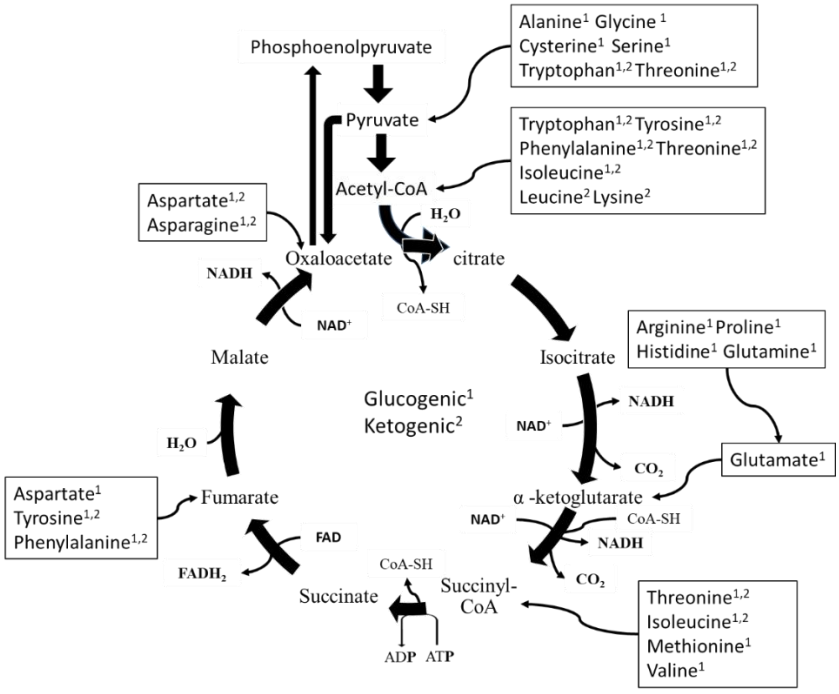

Fig. 1.11: TCA cycle plus amino acids involved on it

When *Saccharomyces cerevisiae* grows on sources from two carbon units such as acetate and ethanol, the TCA cycle cannot generate sufficient amounts of biosynthetic precursors by itself. Therefore, *Saccharomyces cerevisiae* modifies TCA cycle into the glyoxylate cycle, which converts two-carbon units (acetate) into four-carbon units (dicarboxylic acids) by oxidative decarboxylation (Lee *et al.*, 2010; Briggs *et al.*, 2004).

1.6.3.1. Glyoxylate cycle

Glyoxylate cycle is part of the gluconeogenic pathway, which is vital for growth on C2 carbon sources (including ethanol and acetate) and fatty acids (Lee *et al.*, 2010; Briggs *et al.*, 2004). Fundamentally, the glyoxylate cycle is a "modified tricarboxylic acid (TCA) cycle", which bypasses irreversible steps that result in CO_2 loss. The "modified TCA cycle" shares malate dehydrogenase, citrate synthase, and aconitase activities and has key enzymes, namely isocitrate lyase and malate synthase, which convert isocitrate and acetyl-CoA into succinate and malate respectively. Isocitrate lyase breaks down C6-units into succinate and glyoxylate, which generates free CoA-SH and malate by malate synthase with acetyl-CoA (fig. 1.12). Afterwards, malate dehydrogenase continues the cycle and succinate is released (Lee *et al.*, 2010; Kunze *et al.*, 2006). The glyoxylate metabolite names this metabolic pathway, because it allows the conversion of acetyl CoA from various catabolic pathways into 4-carbon units (succinate), which can be used to reload the TCA cycle and/or as precursors for amino acid biosynthesis or carbohydrate biosynthesis (Kunze *et al.*, 2006). The enzymes involved in the glyoxylate

cycle are induced by ethanol and inhibited by glucose. Generally, brewers do not consider glyoxylate cycle, because they claim that the brewing process finishes when sugars are completely consumed (Briggs *et al.* 2004). The understanding of acetyl-CoA formation in different metabolic pathways and, also, how this metabolite is consumed during fermentation is important because acetyl-CoA drives several metabolic activities and as a consequence flavour formation.

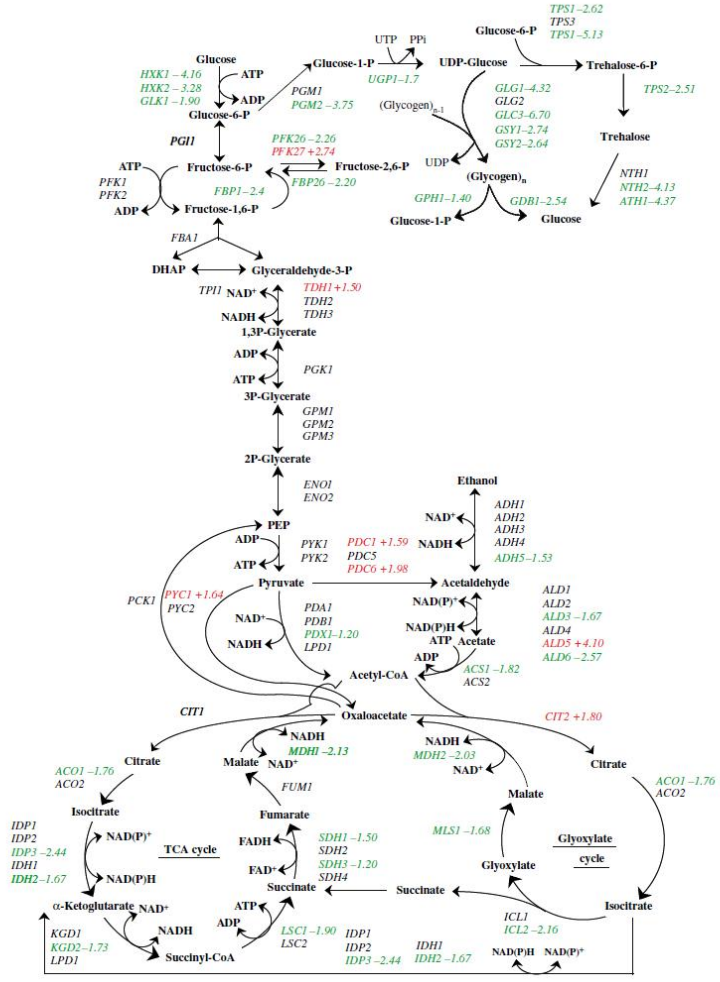

Fig. 1.12: Effect of glucose pulse on expression of genes of the glycolytic, storage carbohydrate and TCA cycle metabolic pathways. Green labels represent a downregulation and red labels represent an upregulation (Reproduced from Kresnowati *et al.* 2006)

1.7. Flavour

Nowadays, a large variety of beer styles exist in the market, ranging from light lager products to very dark and flavoursome ale style beers. Brewers provide a diversity of styles in response to consumer demand. Each beer style has its own particularity due different production processes, raw materials and yeast phenotype/genotype. Flavour production is influenced by malt processing, hops (or alternatives including various herbs or spices), water quality and yeast activity during fermentation.

The flavour-compounds generated can be divided into non-volatile and volatile compounds. Non-volatile compounds are invariably responsible for taste and they include sugars (including mono-, di-, tri-and poly-saccharides), inorganic salts, nucleotides, amino acids, proteins, polyphenols, small peptides and hop-flavours (Zufall and Tyrell 2008, Bailey and Ollis 1986). Volatile compounds are responsible for aroma, due to their greater vapour pressure. Although raw material processing contributes to beer flavour, in this study the emphasis is focused on flavour compounds produced during yeast fermentation. These flavours include higher alcohols and esters, as well as short and medium chain fatty acids, and fatty acids esters. These flavours are described in table 1.1 with their respective flavour descriptors, threshold and beer concentration.

Table 1.1: Flavour descriptors from each compound generated during yeast fermentation. Reproduced from ASBC Methods of Analysis: Beer Flavour Database (2011).

Compounds	**Flavour Descriptors**	**Threshold (g/L)**	**Beer (g/L)**
Ethanol	warming, strong, alcoholic	14	20-80
Glycerol	Viscosity	10	0.4-4
Acetic acid	acid, acetic, sour, pungent, vinegar	1.0	0-2.8
Acetaldehyde	grassy, green leaves, fruity, green apple, apple-skin, paint, sweet, pungent	20	1.2-37
Citric acid	Citrus-like, sour, odourless	3.5	6-240
Succinic acid	Tast-acid, astringevit, bitter	0.13	0.005-0.167
Lactic acid	Acid, neutral, pleasant sour	0.4	0.010-1.362
Higher alcohols		**Threshold (mg/L)**	**Beer (mg/L)**
N-propanol	alcoholic, solvent-like, Fruity	600	4-17
Isobutanol	alcoholic, malty, solvent-like, unpleasant	80	4-60
Isoamyl alcohol	alcohol, banana, sweet, aromatic, malty, vinous, pungent	0.06	5.6-140
Phenyl-2-ethanol	alcohol, flowery, honey-like, roses, sweet	0.04	2.3-100
Short- and medium chain fatty acids		**Threshold (mg/L)**	**Beer (mg/L)**
Isobutyric acid	Sweet, bitter, sour, Rancid, butter, cheesy	-	-
Butyric acid	buttery, rancid, cheesy, butyric	0.9	0.17-2.6
Isovaleric acid	sweat, cheese, rancid, old hops, floral, minty, sweet	0.33	0.1-3.5
Hexanoic acid	goaty, fatty acid, vegetable oil, sweaty, caprylic	8	0.5-8
Octanoic acid	Goaty, fatty acid, vegetable oil, sweat, cheese	13	2-15
Decanoic acid	waxy, caprylic, tallowy, rancid, soapy	10	1-3
Acetate esters		**Threshold (mg/L)**	**Beer (mg/L)**
Ethyl acetate	solvent, fruity, sweet, estery	20	5-50
Isobutyl acetate	Fruity	0.5	0.01-0.8
Isoamyl acetate	banana, sweet, fruity	0.5	0.3-8
Hexyl acetate	sweet, aromatic, perfumed, apple, cherry, pear, floral	3.5	0.003-0.015
Phenyl-2-ethanol acetate	honey, sweet-like, flowery (roses), honey, apple, sweet, flowery	0.2	0.05-2
	Fatty acid esters	**Threshold (mg/L)**	**Beer (mg/L)**
Ethyl butanoate	papaya, butter, sweet, apple, perfumed, fruity	0.4	0.004-0.4
Ethyl isobutyrate	apple, sweet, citrus, fruity, pineapple	5	0.002-0.01
Ethyl hexanoate	sour apple, aniseed, fruity, sweet, estery, solvent, apple, fatty acids	0.17	0.008-1.5
Ethyl octanoate	apple, sweet, fruity, Pineapple, sour apple, soapy	0.30	0.04-1.5
Ethyl decanoate	caprylic, fruity, apple	0.57	0.01-0.5

1.7.1. Acetaldehyde

Although acetaldehyde is not a compound directly investigated in this study, it is important to highlight its role as an intermediate compound in the generation of other metabolites within the cell. Acetaldehyde is a precursor for ethanol and forms the major route (via acetate) (Fig. 1.13) for the production of acetyl CoA, which in turn forms a precursor for ester formation (Saerens *et al.* 2006). In the final product, excess acetaldehyde can be problematic because it gives an unpleasant 'grassy' flavour/aroma. However, in some products acetaldehyde can be desirable when its concentration is above the flavour threshold of 20 ppm, giving the flavour/aroma of green apples (Briggs *et al.* 2004).

In yeast *Saccharomyces cerevisiae*, two enzymes support acetaldehyde dehydrogenases that are localized in: 1) mitochondria, and 2) cytosol. The mitochondrial enzyme requires NAD^+ or $NADP^+$ and K^+ for activity. Similarly, the cytosolic enzyme requires $NADP^+$ and is activated by Mg^{2+} (Fig. 1.13.). (Boubekeur *et al.* 2001, Remize *et al.* 2000). In the brewery, mitochondrial and cytosolic acetaldehyde dehydrogenases are active during the entire fermentation (Boubekeur *et al.* 2001, Remize *et al.* 2000, Briggs *et al.* 2004). The majority of acetaldehyde generated is driven to form ethanol, mainly, due to the Crabtree effect.

1.7.2. Ethanol

Ethanol is the most important industrial compound produced during fermentation. In the brewery, yeast *Saccharomyces cerevisiae* under glucose/carbohydrate repression produce ethanol via catabolic activity.

Proportionally, pyruvate derives from glycolysis and is then decarboxylated into acetaldehyde via pyruvate decarboxylase. Acetaldehyde reduces into ethanol via NAD^+-linked alcohol dehydrogenase (Briggs *et al.* 2004). During ethanol formation, ATP is formed exclusively from glycolysis and alcohol dehydrogenase is the main route for redox regeneration of NAD^+.

During the initial phase of fermentation in the brewery, yeast grows under glucose/carbohydrate repression and in the presence of oxygen. The phenomenon of glucose repression inhibits expression of genes encoding oxidative pathways (oxidative phosphorylation/electron transport chain). During fermentation when glucose is consumed from high to low levels, metabolism is switched proportionally back to the respiratory oxidative phosphorylation although is not enough to avoid the Crabtree effect (Briggs *et al.* 2004).

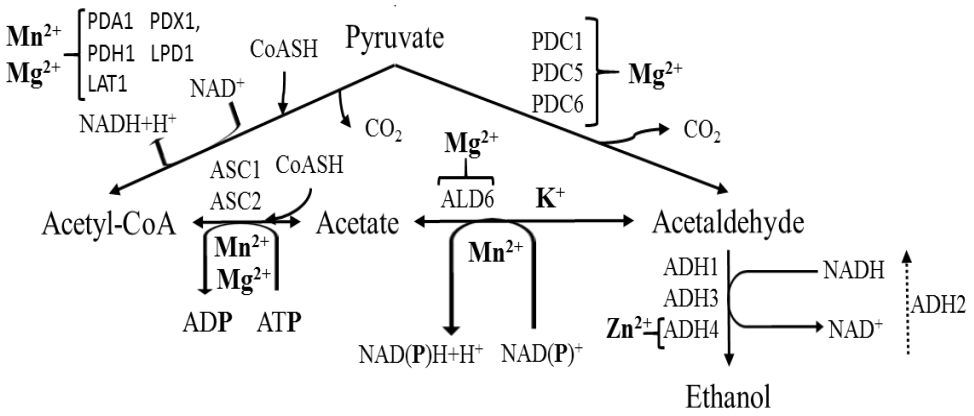

Fig. 1.13: Enzymes of pyruvate catabolism. The genes and enzymes for each step are: PDA1, PDH1, LAT1, PDX1, LPD1, pyruvate dehydrogenase complex (involves magnesium and manganese); PDC1, PDC5, PDC6, pyruvate decarboxylase (magnesium dependent); ADH1, ADH2, ADH3, ADH4, alcohol dehydrogenase (Only ADH4 is zinc-dependent); ALD6, aldehyde dehydrogenase (cytosolic is magnesium dependent and mitochondrial is manganese dependent, in addition potassium is involved); ACS2, acetyl-CoA synthetize (cytosolic is magnesium dependent and mitochondrial is manganese dependent).

Four genes encode yeast alcohol dehydrogenase (ADH), which includes ADH1, ADH2, ADH3, ADH4 and ADH5 (Fig. 1.13). Alcohol dehydrogenases (ADHs) are enzymes responsible for the reversible reduction of aldehydes to alcohol and alcohols to aldehydes (De Smidt *et al.* 2008, Briggs *et al.* 2004). In *Saccharomyces cerevisiae*, the optimal consumption of available carbohydrates occurs because of a balance between fermentative and respiratory carbon metabolism. This balance between fermentative and respiratory metabolism is physiologically controlled by ADH reactions. ADHs control the regeneration of glycosylic NAD, as result it regenerates the redox balance and reduces acetaldehyde to ethanol. ADH1, ADH3, and ADH4 are induced in the presence of glucose. In addition, ADH4 is activated when zinc is present as cofactor. ADH2 is inhibited by glucose but activated in the presence of ethanol (De Smidt *et al.* 2008) and forms a mechanism by which yeast can utilise ethanol as a carbon source by the formation of acetyl-CoA.

1.7.3. Acetyl-CoA

Acetyl-coenzyme A (Acetyl-CoA) is a central and crucial intermediate metabolite (Pietrocola *et al.* 2015). Acetyl-CoA is formed from glucose, fatty acid, and amino acid via catabolism (Shi and Tu 2015). Several metabolic activities involve acetyl-CoA, including the tricarboxylic acid cycle (TCA cycle), the glyoxylate cycle, fatty acid synthesis and β-oxidation, amino acid synthesis and sugar metabolism. Possibly, acetyl-CoA, ATP and NADH share key position in cell metabolic activities, because they can regulate several enzymes activities and gene expression via acetylation/de-acetylation reaction (Pietrocola *et al.* 2015, Galdieri *et al.* 2014). Consequently, acetyl-CoA

regulates flavour generation including higher alcohols, short chain fatty acids, esters and fatty acids esters (Fig.1.14).

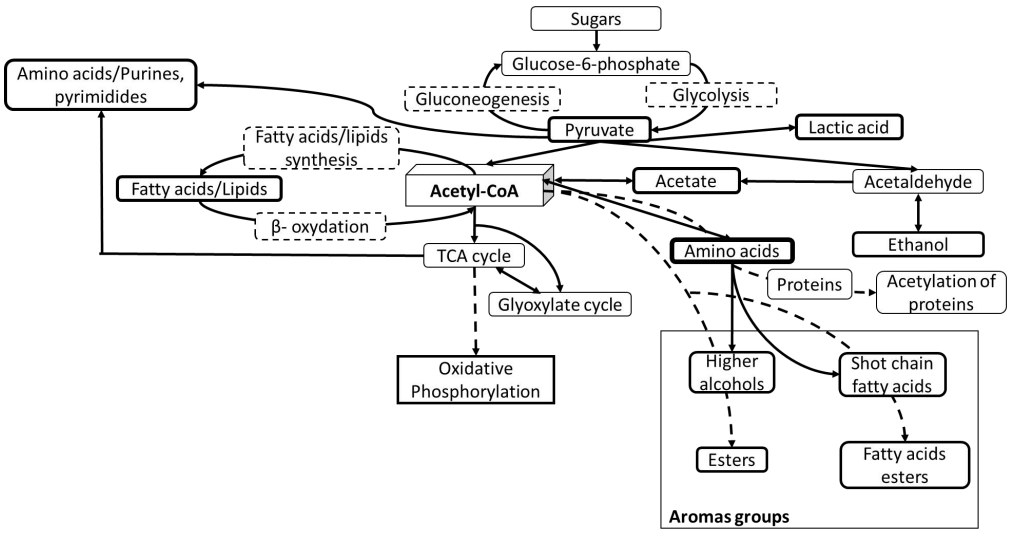

Fig. 1.14: Summary of acetyl-CoA metabolism.

In *Saccharomyces cerevisiae*, acetyl-CoA is spread into four subcellular organelles, the nucleus, mitochondria, cytosol and peroxisomes (Takahashi *et al.* 2006). The localization of acetyl-CoA is important for metabolite generation and secretion. Each organelle has different membrane permeability, and so when acetyl-CoA generates its bio-product precursor, their transport through the membrane may be affected by where they are generated (Kocharin and Nielsen 2013, Takahashi *et al.* 2006). The splitting of acetyl-CoA into each organelle influences the acetyl-CoA utilization into different metabolic pathways.

Acetyl-CoA localized in any organelle is affected by sugar consumption and controls different metabolic pathways. Acetyl-CoA-nuclear is an acetyl donor for histone acetylation (Chen *et al.* 2013, Takahashi *et al.* 2006). While, acetyl-CoA-mitochondrial is produced by the pyruvate dehydrogenase complex and is

fuel for the TCA cycle. On the other hand, the mitochondrial pool of acetyl-CoA is considered small and biochemically isolated; as a consequence mitochondrial acetyl-CoA cannot be used for protein acetylation in the nucleus or cytosol (Takahashi *et al.* 2006). Furthermore, acetyl-CoA-peroxisomal is produced from fatty acids by β-oxidation and afterwards used in the glyoxylate cycle (Kunau *et al.* 1995). As a final point, acetyl-CoA-cytosolic is responsible for formation of flavour-active compounds, which include acetate esters and fatty acid esters.

Acetyl-CoA-cytosolic, is synthesized by Acs1p or Acs2p; however these two enzymes synthesise acetyl-CoA differently due to their transcriptional regulation and kinetic properties (Van den Berg *et al.* 1996). Acs1p is found in mitochondria, nucleus, cytoplasm and peroxisome (Chen *et al.* 2012; Devirgilio *et al.* 1992) and whilst Acs2p is located in the cytoplasm, nucleus and possibly the endoplasmic reticulum (Chen *et al.* 2012; Huh *et al.* 2003). Acs2p is mainly responsible for extra-mitochondrial acetyl-CoA production from ethanol (Takahashi *et al.* 2006). Asc1p is repressed at high sugar concentration (Crabtree effect) and contributes to acetyl-CoA synthesis in presence of non-fermentable sugars or during gluconeogenesis (Kocharin and Nielsen 2013, Kratzer and Schuller 1995). ACS2 prevents, directly and indirectly, the glucose repression of ACS1 (van den Berg *et al.* 1996). When *Saccharomyces cerevisiae* consumes or grows in ethanol and/or acetate rich medium, the glyoxylate cycle occurs in the cytosol (Lee *et al.* 2011) effecting cytosolic acetyl-CoA and consequently the flavour formation.

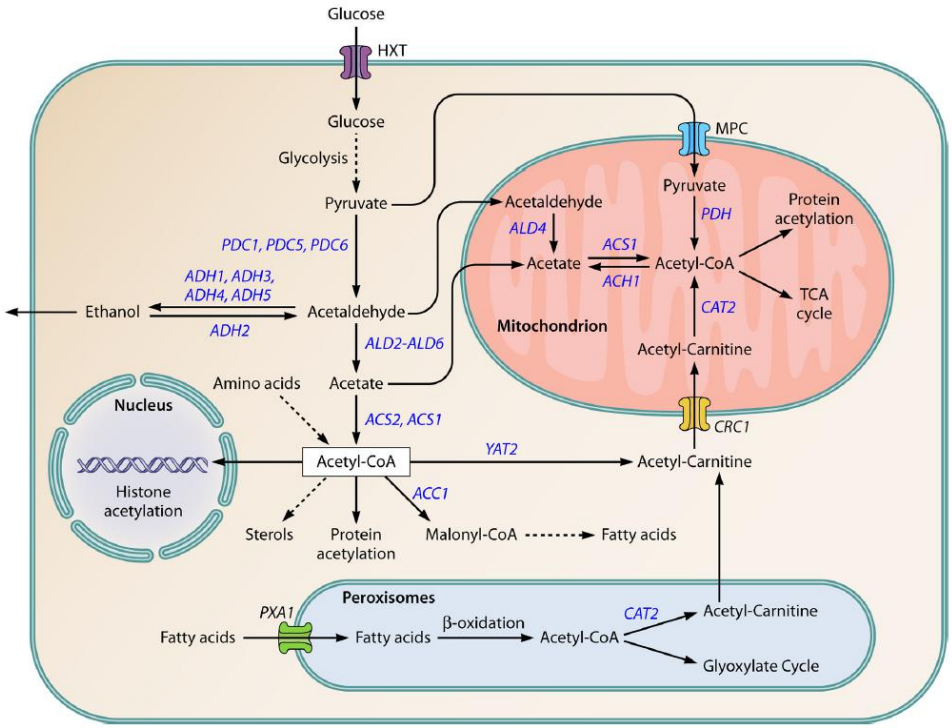

Fig. 1.15: Acetyl-CoA metabolism in budding yeast. Multistep pathways of glycolysis and fatty acid synthesis are indicated by dashed lines (Reproduced from Galdieri *et al.* 2014).

Glucose and ethanol presence have a clear influence on acetyl-CoA generation. Firstly, glucose is the preferred source of carbon and energy for the yeast *Saccharomyces cerevisiae* (Galdieri *et al.* 2014). In the brewery, yeast grows in rich glucose medium under aerobic conditions and cells tend to increase the glycolysis rate converting glucose into pyruvate very rapidly, due to the Crabtree effect. The Crabtree effect inhibits oxidative phosphorylation and only a fraction of pyruvate is transported into mitochondria and converted to acetyl-CoA by the PDH complex. As a consequence, a reduction of the tricarboxylic cycle and respiration rate occur (Heyland *et al.* 2009, Gombert *et al.* 2001). Then, pyruvate is driven to form acetaldehyde by pyruvate decarboxylase (encoded by PDC1, PDC5, and PDC6 genes) (Pronk *et al.* 1996) and the majority of the acetaldehyde is converted into ethanol by alcohol

dehydrogenase (Heyland *et al.* 2009, Gombert *et al.* 2001). PDC1 is responsible for the widely held of the pyruvate decarboxylase. However, under ethanol presence, PDC1 is repressed and as a consequence, acetaldehyde is driven to form acetate by acetaldehyde dehydrogenase (Liesen *et al.* 1996).

The yeast genome encodes five acetaldehyde dehydrogenases, ALD2, ALD3, ALD4, ALD5 and ALD6 (White *et al.* 2003). Ald2p, Ald3p and Ald6p are cytosolic proteins; however, only Ald2p and Ald3p are involved in the synthesis of acetyl-CoA (Galdieri *et al.* 2014, White *et al.* 2003). Ald4p and Ald5p are both mitochondrial proteins but in the presence of glucoseAld4p is inhibited and Ald5p is expressed. In parallel to ethanol formation, small portions of acetaldehyde are converted to acetate (Turcotte *et al.* 2010, Smith *et al.* 2004). Initially, acetate is produced by Ald6p in the cytosol and transported into mitochondria to generate acetyl-CoA (Galdieri *et al.* 2014, Meaden *et al.* 1997, Dickinson *et al.* 1996). During glucose limitation, acetaldehyde is also transported into mitochondria (Fig. 1.15) where it is converted to acetate (Galdieri *et al.* 2014), and then acetyl-CoA.

1.7.4. Higher alcohols

Higher alcohols are often referred to as “fusel alcohols” and their synthesis occurs via anabolic and catabolic pathways (Ehrlich 1904). From anabolic or catabolic processes, alpha-keto acids are intermediate precursors of higher alcohols and short chain fatty acids. However, the most important higher alcohols and short chain fatty acids are synthetized through the Ehrlich metabolic pathway, which is a catabolic process (Hazelwood *et al.*, 2008,

Ehrlich, 1907). Higher alcohols have aliphatic (medium-chain compounds of four or five carbons units) or aromatic side-residues (Tyrosine, tryptophan and phenylalanine), which are from branched-chain amino acids (including leucine, valine, and isoleucine) and aromatic amino acids (including tyrosine, tryptophan and phenylalanine) (Vidal *et al.* 2014).

In the brewery, it is well-known that yeasts produce higher alcohols during fermentation due to initial amino acids content and that they constitute a group of organoleptic compounds which confer alcoholic taste and warming character (Boulton and Quain, 2001). Valine, leucine, isoleucine and phenylalanine are the most important amino acids, which are precursors of isobutanol, 3-methylbutanol, 2-methylbutanol and 2-phenethyl alcohol, and their respective esters. Higher alcohols are immediately secreted into the media, or esterified with acetyl-CoA to synthetize their respective esters because they cannot be used as a carbon source for central metabolism (Vidal *et al.* 2014, Ashraf 2011, Hazelwood *et al.* 2008).

Higher alcohol formation involves a sequence of key reactions in the Ehrlich pathway, which include transamination (transaminase), decarboxylation (decarboxylase) and reduction (dehydrogenase) (Hazelwood *et al.* 2008). The stages of higher alcohols formation are described below and visualized in fig. 1.16.

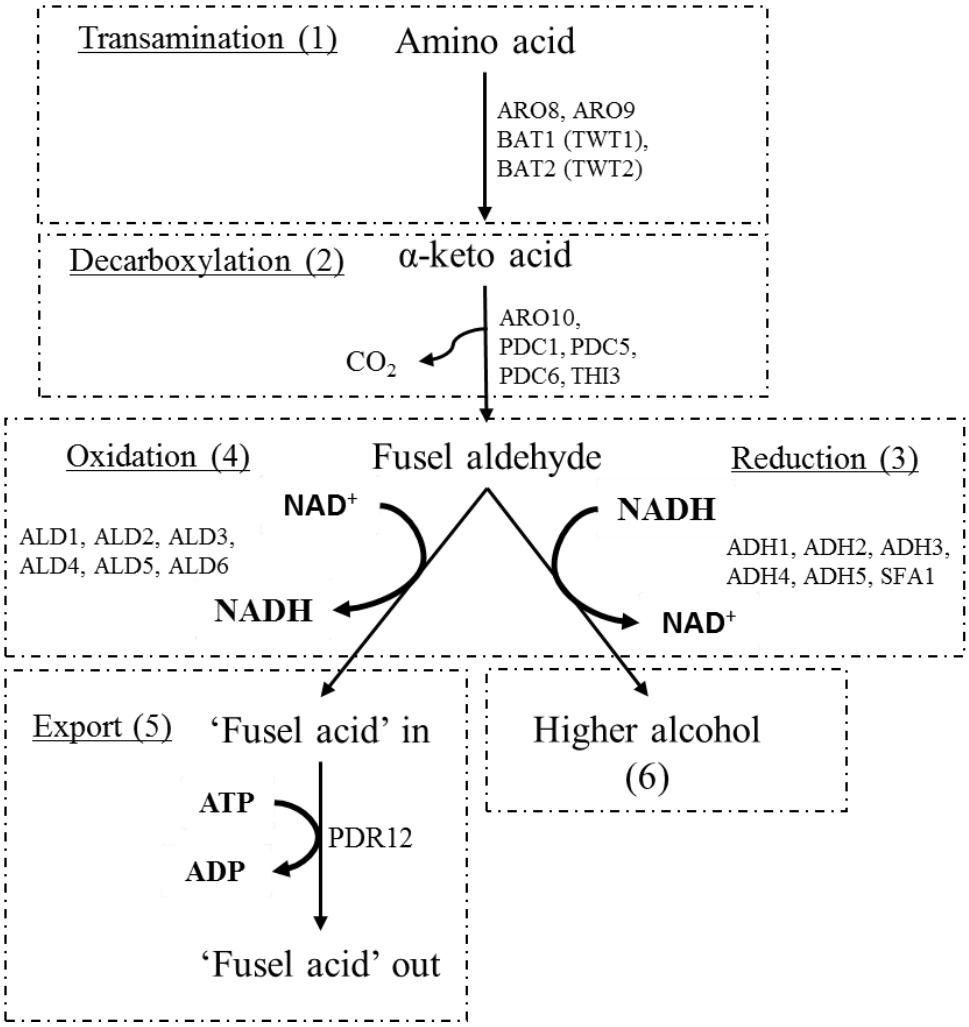

Fig. 1.16: The Ehrlich pathway. Catabolism of branched-chain amino acids (leucine, valine, and isoleucine), aromatic amino acids (phenylalanine, tyrosine, and trytophan), and the sulfur-containing amino acid (methionine) leads to the formation of fusel acids and fusel alcohols. The genes encoding the enzymes of each step are indicated (Hazelwood *et al.* 2008). In addition, were highlighted 6 reaction steps, where steps 1-2 and 4-5 are related to fusel acids formation and steps 1-3 and 6 are related to higher alcohols formation.

Firstly, transamination is the initial reaction of the Ehrlich pathway, which involves four enzymes including BAT1 (TWT1), BAT2 (TWT2), ARO8 and ARO9 (Hazelwood *et al.* 2008). BAT1 (TWT1) is a mitochondria isozyme and BAT2 (TWT2) is the cytosolic isozyme. Mitochondrial isozyme (BAT1 (TWT1)) is highly expressed during exponential growth and inhibited during stationary phase. Conversely, cytosolic isozyme (BAT2 (TWT2)) is inhibited

during exponential growth and highly expressed during stationary phase (Vidal *et al.* 2014, Hazelwood *et al.* 2008). Additionally, Aro8p and Aro9p support amino acid transaminases in the Ehrlich pathway because they have broad-substrate-specificity. During *S. cerevisiae* growth under glucose limitation, ARO9 and BAT2 (TWT2) regulate the Ehrlich pathway, because these genes are overexpressed under glucose limitation and in the presence of the amino acids, which are involved in the Ehrlich pathway (Hazelwood *et al.* 2008).

Second, decarboxylation involves 4 genes (including ARO10, PDC1, PDC5, PDC6 and THI3), which are thiamine diphosphate (ThDP)-dependent. THI3 acts as thiamine diphosphate sensor (Hazelwood *et al.* 2008, Vuralhan *et al.* 2003). During valine catabolism, three isozymes (Pdc1p, Pdc5p, and Pdc6p) will decarboxylate isovaleraldehyde into isobutanol (Dickinson *et al.* 1998). However, at the current time the valine and leucine decarboxylase encoded by the ARO10 (YDR380w) gene is not well characterized (Hazelwood *et al.* 2008, Dickinson *et al.* 2000). In isoleucine catabolism, all decarboxylases encoded by PDC1, PDC5, PDC6, ARO10, and THI3 can convert isoleucine into amyl alcohol (Hazelwood *et al.* 2008, Dickinson *et al.* 2000). Kneen *et al.* (2011) demonstrated that the *Saccharomyces cerevisiae* ARO10 gene is involved in phenylpyruvate decarboxylase and consequently aromatic amino acid catabolism, however it does not affect aliphatic amino acid reduction and these findings reinforced the idea that decarboxylases are ThDP-dependent.

The third and final step in the Ehrlich pathway is reduction, leading to higher alcohol formation. In the yeast cell, oxidoreductases catalyse the formation of higher alcohols from fusel aldehydes. The *Saccharomyces cerevisiae* genome has 16 alcohol dehydrogenases, 6 aldehyde dehydrogenases and 2 broad

spectrum reductases (Hazelwood *et al.* 2008, Dickinson *et al.* 2003); however, the Ehlrich pathway can occur by any of the alcohol dehydrogenases encoded by ADH1, ADH2, ADH3, ADH4, ADH5 or by SFA1 (formaldehyde dehydrogenase). None of the enzymes involved in the final step of the Ehrlich pathway have been shown to play distinct roles in the reduction of individual higher alcohols (Hazelwood *et al.* 2008, Dickinson *et al.* 2003).

Finally, the mechanism responsible for higher alcohol transport in *S. cerevisiae* is diffusion across the lipid bilayer (Hazelwood *et al.* 2008, Lipinski *et al.* 2001) which involves one plasma membrane transporter (PDR12). The function of Pdr12p via Ehrlich pathway is export of fusel alcohols from leucine, isoleucine, valine, phenylalanine, and tryptophan, which is ATP-dependent (Hazelwood *et al.* 2008, Hazelwood *et al.* 2006). Table 1.2 shows amino acids, α-keto acid, fusel aldehyde and higher alcohols (fusel alcohol) involved via Ehrlich pathway. It should be noted that the Ehrlich pathway occurs irrespective of the presence of oxygen, and oxygen concentration does not affect the Ehrlich pathway during fermentation (Vidal *et al.* 2014). However, production of short chain fatty acids (fusel acids) via the Ehrlich pathway might be indirectly affected when oxygen availability is limited (Yu *et al.* 2016, Hazelwood *et al.* 2008).

Table 1.2: Ehrlich pathway intermediates

Amino acids	α-Keto acid	Fusel aldehyde	Fusel alcohol
Valine	α-Ketoisovalerate	Isobutanal or isovaleraldehyde	Isobutanol
Leucine	α-Ketoisocaproate	Isoamylaldehyde	Isoamyl alcohol
Isoleucine	α-Ketomethylvalerate	Methylvaleraldehyde	2-Methylbutanol
Phenilamina	Phenylpyruvate	2-Phenylacetaldehyde	2-Phenylethanol
tyrosine	*p*-Hydroxyphenyl pyruvate	*p*-Hydroxy-phenyl-acetaldehyde	p-Hydroxy-phenyl-ethanol or tyrosol
tryptophan	3-Indole pyruvate	3-Indole acetaldehyde	Tryptophol
Methionine	α-Keto-(methylthio) butyrate	Methional	Methionol

Reproduced from Hazelwood *et al.* (2008)

1.7.5. Short and medium chain fatty acids

Short chain fatty acids (SCFA) are carboxylic acids (four to six carbon units). *Saccharomyces cerevisiae* is able to produce three short chain fatty acids via the Ehrlich pathway. In the brewery, yeast are able to produce isobutyric acid, isovaleric acid and 2-methylbutyric acid via catabolism of the amino acids valine, leucine and isoleucine, respectively (Yu *et al.* 2016, Hazelwood *et al.* 2008). SCFA levels produced during fermentation are very low (Yu *et al.* 2016) and are often considered undesirable off-flavours (Boulton and Quain, 2001). However, SCFA are involved in fatty acids - esters formation and can also affect beer viscosity.

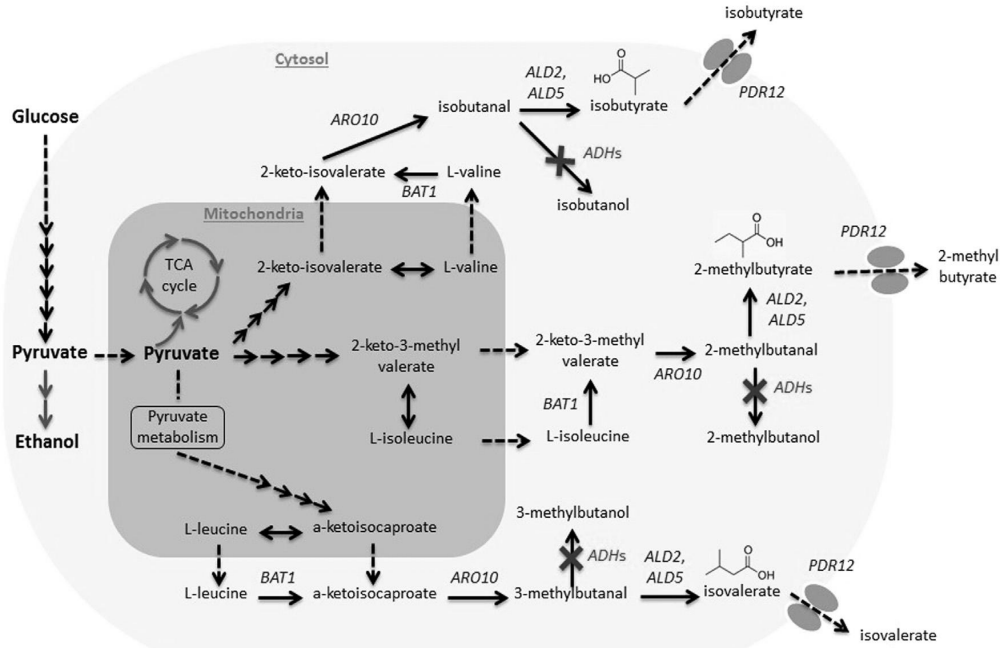

Fig. 1.17. Catabolism of amino acids (isoleucine, leucine and valine) that leads the biosynthesis of Short Chain Fatty Acids (SCFAs) which includes 2-methyl butyric acid (2MBA), isovaleric acid (IVA), isobutyric acid (IBA) via Ehrlich pathway in *Saccharomyces cerevisiae*. SCFAs are excreted into the medium by PDR12 transporter (Reproduced from Yu *et al.* 2016).

Fig. 1.17 illustrates SCFA biosynthesis in *S. cerevisiae* via the Ehrlich pathway (Yu *et al.* 2016). Similar to higher alcohols biosynthesis, SCFA biosynthesis involves a sequence of key reactions, which includes transamination (transaminase), decarboxylation (decarboxylase) and oxidation (oxygenase) (Yu *et al.* 2016, Hazelwood *et al.* 2008). Transamination (transaminase) and decarboxylation (decarboxylase) are performed by BAT1 and BAT2 transaminases to convert amino acids (valine, leucine and isoleucine) to their respective α-keto acids. ARO10, THI3, PDC1, PDC5 and PDC6 encode decarboxylases to convert α-keto acids to their respective short branched-chain aldehydes (Yu *et al.* 2016, Hazelwood *et al.* 2008, Lilly *et al.* 2006, Vuralhan *et al.* 2003). Differentto higher alcohols formation, SCFA involves ALD2, ALD3, ALD4, ALD5 and ALD6 encoded aldehyde dehydrogenases, which

oxidize short branched-chain aldehydes into SCFA. (Yu *et al.* 2016, Pigeau and Inglis 2007). Finally, SCFA is secreted by the same native Pdr12p ATP-dependent transporter involved in the export of higher alcohols (Hazelwood *et al.* 2008, Pigeau and Inglis 2007). Yu *et al.* (2016) demonstrate that when SCFAs are overexpressed ADHs show incomplete pathways.

1.7.6. Esters

During fermentation, flavour-active substances can be identified and divided into 6 main groups: organic acids, higher alcohols, carbonyl compounds, sulfur-containing molecules, phenolic compounds and volatile esters (Saerens *et al.* 2010). Volatile esters are a very important group of compounds produced during yeast fermentation because they are flavour-active (Ashraf 2011, Saerens *et al.* 2010). In beer, esters have a low threshold for detection; they are arguably the most important compounds which compose flavour complex and effect the sensory quality of alcoholic beverages.

Esters are divided into two categories, acetate esters and medium-chain fatty acid ethyl esters (FAEE). The first ester group comprises acetate esters (solvent-like aroma), isoamyl acetate (banana aroma), isobutyl acetate (fruity aroma) and phenyl ethyl acetate (roses, honey). Their formation depends on acetyl-CoA, alcohols and their enzymes involved in synthesis and hydrolysis. The second group includes ethyl hexanoate (aniseed, apple-like aroma) and ethyl octanoate (sour apple aroma) and their formation depends on acetyl-CoA, medium-chain fatty acid (MCFA) and their enzymes involved in synthesis and hydrolysis (Saerens *et al.* 2010). Yeast produces aroma-active esters in the

cytosol and which then diffuse into the wort through the plasma membrane due to their lipid soluble capacity (Saerens *et al.* 2010).

1.7.6.1. Acetate Esters

Ester formation requires two substances (an alcohol and acetyl-CoA) which combine due to the function of their associated enzymes. Ethanol (or higher alcohols) are formed during the catabolic activity of fermentation (Section 1.7.4.). Acetyl-CoA is generated from different pathways and is another key metabolite (Section 1.7.3). However, only cytosolic acetyl CoA is involved in ester formation. Acetate ester synthesis involves alcohol acetyl transferases including AATase I, AATase II and Lg-AATase I, which are encoded by the genes ATF1, ATF2 and Lg-ATF1, respectively (Saerens *et al.* 2010). Whereas ATF1 and ATF2 are found in ales strains (*Saccharomyces cerevisiae*) and lager strains (*Saccharomyces pastorianus*); Lg-ATF1 is present only in lager strains (*S. pastorianus*) (Ashraf 2011, Saerens *et al.* 2010, Yoshimoto *et al.* 1998). In addition, sequencing of the *Saccharomyces cerevisiae* genome has not identified any other genes potentially similar to ATF1 and/or ATF2.

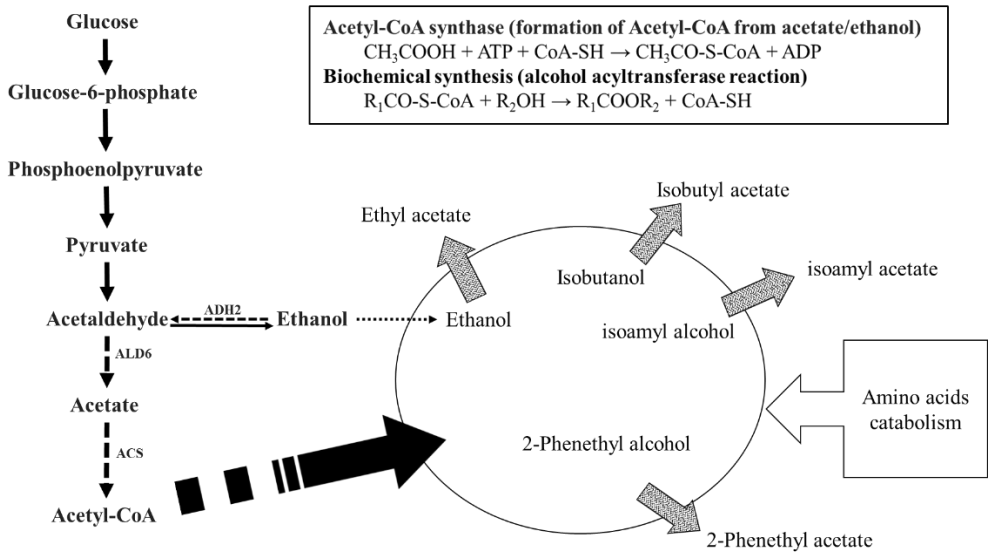

Fig. 1.18. Acetyl-CoA synthetase from acetate/ethanol and Biochemical synthesis of esters through alcohol acyltransferase

ATF1 plays a major role during acetate esters formation and the inhibition or absence of ATF1 can result in a reduction from 60 to 90% in ester concentrations compared to the level in wild-type cells. ATF2 and Lg-AFT1 have only a very limited role in ester formation (Verstrepen *et al.* 2003). Inhibition or disruption of ATF1 and ATF2 affect the acetate ester formation, preventing the formation of isoamyl acetate, amyl acetate, pentyl acetate, hexyl acetate, heptyl acetate, octyl acetate, or phenylethyl acetate (Fig. 1.18) during fermentation (Ashraf 2011, Saerens *et al.* 2010, Verstrepen *et al.* 2003).

1.7.6.2. Fatty acid ethyl esters

Although acetate ester generation is conducted by alcohol acetyl transferases, the formation of ethyl esters does not involve ATF1 and ATF2 genes (Verstrepen *et al.* 2003). Ethyl ester formation involves short- and medium-chain fatty acids, ethanol, acetyl-coA and their enzymes (EEB1 and EHT1).

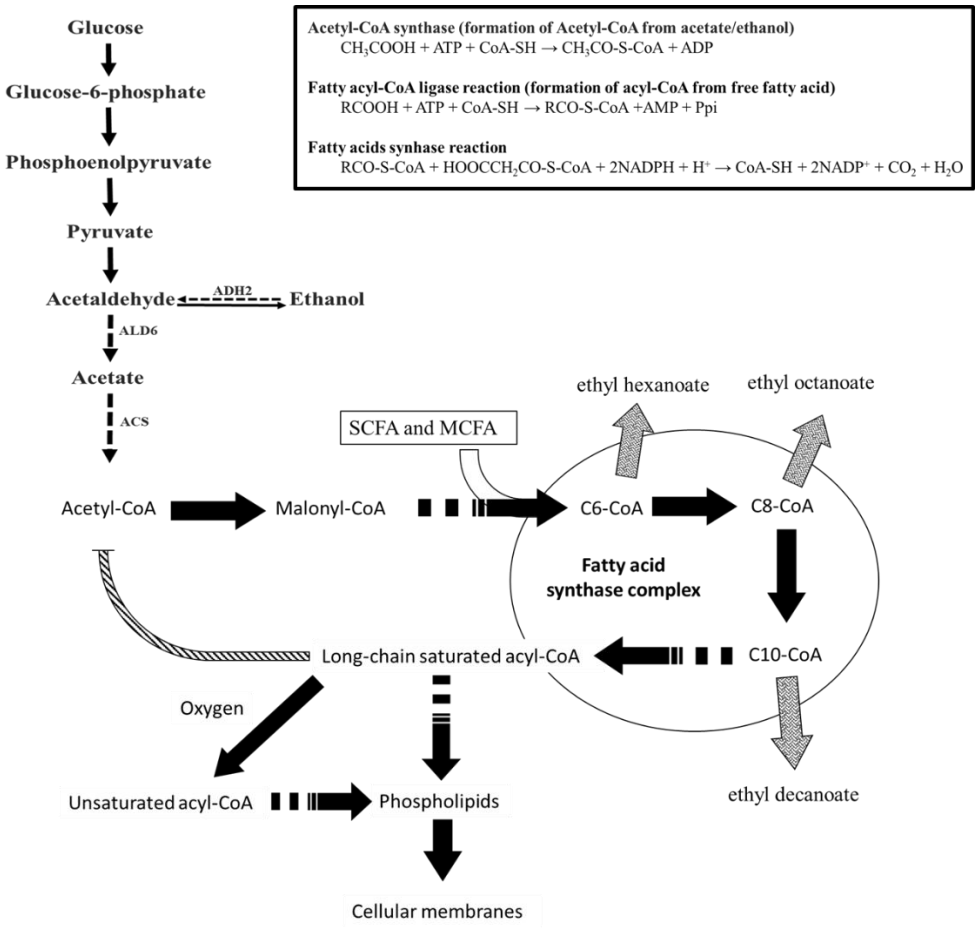

Fig. 1.19. Biosynthesis of fatty acids ethyl esters and its relationship with short chain fatty acids (SCFA), medium chain fatty acids (MCFA) and acetyl-CoA syntetase. In the presence of oxygen, SCFA and MCFA merger with acetyl-CoA initiating fatty acid synthesis, and then long-chain saturated acyl-CoAs are converted to unsaturated acyl-CoAs, which generates saturated, unsaturated fatty acids and as a result, the synthesis of phospholipids, which are then incorporated into cellular membranes. During unsaturated fatty acids formation, acetyl-CoA formation is not inhibit although no longer formation of acetyl CoA from fatty acid (β-oxidation) occurs. As a result, medium-chain fatty acid CoAs are released from the fatty acid synthase complex, which can be converted to the equivalent ester.

Fatty acid ethyl esters (FAEEs) are produced from yeast secondary metabolites. During alcoholic fermentation, volatile medium-chain fatty acid ethyl esters generate desirable fruit aromas (Knight *et al.* 2014). Although, the biochemical synthesis of medium-chain fatty acid ethyl esters is only partly understood, it is known to involve the enzyme acyl-CoA:ethanol O-acyltransferases

(AEATases), encoded by EEB1 and EHT1 (Nancolas *et al.* 2017, Knight *et al.* 2014, Saerens *et al.* 2008; Saerens *et al.* 2006; Dufour *et al.* 2003). EEB1 and EHT1 catalyse a condensation reaction between ethanol and an acyl-CoA. FAEE biosynthesis occurs via acyl-CoA:ethanol O-acyltransferases (AEATases) enzymes, which transfer fatty acyl groups from acyl-CoA to ethanol (Knight *et al.* 2014, Saerens *et al.* 2010, Saerens *et al.* 2006, Mason and Dufour 2000). Then, FAEEs are generated (Fig. 1.19), which includes ethyl hexanoate (pleasant apple-like), ethyl octanoate (aniseed) and ethyl decanoate (floral aromas).

FAEEs synthesis involves both Eeb1 and Eht1 in the esterification process; but Eht1 plays only a minor role (Saerens *et al.* 2006). The role of Eeb1 and Eht1 in esterification and Atf2 in acetylation of sterols, suggest that ester synthesis is a mechanism for cellular detoxification (Knight *et al.* 2014, Saerens *et al.* 2010, Saerens *et al.* 2006).

Under anaerobic conditions, yeasts accumulate free CoA and the primary of AEATases role is to recuperate free CoA from medium-chain acyl-CoAs (Knight *et al.* 2014). Like ethanol, free fatty acids can inhibit cell growth and fermentation and as a result, Eht1 can be sequestered into the lipid particle/mitochondria to stimulate protein interactions with acyl-CoAs and ethanol, and then, FAEEs synthesis is stimulated to detoxify fatty acids and ethanol (Knight *et al.* 2014, Saerens *et al.* 2006).

The formation of all compounds from yeast secondary metabolism are susceptible to several fermentation parameters, which include temperature, dissolved oxygen, wort gravity (includes composition of sugars, amino acids,

vitamins, minerals, lipids, etc and osmotic pressure), specific growth rate, pitching rate, etc.

1.7.7 VDK

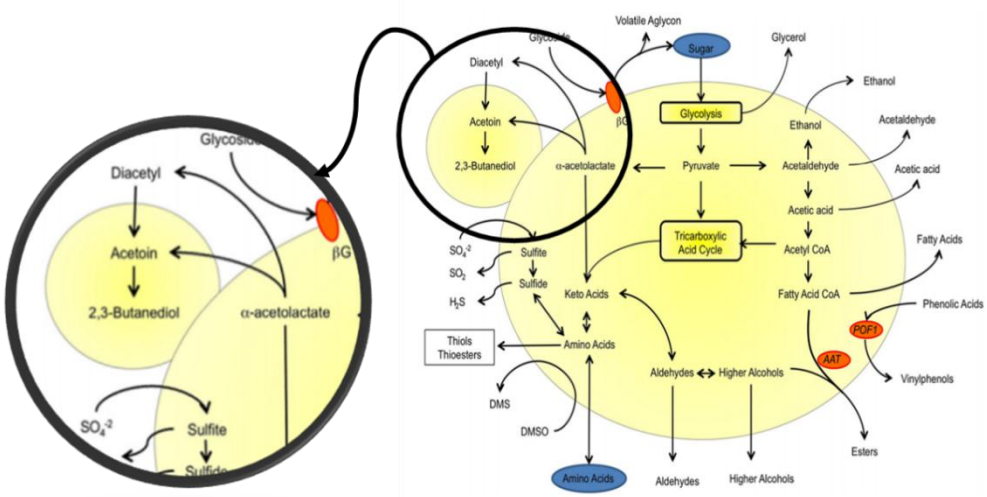

Fig. 1.20: Schematic summarizes the main metabolic pathways of *Saccharomyces* influencing beer quality due to their link to beer flavour (Reproduced from Bokulich and Bamforth 2013).

During fermentation, all brewing yeast produce vicinal diketones (VDKs). The most important vicinal diketones (VDKs): diacetyl (2,3-butanedione) and 2,3-pentanedione, are produced from valine and isoleucine synthesis, respectively. However, they are undesirable for most beers because are responsible for a buttery, butterscotch, or honey-like flavour (Stewart 2017, Bokulich and Bamforth 2013). Diacetyl has a threshold around 0.1–0.2 mg/L (in lagers) and 0.1–0.4 mg/L (in ale) and typically there is a requirement for levels to be below this prior to the end of fermentation (Stewart 2017, Krogerus and Gibson 2013, Bokulich and Bamforth 2013). Although 2,3-pentanedione presents an analogous flavour, it is identified as toffee-like and occurs with threshold around 1 mg/L (Stewart 2017). At detectable concentrations, diacetyl is

acceptable in some beer styles such as Czech Pilsners and some English ales (Stewart 2017, Krogerus and Gibson 2013). However, diacetyl metabolism has been the topic of significant controversy and it may also be generated during beer ageing/beer packaging via the reaction or oxidation of acetoin and 2,3-butanediol (Krogerus and Gibson 2013).

1.8. Overview of essential inorganic elements in flavour formation

After summarize the most important pathway for supporting this work, which included driving carbon and essential inorganic element individual role. Therefore, this section summarizes the importance of essential inorganic elements and as a consequence their possible influence in the flavour formation. Table 1.3 gives an overview of mineral requirement in yeast cell and as the many essential inorganic elements, which perform biochemical forms, functions and enzymes activations; and as a consequence, the possible influence of minerals in flavour formation.

Table 1.3: Summary of biochemistry forms and function of minerals

Mineral	Major forms	Functions	Enzyme activation
Nitrogen	Amino acids and ammonia-nitrogen	Growth, structure, protein structure, amino acid recycling	Enzymes involved in Ehrlich pathway and **flavour formation**
Phosphorus	Phosphate	Sugar phosphates, phospholipids, ATP, RNA, DNA	ATP generation in cytosol. Mg-ATP link is an essential co-factor in enzymes that hydrolase ATP for energy generation, **Flavour**
Potassium	Free ions	Ion currents, osmotic balance	Transferases/ Pyruvate kinase Aldehyde dehydrogenase **Flavour**
Magnesium	Complex with ATP	Enzyme cofactor, ATP activation, substrate binding	Transferases/ ATPases, Kinases, Enolase, hydrolysis, acetyl CoA synthetase, glutamine synthetase **Flavour**
Iron	non-heme	Oxygen binding, electron transport	Oxidoreductases/ Oxidative enzymes electron-transport (redox) enzymes, oxygenases, dismutases
Zinc	Protein-bound, Substrate binding structural stability	Metalloenzymes, cell signaling	Lyases, ligases/ Hydrolases, synthases, Alcohol dehydrogenase (ADH4) and Cu-Zn superoxide dismutase (SOD1)
Copper	Protein-bound, Dioxygen activation	Cu-metalloenzymes	Oxidoreductases/ Cu-Zn superoxide dismutase (SOD1) electron-transport (redox) enzymes, oxygenases, dismutases
Manganese	Protein-bound, Intramolecular shifting	Mn-metalloenzymes	Oxidoreductases/ Mn superoxide dismutase (SOD2) electron-transport (redox) enzymes, oxygenases, dismutases

Essential inorganic elements (including nitrogen, potassium, phosphorous and magnesium and heavy metals such as iron, zinc, copper and manganese) can impact in flavour formation during fermentation because they influence yeast physiology, growth and as a consequence metabolic activities. The amount of these compounds (mainly heavy metals) in the end product, might contribute to destabilization of flavour compounds (Wietstock *et al.* 2015, Zufall and Tyrell 2008), which is recognized as an industrial problem.

1.9. Statistical and design support

Considering the complexity of measuring eight essential inorganic elements and understanding their individual influence and interactions, therefore, multivariable analysis were used in this work. This section summarizes the general understanding about these techniques, which have been applied to bioprocesses. Bioprocesses involve multiple independent variables and can inhibit application of several engineering principles. Likewise multiple dependent variables can be problematic for describing what is going on during bioprocess (Deepak *et al.* 2008). Several statistical analyses have been used as basic tools for authentication and classification of analytical data; however, two advanced statistical tools have been used for authentication, classification, trends, correlations and optimization among an original set of variables. In this thesis, two statistical techniques were applied to describe process phenomena and their interactions, they are the multivariate method: 1) Principal Component Analysis (PCA) and 2) Response Surface Methodology (RSM) for experimental design.

1.9.1. Principal component analysis (PCA)

A large group of qualitative and quantitative variables from different populations can be analysed using a group of multivariate techniques simultaneously. Multivariate method (MA) is a statistical group, which has several available tools to analyse a large amount of data (Ribeiro *et al.* 2015). MA allows for greater discriminating power, eliminating those difficult to measure variables and those that contribute towards explaining variation (Da Silva *et al.* 2016). MA permits for simultaneous, consistent and useful interpretations using correlations among a large number of variables (Ferreira *et al.*, 2009). Among multivariate methods, principal component analysis (PCA) is the most popular best established multivariate technique and has been used in almost all scientific areas (Abdi and Williams 2010). In recent years, PCA has been used in food science and brewing science, and specifically in investigations into food/brewing flavour.

PCA is a technique that models covariance through linear transformation from an original set of variables to a significantly smaller group of components and has been used to describe those variables that explain the total variation. PCA permits the estimation of interactions between variables (which are, generally, inter-correlated) reducing an original set of variables to a smaller number of independent parameters, simplifying interpretation (Ribeiro *et al.* 2015, Abdi and Williams 2010).). Although multivariate techniques were developed for solving very specific problems, they techniques became very popular due to the size reduction of an original data set.

1.9.2. Response surface methodology

Over the years, science and industry have optimized their processes using the one-factor-at-a-time (one variable input) technique. This technique changes only one input variable and keeps all other input variables constant. Although one-variable-at-a-time has been used for years by almost all scientific areas, it has several disadvantages (Myers *et al.* 2009, Bezerra *et al.* 2008, Barros Neto *et al.* 2003). Firstly, it increases the number of experiments required, and hence experimental time and consumption of materials/reagents, as a result it increases the cost of the experimental process. Secondly, it does not include the interactive effects between input variables, as a consequence, does not fully describe the effects of input variables on the response (Khuri and Mukhopadhyay 2010, Bezerra *et al.* 2008). In order to overcome this problem, process optimization studies have used response surface methodology (RSM), which is a multivariate statistical technique (Bezerra *et al.* 2008, Bas & Boyacı 2007, Barros Neto *et al.* 2003). RSM differs from one-variable-at-a-time, because it includes the interactive effects between the input variables, and as a consequence, it can describe the effects of input variables on the response.

"Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving and optimizing processes" (Myers *et al.* 2009). In addition, it is a universal language to set, perform and analyse experiments. It has been used in different fields including engineering, business, industry and academia (Deepak *et al.* 2008, Myers and Montogomery, 2002) to optimise input variables and describe their potential influence on performance, quality and quantity characteristics of products and process. Furthermore, RSM has been successfully applied to model and

optimize biochemical and biotechnological processes related to food systems (Myers & Montgomery 2002, Anjum e al. 1997). Table 1.4 shows how to select an experimental design by the number of variables.

Table 1.4: Selecting experimental design by input variable number

Variable number	**Comparative**	**Screening**	**Response Surface**
1	1-factor completely randomized design	-	-
2-4	Randomized block design	Full or fractional factorial	Central composite, Box Behnken, or D-optimal
5 or more	Randomized block design	Full or fractional factorial	Screen first to reduce number of factors

First-order designs (equation 1) are commonly used 2^k factorial (k is a number of variables), 2^{k-n} fractional factorial (k is a number of variables and n is a fractional resolution), Plackett-Burman and simplex. Second-Order design (equation 2) are most common are 3^k factorial, central composite, Box-Behnken and D-Optimal designs.

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \in \qquad (1.1)$$

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ij} x_i^2 + \sum \sum_{i>j=1}^{k} \beta_{ii}\, x_i x_j + \in \qquad (1.2)$$

RSM is based on the adjustment of a linear or polynomial equation to the experimental data, which can describe the phenomena from an original data set

by statistical previsions (including evaluation of main and interactive effects). The effect of interactions over 2 factors such as XYZ (X = factor 1, Y = factor 2, Z=factor 3) can be negligible (Jaynes *et al.* 2012, Wu and Hamada 2009, Montogomery, 2002) because they generally have a low-order interaction compared with the main effects (Jaynes *et al.* 2012).

Yeasts are responsible for fermenting and generating a large group of compounds, which most of them are formed during growth. Several studies have been conducted and all suggest that carbohydrates and amino acids are the key sources of flavour formation during yeast alcoholic fermentation (Zhuang *et al.* 2015, Bilverstonem *et al.* 2015, Vidal *et al.* 2014, Fairbairn 2012, Zufall and Tyrell 2008, Francis & Newton 2005; Swiegers *et al.* 2005); another group of studies have evaluated the influence of some inorganic elements such as ammonia-nitrogen, inorganic-phosphorus, potassium, magnesium and heavy metals for fermentation performance and ethanol production (Wietstock *et al.* 2015, Silva *et al.* 2008, Silva *et al.* 2006, De Freitas *et al.* 2003, Blackwell *et al.* 1998, Szczypka *et al.* 1997, Crapo *et al.* 1992, Turrens and Boveris 1980). However, none of the studies has evaluated the yeast nutrition considering carbohydrates consumption and essential inorganic elements presence on yeast-flavour formation. Furthermore, any study also has used response surface method for evaluating the individual influence and interactions between inorganic elements (inputs) and as a result flavour compounds (outputs).

1.10. GENERAL OBJECTIVES

This thesis generates new knowledge about the role of yeast nutrition on flavour formation during fermentation, specifically related to the influence of essential inorganic elements on yeast flavour formation. All information generated can be applied in an industrial setting to make better informed and more accurate processing decisions (including nutritional supplementation). In the long-term, industries may be able to apply this knowledge in real-time to identify and resolve processing problems related to nutritional deficiency, by measuring the flavour composition.

1.10.1. SPECIFIC OBJECTIVES

Chapter 3: This chapter aims to differentiate and characterise *Saccharomyces* yeast strains according to temperature adapted growth, growth on different carbohydrate sources, DNA fingerprinting and mineral composition.

Chapter 4: This chapter generates a more complete understanding of yeast flavour formation. In addition, the competition between formation of higher alcohols and short/medium chain fatty acid, which is correlated to exponential phase of growth curve; therefore, the consequent effects on ester formation, including acetate esters and fatty acid esters.

Chapter 5: This chapter generates a new understanding of the role of the essential inorganic element and their effect on yeast-flavour formation during supplemented fermentations. Anticipating a complete

fundamental understanding of “the role of essential inorganic elements on yeast flavour production”.

Chapter 6: This chapter aims to create a more complete synthetic sweet wort for comparison to beer sweet wort during yeast fermentation. In addition, synthetic sweet wort supports the understanding of the role of essential inorganic elements in brewery growth/flavour formation and, as a result, a tool was created for long-term fermentation, physiological and flavour studies.

Chapter 7: This chapter generates a new knowledge of the role of inorganic-phosphate, potassium, magnesium as independent variables (input) on flavour formation as dependent variables (output) during alcoholic fermentation using RSM, which considers significance, prediction, optimization and mathematical model for fermentation control. As a result, the optimization of experimental explains the interactions and role of inorganic phosphate, potassium, magnesium on yeast flavour production.

CHAPTER II
METHODOLOGY

2. CHAPTER II: METHODOLOGY

This chapter is divided five steps: 1) Medium, solutions and wort, 2), Microorganisms assays, 3) Fermentations and supplementation design, 4) fermentations and design, 5) instrumental analysis, and 6) Processing data.

2.1. Media, solutions and wort

All media and solutions were prepared using materials supplied by Fisher Scientific, UK. All media were autoc1aved at 121°C at 15 psi for 15 min immediately after preparation.

2.1.1. YPD, YPD agar and cryoprotectant solution

2.1.1.1. YPD

YPD (1% of yeast extract, 2% of peptone and 2% of D-glucose) was used in the most of experiments for yeast growth. YPD was adjusted up to 1litre using RO (reverse osmosis) water, sterilised (steam, 121°C) and stored at 22°C (room temperature).

2.1.1.2. YP + SCS (specific carbon source)

YP + SCS (specific carbon source) was prepared at a concentration of 2% (specific carbon source). The carbon sources used were glucose, fructose, galactose, xylose, mannose, rhamnose, sucrose, maltose, lactose, melibiose and melizitose. All media were adjusted up to 1litre using RO (reverse osmosis) water and sterilised (steam, 121°C) and stored at 22°C (room temperature).

2.1.1.3. YPD agar

Dissolving 1% of yeast extract, 2% of peptone, 2% of D-Glucose and 2% agar adjusted up to 1litre using RO (reverse osmosis) water.

2.1.1.4. Cryoprotectant solution

Cryoprotectant solution consisted of 1ml YPD plus 0.2 mL of glycerol (Sigma, UK) to maintain cell viability.

2.1.2. Methylene blue viability assessment

100 mL of methylene blue (0.01 g/L stock solution) was dissolved in sodium citrate solution (2% w/v) to a final concentration of 0.01 % (Pierce, 1970).

2.1.3. Sweet wort

Sweet wort (SG 1.054 and pH 5.42) was produced from an all malt within the International Centre for Brewing Science (ICBS) at The University of Nottingham. The characterization involved specific gravity, pH, carbohydrates (Table 2.1) and mineral composition (Table 2.2) are presented below:

Table 2.1: Beer sweet wort carbohydrates (including maltotetrose+dextrin, Maltotriose, Maltose, Sucrose, glucose and fructose) concentration (g/L), each value is the mean of 3 replicates and errors shown by standard deviation.

Compounds	Concentration (g/L)	Percentage
Maltotetrose+dextrin	17.95±0.00	13.81
Maltotriose	18.06±0.40	13.90
Maltose	77.59±2.20	59.68
Sucrose	2.35±1.10	1.81
Glucose	12.46±0.20	9.59
Fructose	1.59±0.02	1.22
Total	130.00±1.90	100.00

Table 2.2: Beer wort mineral composition by ICPMS

Minerals	Unit	Concentration
Group 1		
Nitrogen (N)*	mg/L	-
Phosphorus (P)		256.50±24.40
Potassium (K)		554.96±78.75
Magnesium (Mg)		78.84±8.40
Calcium (Ca)		159.44±26.80
Iron (Fe)		0.08±0.05
Copper (Cu)		0.06±0.01
Zinc (Zn)		0.00±0.00
Manganese (Mn)		0.00±0.00
Group 2		
Sulfur (S)	(mg/L)	197.73±29.20
Sodium (Na)		12.61±1.40
Aluminium (Al)		0.17±0.10
Boron (B)		0.08±0.02
Rubidium (Rb)		0.10±0.01
Strontium (Sr)		0.16±0.02
Group 3		
Barium(Ba)	µg/L	25.75±3.90
Titanium (Ti)		41.63±10.20
Chromium (Cr)		8.04±1.40
Cobalt (Co)		0.40±0.05
Nickel (Ni)		10.32±5.20
Lithium (Li)		3.18±0.40
Beryllium (Be)		0.08±0.05
Vanadium (V)		0.37±0.10
Gallium (Ga)		0.24±0.05
Arsenic (As)		0.60±0.20
Selenium (Se)		0.58±0.20
Molybdenur (Mo)		2.72±0.90
Silver (Ag)		0.07±0.04
Cadmium (Cd)		0.53±0.30
Caesium (Cs)		0.07±0.03
Thallium (TI)		0.00±0.00
Lead (Pb)		4.00±3.44
Uranium (U)		0.07±0.02

*inorganic elements not analysed

2.1.4. Essential inorganic elements: supplementation solutions

Each solution was individually prepared using 30.00g of $(NH_4)_2SO_4$ (nitrogen), 10 g of KCl (potassium), 10 g of $Na_2PO_4.H_2O$ (phosphorous), 10 g of $MgCl_2$ (magnesium), 10 g of $FeSO_4.7H_2O$ (iron), 15 g of $ZnSO_4.7H_2O$ (zinc), 10 g of $CuSO_4.5H_2O$ (copper) and 10 g of $MnSO_2.4H_2O$ (manganese). All these solutions were individually prepared in 100ml in volumetric flasks and sterilised (steam, 121°C) and stored at 22°C (room temperature).

2.1.5. Synthetic sweet wort

Table 2.3 shows the synthetic wort composition including carbohydrates, amino acids, essential inorganic elements (micro inorganic nutrients and metal ions), vitamins and lipids. The carbon source and essential inorganic elements composition were measured (Section, 2.1.3. Table 2.1 and 2.2); and amino acids, vitamins and lipids were raised from literature data. All data was compared to beer sweet wort to develop a synthetic sweet wort (Table 2.3).

Table 2.3: Synthetic wort composition including carbohydrates and amino acids, essential inorganic elements (micro inorganic nutrients and metal ions), vitamins, lipids.

Class		Compounds		Synthetic wort		Percentage (%)
Carbon source (g/L)		Maltotetrose		-		10.00
		Maltotriose		-		
		Maltose		75		
		Sucrose		3		
		Glucose		20		
		Fructose		2		
[1,2]Amino acids (mg/L)		L-Aspartic acid		135.00		0.31
		L-Threonine		93.60		
		L-Serine		75.00		
		L-Asparagine		257.20		
		L-Glutamine		10.40		
		L-Glutamic acid		155.60		
		L-Proline		545.80		
		Glycine		56.80		
		L-Alanine		176.80		
		L-Valine		187.20		
		L-Methionine		46.80		
		L-Isoleucine		99.20		
		L-Leucine		243.60		
		L-Tryosine		160.40		
		L-Phenylalanine		191.20		
		L-Tryptophane		84.60		
		L-Lysine hydrochloride		224.40		
		L-Histidine hydrochloride		101.8		
		L-Arginine hydrochloride		276.80		
		[3]YAN	[3]FAN	3122.20	3122.20	
			[3]NH3-N **by** $(NH_4)_2SO_4$	-		
Essential inorganic elements	**Micro inorganic nutrients (mg/L)**	Potassium (K) by KCl		1000 (524.50 mg of ion)		0.31 (0.10 of ions)
		Phosphorous (P) by $Na_2PO_4.H_2O$		1300 (253.35 mg of ion)		
		Magnesium (Mg) by $MgCl_2$		300 (76.57 mg of ion)		
		Calcium (Ca) by $CaSO_2.2H_2O$		550 (156 mg of ion)		
		Iron (Fe) by $FeSO_4.7H_2O$		0.20 (0.04 mg of ion)		
	Metal ions (mg/L)	Zinc (Zn) by $ZnSO_4.7H_2O$		0.70 (0.15 mg of ion)		
		Copper (Cu) by $CuSO_4.5H_2O$		0.20 (0.05 mg of ion)		
		Manganese (Mn) by $MnSO_2.4H_2O$		0.20 (0.06 mg of ion)		
[4,5,7]Vitamins (g/L)		Biotin		0.01		0.021
		Vitamin B1 (Thiamine Hydrochloride)		0.05		
		Vitamin B2 (Riboflavin)		0.05		
		Vitamin B5 (Calcium Pantothenate)		0.05		
		Vitamin B6 (Pyridoxine Hydrochloride)		0.05		
[4,8]Lipids (ml/L)		Tween 80		0.1		0.03
		Linoleic acid		0.2		

[1]Thompson *et al.* (1973), [2]Taidi *et al.* (2003). [3]$YAN = (FAN) + (NH_3 - N)$. YAN (Yeast Assimilable nitrogen) is a sum of FAN (Free Amino Nitrogen) and NH3-N (Ammonia Nitrogen),[4] Henschke and Jiranek (1993) cited Fairbairn (2012); [5]Jones and Pierce (1964), [7]Hucker *et al.* (2016), [8]Anness and Reed (1985)

2.1.5.1. Essential inorganic elements solutions for the preparation of synthetic sweet wort

To prepare the synthetic sweet wort with quantities of essential inorganic elements described in the table 2.3. To supplement the necessary quantity of essential inorganic elements, solution were previously prepared: 25 g/L of KCl (Potassium), 32.50 g/L of $Na_2PO_4.H_2O$ (Phosphorous), 7.50 g/L of $MgCl_2$ (Magnesium), 0.10g/L of $FeSO_4.7H_2O$ (iron), 0.33g/L of $ZnSO_4.7H_2O$ (zinc), 0.10g/L of $CuSO_4.5H_2O$ (copper), 0.10/L g of $MnSO_2.4H_2O$ (manganese). All these solutions were prepared in 100ml in volumetric flasks and sterilised (steam, 121°C). Therefore, 4 mL of potassium, phosphorus, and magnesium; and 200µl of iron, zinc, copper and manganese were previously prepared from their respective solution were added the medium.

2.2. Microorganism assays

2.2.1. Yeast Strains and Storage

Two yeast *Saccharomyces cerevisiae* strains (NCYC2592 and M2) and one *Saccharomyces pastorianus* (W34/70) were used in these study. All strains used in this study were fresh yeast, which were laboratory-grown at 25°C at 150 RPM for 24 hours. Stock cultures of each strain were prepared and maintained in: 1) Cryovials (Nalgene Nunc International, UK) stored at -80°C. A loop full of yeast grown on YPD agar was re-suspended in cryoprotectant solution containing 1ml YPD plus 20 % (v/v) glycerol (Sigma, UK) to maintain cell viability; and 2) using a small aliquot of the stock cryopreserved culture, which were propagated in 10 mL and 250 mL of YPD at 25°C for 3

days each. Therefore, YPD agar slopes were prepared and maintained as stock culture at 4 °C during 2 months for use in subsequent experiments.

2.2.2. Microrganism propagation

The fermentation used three yeast *Saccharomyces*, which two yeast *Saccharomyces cerevisiae* strains (NCYC2592 and M2) and one *Saccharomyces pastorianus* (W34/70). Each microorganism were grown by incubation in YPD during 24 hours at 30°C. The amount of yeast pitched into the wort was ~ $1.5x10^7$ cells/mL.

2.2.3. Viability assays

Yeast suspension (0.5 ml) was mixed with methylene blue solution (0.5 ml) and after 5 min at room temperature examined microscopically (Leitz, Diaplan Microscope, Germany at x400 magnification). Images were captured using a digital camera attached to the microscope. Viability was evaluated using Aber countstar instruments connected to a computer for data acquisition via countstar software. The countstar instrument is used with specific disposable slides containing 5-sample chambers and require 20µl for each sample (Aber countstar Instruments specifications).

2.2.4. Spot plate assays

Cells were grown overnight in 10 ml of YPD at 25 °C with orbital shaking at 150 rpm. Spot plate (fig. 2.1) tests were performed using sequential dilutions (Table 2.4) of pre-growth yeast from $1.5x10^7$ to $1.5x10^3$ cell/mL ($1.5x10^4$ to 15 cell/10µl) at 15°C, 22°C, 25°C, 30°C, 37°C and 40°C during 72 h. Pictures were captured in a dark incubator under UV light using VisionWorks®LS analysis software edited using adobe Photoshop.

Table 2.4: Serial dilution for spot plates assay

Yeast suspension + RO water		Cell number/mL	cell/10µl
S1	1 + 0	15000000	150000
S2	0.1+0.9	1500000	15000
S3	0.1+0.9	150000	1500
S4	0.1+0.9	15000	150
S5	0.1+0.9	1500	15

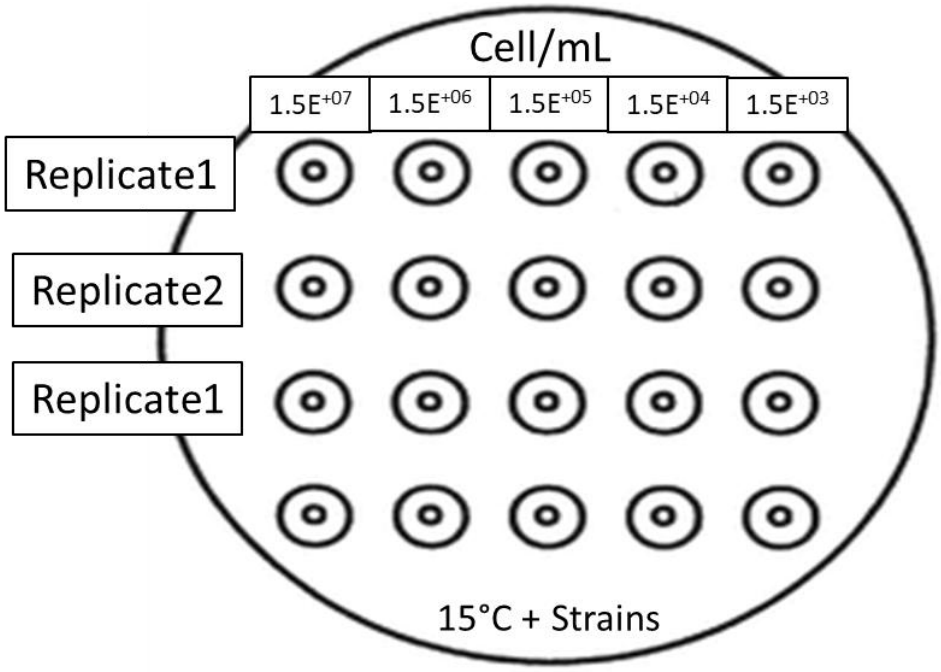

Fig. 2.1: Add spot plates preparation picture (paper with spots plates design)

2.2.5. Measurement of yeast growth

Yeasts growth measurement were performed in 96 well plates covered with low evaporation film (Costa®) and monitored using a Tecan (Mannedorf, Switzerland) Infinite M200 Pro plate reader (OD600 nm). Tecan measurements were divided into four growth assays: 1) Ale and lager strain differentiation based on growth temperature, 2) yeast growth on different carbon source, 3) essential inorganic elements growth assay and 4) Yeast growth during vessel fermentations.

Ale and lager strain differentiation based on growth temperature. Yeast growth were measured in 96-well plates at 22°C, 25°C, 30°C, 37°C and 40°C during 72 h. Yeasts were inoculated in YPD.

Ale and lager strain differentiation based on carbon source utilization**.** Yeast growth was measured in 96-well plates. Individual strains were inoculated on different carbon source during 72 h at 25°C. YP (2% bacteriological peptone, 1% yeast extract) + 2% of individual carbon source (glucose, fructose, galactose, xylose, mannose, rhamnose, sucrose, maltose, lactose, melibiose, melizitose).

Essential inorganic elements growth assay. Yeast growth were measured in 96-well plates with different concentration of essential inorganic elements during 72 h at 30°C.

Yeast growth during vessels fermentation. Yeast growth were measured in 96-well plates at 22°C parallels to fermentation vessels (described at sub-topic 2.3.1.) for measuring growth in fermentation vessels.

All Tecan measurement had the amount of yeast inoculated ~ $1.5x10^7$ cells/mL. Furthermore, all growths were classified and tabled as exemplified into the table 2.5 below:

Table 2.5: Growth intensity classification using Tecan growth curves.

OD600	Growth intensity	Representation
0.80-1.00	very intense growth	++++
0.60-0.80	intense growth	+++
0.40-0.60	moderate growth	++
0.3-0.40	very low growth	+
0-0.30	no growth	-

Table 2.6: Distribution of essential inorganic elements in 96-well plates for growth assay. Zero levels means the absence, 0.5x levels means half amount of inorganic elements found in original wort; 2x levels means the supplementation with double amount of inorganic elements found in original wort (Section 2.2.5)

		Plate column											
		1	2	3	4	5	6	7	8	9	10	11	12
Plate raw	A	BW1	BW2	BW3	N01	N02	N03	N21	N22	N23	N31	N32	N33
	B	SW Free1	SW Free2	SW Free3	P01	P02	P03	P21	P22	P23	P31	P32	P33
	C	SW Full 11	SW Full 12	SW Full 13	K01	K02	K03	K21	K22	K23	K31	K32	K33
	D	SW Full 21	SW Full 22	SW Full 23	Mg01	Mg02	Mg03	Mg21	Mg22	Mg23	Mg31	Mg32	Mg33
	E	BW1 NY	BW2 NY	BW3 NY	Cu01	Cu02	Cu03	Cu21	Cu22	Cu23	Cu31	Cu32	Cu33
	F	SW Free1 NY	SW Free2 NY	SW Free3 NY	Fe01	Fe02	Fe03	Fe21	Fe22	Fe23	Fe31	Fe32	Fe33
	G	SW Full 11 NY	SW Full 12 NY	SW Full 13 NY	Zn01	Zn02	Zn03	Zn21	Zn22	Zn23	Zn31	Zn32	Zn33
	H	SW Full 21 NY	SW Full 22 NY	SW Full 23 NY	Mn01	Mn02	Mn03	Mn21	Mn22	Mn23	Mn31	Mn32	Mn33
					zero and replication			0.5x and replication			2x and replication		

BW - beer wort, SW - Synthetic wort, SW free - Synthetic wort without essential traces elements (medium recommended by Taidi *et al.* 2003), NY - no yeast

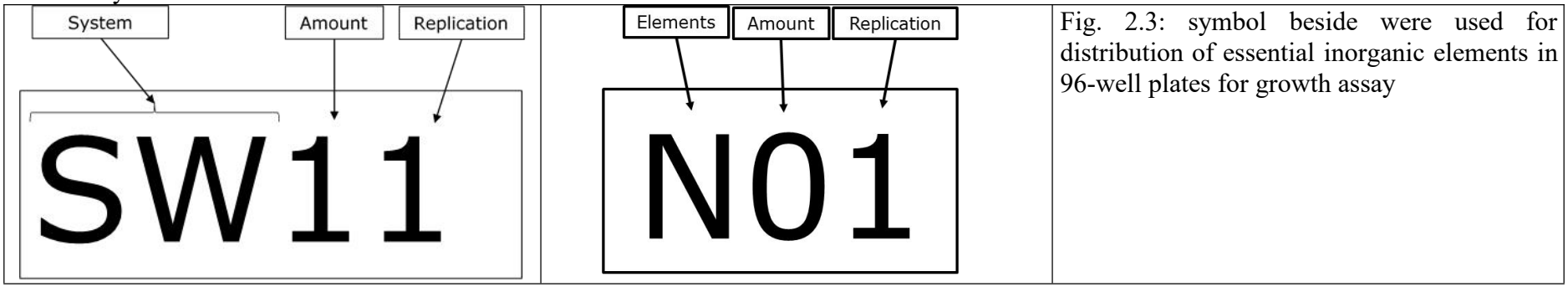

Fig. 2.3: symbol beside were used for distribution of essential inorganic elements in 96-well plates for growth assay

2.2.6. DNA assay

2.2.6.1. DNA extraction

DNA was prepared from 10ml YPD incubated at 25°C at 150 RPM for 24 h. 2 ml of the culture was centrifuged in a 2.5 ml Eppendorf tube (5000 rpm, 5 min). 400 µl of lysis buffer (Tris 10 mM, pH 7.6, EDTA 1 mM, NaCl 100 mM, Triton X-100, 2% w/v, sodium dodecyl sulphate (SDS) 1% w/v), 400 µl of phenol/chloroform/iso-amyl alcohol (25:24:1 v/v), and 600 µl of glass beads were added to the pellet. The mixture was vortexed for 4 min. Then 200 µl of Tris EDTA (pH 7.6) buffer was added, and the mixture centrifuged for 5 min at 6000 rpm. 500 µl of chloroform:isoamyl alcohol (98:2 v/v) was added to the upper phase and after gentle agitation, the mixture was submitted to centrifugation (14000 rpm for 2 min). Two volumes of ethanol were added to the aqueous phase. After centrifugation (14000 rpm, 5 min), the nucleic acid pellet was dissolved in 10mM TE buffer pH 8.0.

2.2.6.2. DNA amplification

PCR amplifications were carried out in 25 µl reaction volumes containing 5-20ng yeast DNA, 10mM Tris pH, 9.0, 50 mM KCl, 0.1% Triton X-100, 0.2 mg/ml gelatin, 200 mM of each dNTP, 2.5 mM $MgCl_2$ and 1 µM for each oligonucleotide primer of the delta12 family and delta21 family. Primers are delta12 (5'-TCAACAATGGAATCCCAAC-3') and delta21 (5'-CATCTTAACACCGTATATGA-3').

Amplification reactions were performed with a Stratagene thermal cycler using the following programme: 4 min at 98°C followed by 38 cycles of 10s at 98°C, 30 s at 50°C and 90s at 72°C and the finishing step of 10min at 72°C.

2.2.6.3. Electrophoresis

Amplification products were separated by electrophoresis on 15 cm of 2% agarose gels submitted to 75mV for 1.5h in 1xTBE buffer and fluorescent ethidium bromide. The stained bands were photographed under UV light using VisionWorks®LS analysis and edition was treated using adobe Photoshop.

2.3. Fermentations design

2.3.1. Fermentation vessels

Miniature fermentation vessels (FVs) were conducted using 180 mL sterile Wheaton glass serum bottles (Sigma-Aldrich, U.K.). 100 ml aliquots of brewery sweet wort (SG 1.054, pH 5.42) or synthetic sweet wort (SG 1.054, pH 5.41). Yeast was pitched $1.5x10^7$cell/mL and magnetic stirrers were used for agitation at 200 rpm. The fermentation vessels were sealed with a rubber septum and a metal crimp. A hypodermic needle was then inserted through the septum. A Bunsen valve, attached to the needle, allowed the exit of gas from the vessel (Fig.2.4). Fermentations were performed in triplicate monitoring CO_2 by weight loss from the vessels at 22°C during 72 h.

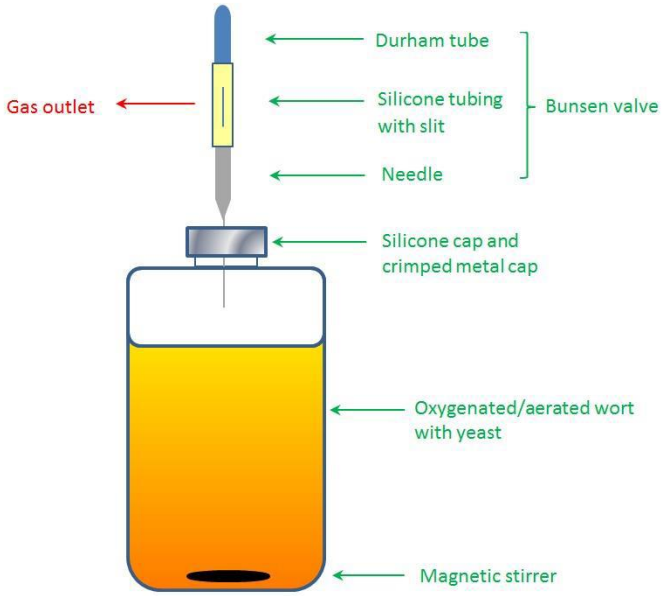

Fig. 2.2: 180 ml fermentation vessel schematic

2.3.2. Supplementation design

Fermentations experiment were conducted using beer sweet wort (SG 1.054 and pH 5.42) as a reference (T0) plus 9 supplementation treatments. Supplementation treatments were conducted by adding to the fermentation vessels; ammonium nitrogen (T1, 63.50 mg/L), phosphorus (T2, 19.50 mg/L), potassium (T3, 52.50 mg/L), magnesium (T4, 25.53 mg/L), copper (T5, 25.50 mg/L), zinc (T6, 34.10 mg/L), iron (T7, 20.10 mg/L), manganese (T8, 27.75 mg/L), T9 (a composite mixture of all nutrients). Each quantity described above was achieved by adding 0.1mL (100µL) from the pre-prepared solution into the treatment vessel (Section 2.2).

After fermentation, samples were transferred into 50 mL falcon tubes and centrifuged at 4000 RPM for 4 min to separate the supernatant. 25 mL of each individual supernatant was transferred into fresh 50 mL falcon tubes and kept frozen. These samples were defrosted 1h before HPLC or GC-MS protocols.

2.4. Surface response method

Evaluation of the effect of essential inorganic elements (inorganic phosphate, potassium, magnesium) were carried out using response surface methodology (Myers and Montgomery 2002, Montgomery 2001). A three-factor and a three level face-centered cube design (D-optimal) presented at table 2.5 consisting of twenty-eight experimental runs were employed including three replicates at the center point (table 2.6). The effects of unexplained variability in the observed response due to extraneous factors were minimized by randomizing the order of experiments. The samples were measured for CO_2 loss, yeast growth, glycerol, acetic acid, ethanol and flavours.

2.4.1. Data analysis

Experimental data were fitted to a second-order polynomial model and regression coefficients obtained using Design expert (version 6.0.5) software.

Table 2.7 shows levels of the matrix of D-Optimal cubic planning with 3 replicas at the midpoint, used in this study. D-optimal cubic plan for 3 variables plus 3 replicas at the midpoint.

Table 2.7: Levels of the matrix of D-Optimal cubic planning with 3 replicas at the midpoint.

Parameters			Coded						
			-1	-0.5	-0.33	0	0.33	0.5	1
			Uncoded						
Inorganic phosphate	mg/L	Values	0	125	166.67	250	333.33	375	500
Potassium			0	250	333.33	500	666.67	750	1000
Magnesium			0	40	53.33	80	106.67	120	160

Table 2.8 shows the real value of the matrix of D-Optimal cubic planning with 3 replicas at the midpoint. The central points are represented by runs 26, 27 and 28, which simulate beer wort.

Table 2.8: The real values of the matrix of D-Optimal cubic planning with 3 replicas at the midpoint.

Run	Pi	K	Mg
1	500	180	116.8
2	500	1000	160
3	500	0	0
4	500	650	32
5	500	1000	0
6	500	0	160
7	500	1000	103.646
8	500	515	160
9	500	0	55.2
10	157.5	1000	160
11	157.5	750	0
12	157.5	250	160
13	157.5	0	21.6
14	0	600	160
15	0	1000	84.8
16	0	1000	0
17	0	0	0
18	0	415	24
19	0	0	160
20	0	1000	160
21	0	0	96.8
22	350	755	132
23	350	1000	36
24	350	300	0
25	350	0	160
26	250	500	80
27	250	500	80
28	250	500	80

2.5. Instrumental analysis

2.5.1. HPLC protocol

Maltotretraose, maltotriose, maltose, sucrose, glucose, fructose, glycerol, acetic acid and ethanol concentrations were quantified by high-performance liquid chromatography (HPLC). HPLC analyses were conducted using 2 mL of pre-centrifuged/filtered samples pipetted into a 2 mL HPLC vials (SLS Ltd, Nottingham, UK). The HPLC system included a Jasco AS-2055 Intelligent auto sampler (Jasco, Tokyo, Japan) and a Jasco PU-1580 Intelligent pump (Jasco). The chromatographic separation was performed on a Rezex ROA H^+ organic acid column, 5 µm, 7.8 mm × 300 mm (Phenomenex, Macclesfield, UK), at 22 C ambient temperature. The mobile phase was 0.005N H_2SO_4 with a flow rate of 0.5 ml/min. For detection, a Jasco RI-2031 Intelligent refractive index detector (Jasco) was employed. Data acquisition was taken via the azur software (version 4.6.0.0, Datalys, St Martin D'heres, France) and concentrations were determined by peak area comparison with injections of authentic standards. The injected volume was 10 µl and analysis was completed in 28 min. All chemicals used were analytical grade (>95% purity, Sigma-Aldrich, UK).

2.5.2. Liquid-liquid extraction and GC-MS protocols

2.5.2.1. Liquid-liquid extraction

The analysis of the fermentation samples were conducted with aliquots (10 mL) containing 100 µl of 0.01% hexanol (Internal standard) and extracted 5 times

with 2mL of diethyl ether for 1 h at room temperature (22°C). After leaving the mixture to stand for 1 min, the higher diethyl ether layer was collected in 0.3 mL GC-MS glass vials (screw top, fixed insert vials; Chromacol Ltd, Herts, UK) and stored at -20 ºC for analysis by GC-MS.

2.5.2.3. GC-MS Analysis

Gas chromatography and mass spectrometry (GC-MS) analyses were conducted using 2 mL of the ether layer was pipetted into a 2 mL GC vials (SLS Ltd, Nottingham, UK) and 1.5 µL of the each sample was injected into the injector port of a Trace 1300 Series GC (Thermo Scientific, Massachusetts, USA) in a splitless using an AS 3000 autosampler (Thermo Scientific, Massachusetts, USA). The column was ZB WAX, 30 m x 0.25 mm i.d. x 0.25 µm film thickness (Phenomenex, Macclesfield, UK). The temperature programme for the oven was kept at 40 °C for 2 min after injection and then ramped at 8 °C/min to 250 °C over 2 min. Method intakes such as carrier pressure (10 psi) and splitless time (0.5 minutes). Analytes were detected in triplicate using an ISQ mass spectrometer (Thermo Scientific, Massachusetts, USA) operating in full scan mode from 35 to 300 m/z at 1.8 scans/s. The peak area was identified and considered formula, mass weight and retention time to calculate the quantity of each compound.

All compounds analysed using GC-MS are divided into groups: 1) Higher Alcohols (n-propanol, isobutanol, isoamylic alcohol, phenyl-2-ethanol); 2) Fatty Acids (isobutyric acid, butyric acid, isovaleric acid, hexanoic acid, octanoic acid, decanoic acid, dodecanoic acid); 3) Acetate Esters (ethyl acetate,

isobutyl acetate, isoamyl acetate, hexyl acetate, phenyl-2-ethanol acetate); 4) Fatty Acid Esters (ethyl butyrate, ethyl isobutyrate, ethyl hexanoate, ethyl octanoate, ethyl decanoate); 5) diacetyl precursor (acetoin and 2,3-butanediol).

2.5.3. ICP-MS protocol

Inductively coupled plasma mass spectrometry (ICP-MS) protocol were divided in three steps: 1) Sample preparation for mineral analysis (Sub-section 2.5.3.1), sample digestion (Sub-section 2.5.3.2) and ICPMS analysis (Sub-section 2.5.3.3)

2.5.3.1. Sample preparation for mineral analysis

Solid samples were taken 0.25 g of dried yeast. The fermented samples were centrifuged 50 ml of sample, separated of liquid phase (liquid sample analysis) and was added in 5 mL of HNO_3 (10098862 - Nitric Acid 68%, d=1.42, Primar Plus, for Trace Metal Analysis; Fisher chemical; CAS No: 7697-37-2; HDPE plastic bottle; 2.5L).

Liquid samples, taken 1 mL of the sample and was added in 4 mL of HNO_3 (10098862 - Nitric Acid 68%; d=1.42, Primar Plus, for Trace Metal Analysis; Fisher chemical; CAS No: 7697-37-2; HDPE plastic bottle; 2.5L).

2.5.3.2. Sample digestion

All samples (Solid or liquid) were kept to react for 24 h at 22°C (room temperature). The samples were exposed to microwave heat (Multiwave PRO,

Anton Paar) using the following temperature program: power (1,500W), heating to 140°C in 10 min, holding the temperature at 140°C for 30 min, and cooling down for 30 min. Digested samples were transferred into fresh tubes and adjusted up to 20 ml using milliQ water. 1 ml of diluted samples were transferred into ICPMS tubes and adjusted up to 10 ml using milliQ water.

2.5.3.3. ICP-MS analysis

ICPMS instrument, the following parameters were used: Plasma power, 1,550 W; cool gas (14 L/min), argon gas flow rates, auxiliary at 0.8 L/min and nebulizer at 0.4 L/min; sample flow rate, 4.0 mL/min. Multi-element analysis of diluted solutions was undertaken by ICP-MS (Thermo-Fisher Scientific iCAP-Q; Thermo Fisher Scientific, Bremen, Germany). The instrument was run employing three operational modes, including (i) a collision-cell (Q cell) using He with kinetic energy discrimination (He-cell) to remove polyatomic interferences, (ii) standard mode (STD) in which the collision cell is evacuated and (iii) hydrogen mode (H_2-cell) in which H_2 gas is used as the cell gas. Samples were introduced from an autosampler (Cetac ASX-520) incorporating an ASXpress™ rapid uptake module through a PEEK nebulizer (Burgener Mira Mist). Internal standards were introduced to the sample stream on a separate line via the ASXpress unit and included Ge (10 µg L^{-1}), Rh (10 µg L^{-1}) and Ir (5 µg L^{-1}) in 2% trace analysis grade (Fisher Scientific, UK) HNO_3. External multi-element calibration standards (Claritas-PPT grade CLMS-2 from SPEX Certiprep Inc., Metuchen, NJ, USA) included Ag, Al, As, Ba, Be, Cd, Ca, Co, Cr, Cs, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Se, Sr, Tl,

U, V and Zn, in the range 0 – 100 µg L^{-1} (0, 20, 40, 100 µg L^{-1}). A bespoke external multi-element calibration solution (PlasmaCAL, SCP Science, France) was used to create Ca, Mg, Na and K standards in the range 0-30 mg/L. Phosphorus, boron and sulphur calibration utilized in-house standard solutions (KH_2PO_4, K_2SO_4 and H_3BO_3). In-sample switching was used to measure B and P in STD mode, Se in H_2-cell mode and all other elements in He-cell mode. Sample processing was undertaken using Qtegra™ software (Thermo-Fisher Scientific) utilizing external cross-calibration between pulse-counting and analogue detector modes when required.

2.7. Statistical analysis

2.7.1 Basic statistics

All data comparing among growth curves, CO_2 loss weight, yeast total/viability, flavour (including carbohydrates, ethanol, glycerol), aromas compounds, mineral composition were analysed using ANOVA, Tukey test and standard deviation to identify whether a significant difference ($p<0.05$) and/or similarities using XLSTAT/Microsoft excel®.

2.7.2. Principle Component Analysis (PCA)

Principle Component Analysis (PCA) to identify whether a significant difference ($p<0.05$) and similarities present for each compound among each supplementation treatment was generated using XLSTAT/Microsoft excel®.

2.7.3. Response Surface Method

Response surface method (RSM) experiment used computational support (Design expert version 6.0) for assessing the data using regression models, analysis of variance (ANOVA), interactions of model by Pareto plot and response surface. Math model were created using software regression model adjust for each compound and compound groups.

Design expert (version 6.0.5) software were used to analyse the experimental data adjusting into response surface regression. Experimental data were adjusted into a second-order polynomial model and regression coefficients obtained. The generalized second-order polynomial model (equation 2.1) used in the response surface analysis was as follows:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ijz} x_i^2 + \sum \sum_{i>j=1}^{k} \beta_{ii}\, x_i x_j x_z + \in \qquad 2.1$$

where β_0, β_i, β_j, β_z, β_{ii}, β_{ij}, β_{iz}, β_{jz} and β_{ijz} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively, and X_i, X_j and X_z are the independent variables (inorganic phosphate, potassium and magnesium, respectively). The design expert software was used to generate response surfaces and contour plots while holding a variable constant in the second-order polynomial model. The results were showed by response surfaces and was used to compute for the estimation of the optimum response.

2.7.3.1. Verification of model

Optimal conditions for the flavour compounds from synthetic sweet wort depended on presence of essential inorganic elements (inorganic phosphate, potassium and magnesium) were obtained using the predictive equations of RSM. The experimental and predicted values were compared in order to determine the validity of the model.

CHAPTER III:

CHARACTERISATION OF BREWING YEAST STRAINS

3. CHAPTER III: CHARACTERISATION OF BREWING YEAST STRAINS

3.1. Introduction

In the brewery, yeast are generally *Saccharomyces* genus. *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* are used to produce ale and lager, respectively (Walker and Stewart 2016, Spitaels *et al.* 2014). Understanding the behaviour of different yeast via the utilization of different carbon source, nutrients and, consequently, growth and the production of desirable flavours are important for process control (Walker and Stewart 2016, Boulton and Quain 2001). Therefore, to assess and characterize all yeast strains: (3.1.1) permissive growth temperature; (3.1.2) capacity to grow on different carbon source; (3.1.3) DNA fingerprint (physiologic differentiation); and (3.1.4) mineral contents.

First, brewing yeast differentiation by permissive growth temperature is supported by the fact *Saccharomyces* can or can not grow at 37 °C (Parker *et al.* 2015, Kurtzman *et al.* 2011). *S. cerevisiae* can grow at 37°C and other species the other brewing associated species such as *S. pastorianus* cannot grow at this elevated temperature (Parker *et al.* 2015). Second, brewery yeast can assimilate the majority of carbohydrates including glucose, fructose, sucrose, maltose, galactose, mannose, raffinose and occasionally trehalose but they cannot assimilate dextrins (Stewart 2016, Briggs *et al.* 2004, Boulton and Quain 2001). However, ale and lager strains can be differentiated because of their ability to consume melibiose (disaccharide). The ability of lager strains to consume melibiose and a lacking of ale strain to utilize melibiose (Zaragoza *et al.*, 2002, Daran *et al.* 1997). Third, brewing yeast are Ale strains (*S.*

cerevisiae), which are taxonomically represented as *S. cerevisiae* and lager strains (*S. pastorianus*) involves a diverse variety of contributions from *S. cerevisiae*, *S. bayanus* and *Saccharomyces bayanus* var. *uvarum* (Rowen *et al.*, 1992, Dunn and Sherlock, 2008). Therefore, lager and ale strains are "producing yeasts" and their physiological differentiation can evaluate using polymerase chain reaction (PCR) and DNA fingerprinting. This is a one of the methods used for yeast physiological differentiation. Finally, mineral composition of brewing yeasts is an important parameter for this thesis study because yeasts are composed of several minerals, which influence the properties and metabolic activities of yeast. The different amount of inorganic elements is influenced by their physiological characteristics and consequently, contribute to growth, fermentation control and the quality of final product (flavours).

This chapter assesses three-yeast strains for their differentiation. In order to obtain an overview of the genetic/physiologic characteristics of the brewing lager and ale yeast strains selected, which were used as representative strains for this thesis.

3.2. Material/Method/Experimental design

Two ale yeast strains, designated NCYC2592 and M2, and one lager strain, designated W34/70 were used in this study (Section 2.1). In order to confirm the lager and ale phenotype of NCYC2592, W34/70 and M2, traditional and modern methods were applied. Four different parameters were deployed based on: (3.2.1) permissive growth temperature; (3.2.2) capacity to grow on

different carbon source; (3.2.3) DNA fingerprint (physiological differentiation); and (3.2.4) mineral contents.

3.3. Results

3.3.1. Permissive Growth Temperature

Traditional differentiation of ale and lager strains is measured using temperature assay, which considers that lagers strains cannot grow at 37°C, whereas ale strains can grow over 37°C (Kopeck *et al.* 2016, Boulton and Quain 2001). Fig. 3.1 illustrates yeast permissive growth temperature using sequential dilutions from $1.5x10^7$ to $1.5x10^3$ cell/mL ($1.5x10^4$ to 15 cell/10µl). NCYC2592 and M2 can grow at 37°C, on the other hand, W34/70 cannot grow at 37°C. NCYC2592 can grow at 40°C and W34/70 and M2 cannot grow at 40°C. All strain evaluated grow similarly at 22, 25°C and 30°C. Additionally, all strains grow similarly at 15°C; however, when were inoculated lower cell concentrations from $1.5x10^4$ to $1.5x10^3$ cell/mL, W34/70 exhibited better growth than NCYC2592 and M2.

Fig. 3.2 illustrates yeast growth curves at 22, 25, 30, 37 and 40°C during 72 hours using optical density (OD600). Similarly as Fig.3.1, NCYC2592 and M2 can grow at 37°C, on the other hand, W34/70 cannot grow at 37°C. Also, NCYC2592 can grow at 40°C and W34/70 and M2 cannot grow at 40°C. The growth using OD600 were from 0 to 1. All strains grow optimally at 25°C (0.95 OD). NCYC2592 and M2 grow similarly at 22 and 37°C (0.88 OD). W34/70 show a negligible growth (0.20 OD) at 37°C, but an intense growth (0.88 OD) at 22°C.

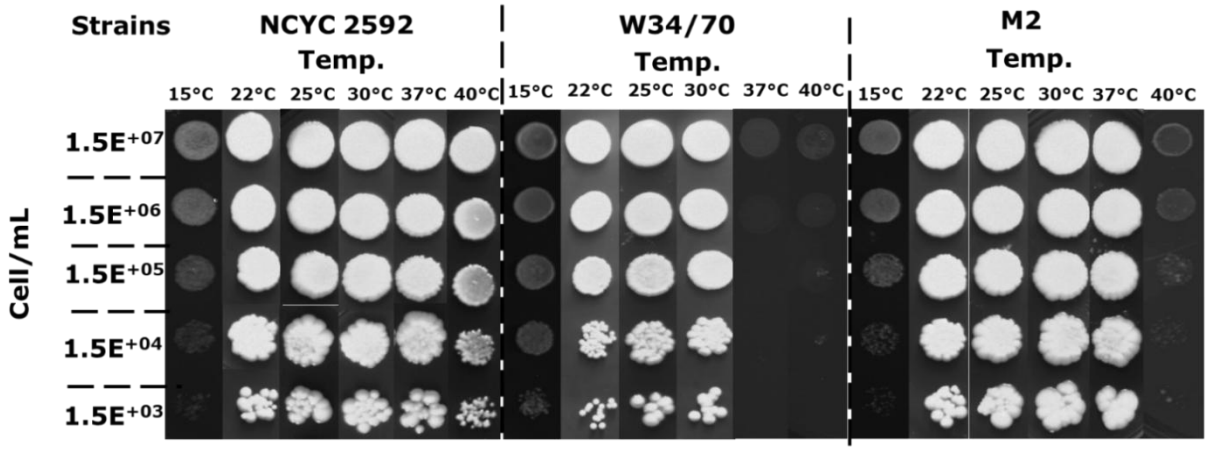

Fig. 3.1: NCYC2592, W34/70 and M2 colonies. All spot plates were carried out at 15, 22, 25, 30, 37 and 40°C during 72 hours. Spot plates carried using sequential dilutions from $1.5x10^7$ to $1.5x10^3$ cell/mL ($1.5x10^4$ to 15 cell/10µl) and pictures were captured in dark incubator under UV light using VisionWorks®LS Analysis Software editing using adobe Photoshop.

Table 3.1 shows the yeast ability to grow in different temperature and simplifies the data from Fig. 3.1 and Fig. 3.2. Therefore, five growth levels were considered: OD 0.80-1.00 (++++ very intense growth); OD 0.60-0.80 (+++ intense growth); OD 0.40-0.60 (++ moderate growth); OD 0.3-0.40 (+ very low growth); and OD 0-0.30 (- no growth). Furthermore, any growth under 0.40 OD were ignored because cannot be visualised on the spot plate (Fig. 3.1).

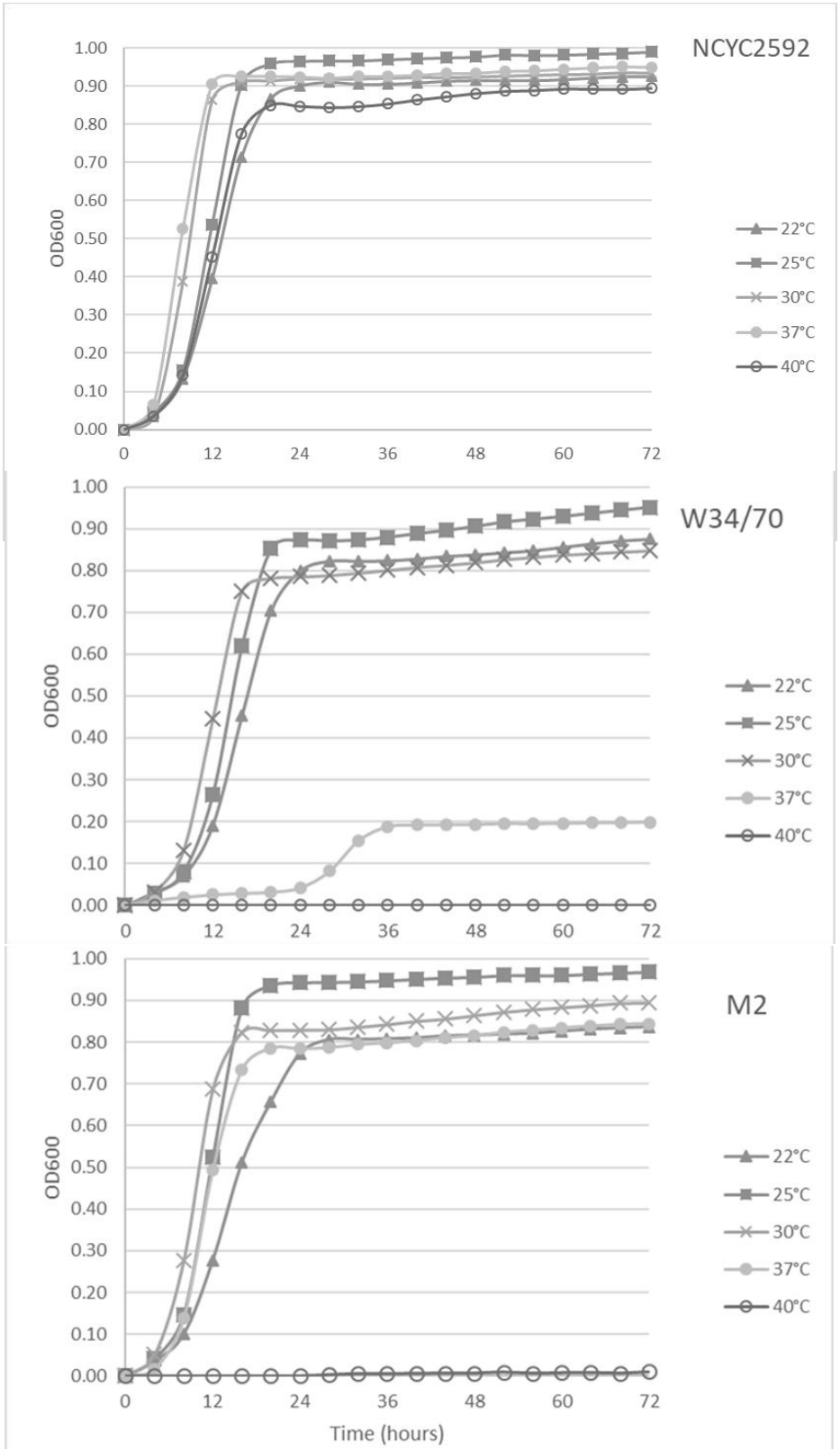

Fig. 3.2: NCYC2592, W34/70 and M2 growth curves. All growth curves were carried out at 22, 25, 30, 37 and 40°C during 72 hours. The growth curves were carried out using OD_{600} and results are the average of three replicates experiments with standard deviations indicated by error bars.

At 22°C, W34/70 and M2 grow similarly and NCYC2592 grow significantly better than the other two strains. At 25°C, all strains grow similarly. At 30°C, NCYC2592 (0.93 OD) grow better than M2 (0.89 OD) and W34/70 shows similar growth (0.85 OD) to both other strains. At 37°C, NCYC2592 and M2 can grow and differ from W34/70, which did not grow (0.20 OD). At 40°C, NCYC2592 can grow and M2 cannot grow.

Table 3.1: Yeast ability to grow in different temperature

Temp.	OD600			Growth intensity		
	NCYC2592	W34/70	M2	NCYC2592	W34/70	M2
22°C	0.92^{a}	0.88^{b}	0.84^{b}	++++	++++	++++
25°C	0.99^{a}	0.95^{a}	0.97^{a}	++++	++++	++++
30°C	0.93^{a}	0.85^{ab}	0.89^{b}	++++	++++	++++
37°C	0.95^{a}	0.20^{c}	0.84^{b}	++++	-	++++
40°C	0.89^{a}	0.00^{b}	0.01^{b}	++++	-	-

Data from triplicate measurements presented in average with stardard deviation. Data with different letters are significantly different at $p < 0.05$.
++++ very intense growth (OD 0.80-1.00)
+++ intense growth (OD 0.60-0.80)
++ moderate growth (OD 0.40-0.60)
+ very low growth (OD 0.3-0.40)
- no growth (OD 0-0.30)
*Any growth under 0.40 OD can be ignored because it cannot be visualised on the spot plate.

3.3.2. The growth of yeast on different carbon sources

To evaluate the growth of yeast on different carbon sources, were used YP (1% of yeast extract and 2% of peptone) + SCS (2% of specific carbon source). The carbon sources used were glucose, fructose, galactose, xylose, mannose, rhamnose, sucrose, maltose, lactose, melibiose and melizitose (Chapter 2, Section 2.1.1.1.). The growth curves were carried out using OD600 at 25°C, which was the optimal growth temperature for all strains used in this study.

Fig. 3.2 illustrates yeast growth on different carbon sources during 72 hours using optical density (OD600). Table 3.2 shows five growth levels were

considered the intensity of growth but here the intensity of yeast growth were evaluated on different carbon sources with a concentration of 2% and simplifies the data from the Fig. 3.3. All strains did grow as a very intense growth (OD 0.80-1.00) when inoculated in medium, which the carbon source were glucose or fructose. When the carbon source was galactose, mannose, sucrose or maltose, all strains showed growth between very intense growth (OD 0.80-1.00) and intense growth (OD 0.60-0.80). W34/70 grow on melibiose (0.81 OD) or rhamnose (0.72 OD), which are classified as a very intense and an intense growth, respectively. NCYC2592 and M2 can not grow on melibiose or rhamnose. All strains can not grow on xylose and performed a very low growth when inoculated on melizitose. Using lactose as a carbon source, NCYC2592 (0.55 OD) performed a very low growth; however, W34/70 (0.60 OD) and M2 (0.75 OD) performed a intense growth.

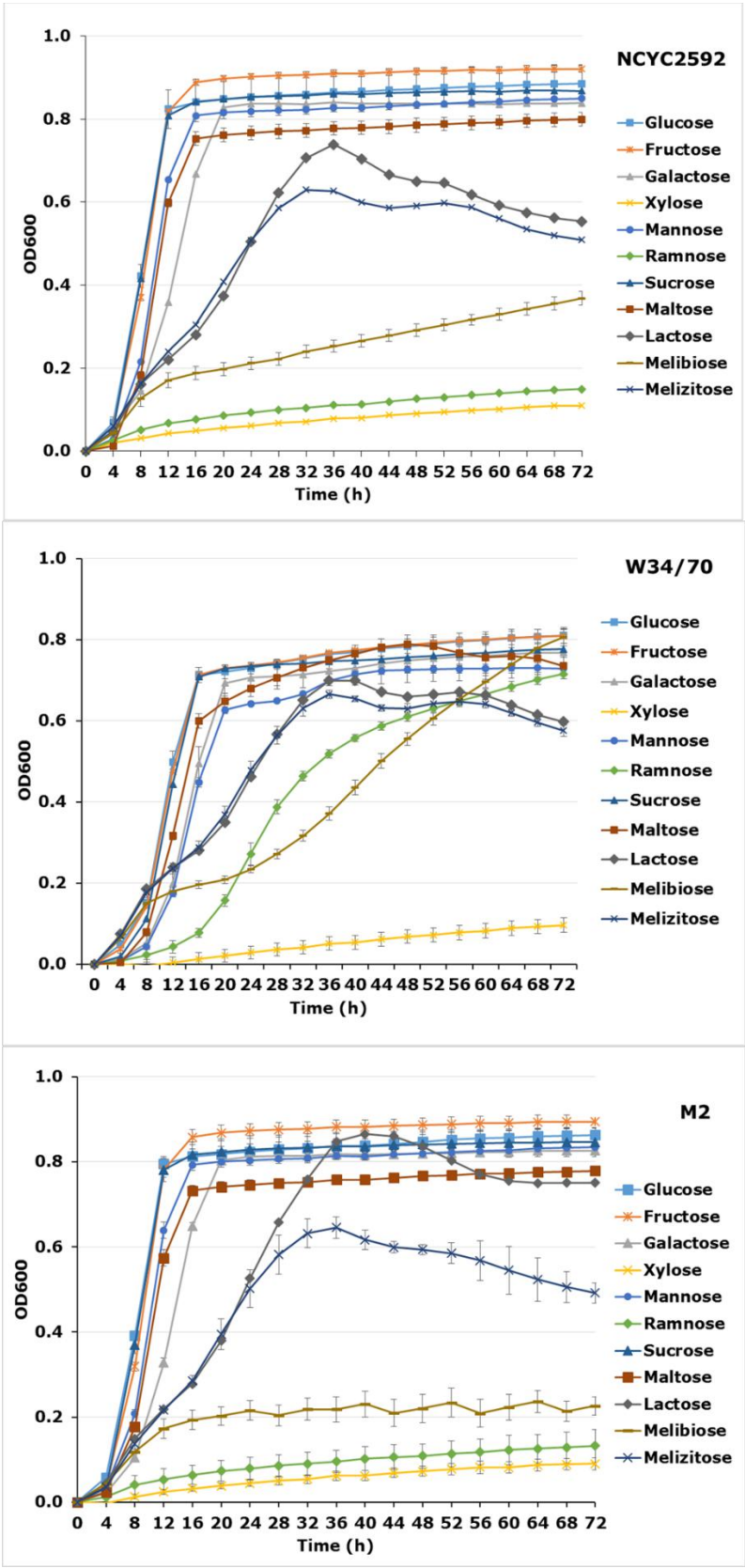

Fig. 3.3: NCYC2592, W34/70 and M2 growth curves. All growth curves and spot plates were carried out on different carbon source at 25°C during 72 hours. The growth curves were carried out using OD_{600} and results are the average of three replicates experiments with standard deviations indicated by error bars.

Table 3.2: Yeast growing in different carbon sources with a concentration of 2%

Carbon source	OD600			Growth intensity		
	NCYC2592	W34/70	M2	NCYC2592	W34/70	M2
Glucose	0.89^{a}	0.81^{b}	0.86^{ab}	++++	++++	++++
Fructose	0.92^{a}	0.81^{b}	0.89^{a}	++++	++++	++++
Galactose	0.84^{a}	0.77^{b}	0.83^{a}	++++	+++	++++
Xylose	**0.11^{a}**	**0.10^{a}**	**0.09^{a}**	**-**	**-**	**-**
Mannose	0.85^{a}	0.73^{b}	0.83^{a}	++++	+++	++++
Rhamnose	**0.15^{b}**	**0.72^{a}**	**0.13^{b}**	**-**	**+++**	**-**
Sucrose	0.87^{a}	0.78^{b}	0.85^{a}	++++	+++	++++
Maltose	0.80^{a}	0.74^{b}	0.78^{ab}	+++	+++	+++
Lactose	0.55^{c}	0.60^{bc}	0.75^{a}	++	+++	+++
Melibiose	**0.38^{b}**	**0.81^{a}**	**0.23^{c}**	**++**	**++++**	**-**
Melizitose	0.51^{a}	0.58^{a}	0.49^{a}	++	++	++

Data from triplicate measurements presented in average with standard deviation. Data with different letters are significantly different at $p < 0.05$.
++++ very intense growth (OD 0.80-1.00)
+++ intense growth (OD 0.60-0.80)
++ moderate growth (OD 0.40-0.60)
+ very low growth (OD 0.3-0.40)
- no growth (OD 0-0.30)
*Any growth under 0.40 OD can be ignored because it cannot be visualised on the spot plate.

W34/70 (*S. pastorianus*) grows significantly less than other two *S. cerevisiae* (NCYC2592 and M2, ale strains) on all carbohydrates used in this work, except rhamnose and melibiose. W34/70 can grow rhamnose and melibiose as single carbon source. As a result, can support the diferentiation and confirms that NCYC2592 and M2 are ale strains (*Saccharomyces cerevisiae*) and W34/70 is a lager strain (*Saccharomyces pastorianus*).

3.3.3. Differentiation of yeast strains

DNA fingerprint is a technique associated with genetic engineering and contributes for verification of DNA compatibilities. In principle, DNA is a molecule finds up in cell's nucleus and has all genetics information of each being different from individual to individual. The standard of bands are unique for each individual and similarities proves genetic approaching. According to Legras and Karst (2003) is possible to identify strains *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* using delta12-delta21 primers for DNA fingerprint (Chapter 2, section 2.2.6.).

Fig. 3.4 displays electrophoresis patterns carried with different yeasts with delta12-delta21 primers. Results were obtained using agarose electrophoresis and illustrates the detection of intense and faint bands, which supports the different number and size of fragments found. NCYC2592 and M2 possess intense and similar bands from 140 bp up to 400 bp, which were expected because their capacity of growth at 37°C and their no capacity to grow on melibiose that was proved in the last to sections. However, M2 possesses five intense bands over 400bp that differ to NCYC2592 and W34/70. On the other hand, W34/70 possesses other faint bands below 140 bp and above 500 bp, which differs totally to other two strains used in this study. These three-yeast strains presented different DNA fingerprint, which confirms that they are genetically different.

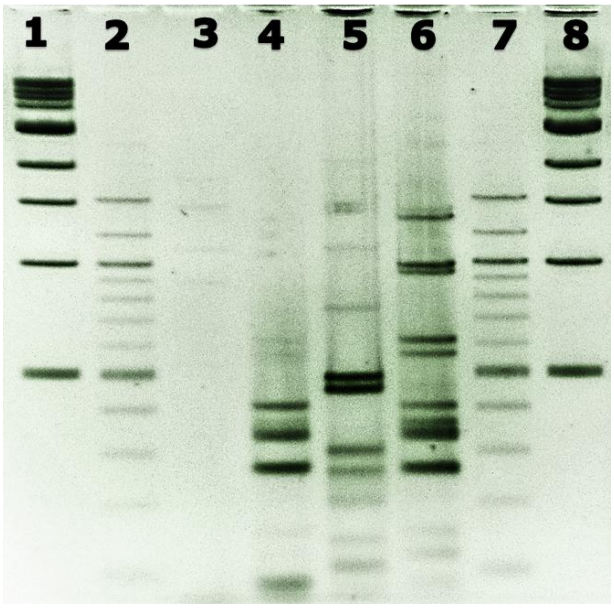

Fig. 3.4: Electrophoretical patterns carried for different yeasts with delta12-delta21 primers. Lanes 1-8 of gel: 1, molecular mass marker (1Kb ladder); 2, molecular mass marker (100pb ladder); 3, negative control; 4, *S. cerevisiae* NCYC2592; 5, *S. pastotianus* W34/70; 6, *S. cerevisiae* M2; 7, molecular mass marker (100pb ladder); 8, molecular mass marker (1Kb ladder). Pictures' capture in dark incubator under UV light using VisionWorks®LS Analysis Software editing using adobe Photoshop.

3.3.4. Yeast mineral composition.

The analysis of the mineral components in brewing yeast was measured using ICPMS (Chapter 2, section 2.5.3). Table 3.3 shows yeast mineral composition of NCYC2592, W34/70 and M2 yeast strains, which was divided into 3 groups: Group 1 has nine nutrients (presented in ppm - mg/Kg), which are the most important metabolic minerals for yeast health and growth. Group 2 (presented in ppm - mg/Kg) includes a very important group of elements, which are responsible for metabolic activities and help driving elements from group 1 into yeast cell, but if the intakes of element of group 2 do not occur, it might not affect yeast growth. Finally, group 3 (presented part per billion - µg/Kg) involves mineral elements, which are in very low presence in the yeast and wort.

Table 3.3: Mineral composition of NCYC2592, W34/70 and M2 strains.

Minerals		Yeasts stain		
Group 1		NCYC 2592	W34/70	M2
Nitrogen*	mg/kg	-	-	-
Phosphorus		19,405^{ab}±437	16,912^{b}±506	22,656^{a}±2,007
Potassium		27,366^{a}±472	18,064^{b}±243	17,281^{b}±1,435
Magnesium		1,109^{a}±20	1,056^{a}±12	1,063^{a}±85
Calcium		290^{b}±3	349^{b}±7	635^{a}±50
Iron		159^{a}±3	164^{a}±2	144^{a}±13
Copper		1.86^{c}±0.04	4.4^{b}±0.1	5.9^{a}±0.5
Zinc		304^{b}±17	508^{a}±7	470^{a}±4
Manganese		249^{a}±184	603^{a}±137	245^{a}±85
Group 2				
Sulphur	mg/kg	4,565^{a}±118	4,777^{a}±80	3,528^{b}±277
Sodium		3,492^{b}±88	15,742^{a}±379	13,604^{a}±1,232
Aluminium		0.7^{a}±0.1	0.4^{a}±0.2	0.3^{a}±0.0
Boron		0.74^{a}±0.03	0.53^{b}±0.01	0.51^{b}±0.07
Rubidium		7.2^{a}±0.2	6.68^{a}±0.06	7.8^{a}±0.7
Strontium		1.84^{b}±0.05	2.14^{b}±0.03	2.85^{a}±0.25
Group 3				
Barium	µg/kg	323^{a}±16	215^{c}±8	267^{b}±22
Titanium		118^{b}±5	137^{b}±11	199^{a}±16
Chromium		15^{a}±2	25^{a}±2	23^{a}±21
Cobalt		2,987^{b}±56	6,356^{a}±67	3,102^{b}±248
Nickel		51^{a}±7	49^{a}±6	28^{a}±11
Lithium		91^{c}±2	157^{a}±6	127^{b}±13
Beryllium		0.7^{a}±0.6	0.4^{a}±0.1	0.7^{a}±0.4
Vanadium		153^{a}±5	116^{b}±8	102^{b}±7
Gallium		10^{a}±7	20^{a}±4	6.8^{a}±2.4
Arsenic		107.4^{a}±3.6	60.2^{b}±5.4	54.2^{b}±4.7
Selenium		140.9^{a}±6.5	132.7^{a}±0.6	125^{a}±9
Molybdenum		32.8^{a}±0.9	34.5^{a}±0.7	33.4^{a}±5.1
Silver		1.4^{a}±0.6	1.8^{a}±0.1	1.7^{a}±0.6
Cadmium		28.3^{c}±2.2	59^{a}±2	41^{b}±2
Caesium		57^{c}±1	72^{b}±1	92^{a}±6
Thallium		74^{a}±2	25.7^{b}±0.5	24^{b}±2
Lead		6^{a}±1	0.8^{a}±0.6	3.0^{a}±2.8
Uranium		1.9^{b}±0.1	3.00^{a}±0.03	2.0^{b}±0.2

*Ammonium nitrogen was not analysed

**Data highlighted in orange colour represent the similarities among three yeast strains evaluated.

Data from triplicate measurements presented in average and standard deviation. Data with different letters are significantly different at $p < 0.05$.

Table 3.3 shows the mineral composition of NCYC2592, W34/70 and M2 strains. W34/70 has a lower and significant different quantity of phosphorus (16.9±0.5 g/kg) than M2 (22±2 g/kg) but a similar quantity comparing to NCYC2592 (19.4±0.4 g/kg). NCYC2592 has higher concentration of potassium (27.3±0.4 g/kg) than W34/70 (18.1±0.2 g/kg) and M2 (17±1 g/kg). M2 has a significant and higher concentration of calcium (635±50 mg/kg) than NCYC2592 (290±3 m/kg) and W34/70 (349±7 mg/kg). Observing the quantification of copper, all strains contain different concentration. M2 (5.9±0.5 mg/kg) has a higher quantity than W34/70 (4.4 mg/kg) and NCYC2592 (1.86±0.04 mg/kg). NCYC2592 contains lower quantity of zinc (304±17 mg/kg) than W34/70 (508±7 mg/kg) and M2 (470±4 mg/kg). When compared the quantity of other essential inorganic elements (magnesium, iron and manganese), all strains did not differ.

3.3.5. Overall of yeast differentiation

Principal component analysis (PCA) shows that all yeasts used in this study are very different when compared their permissive growth temperature and mineral content. It was noticed that exist correlations between permissive growth temperature and mineral content. All strains studied possess all mineral illustrated in the Fig. 3.5 and table 3.3. Fig. 3.5 illustrates that the first component separates the traditional lager (W34/70) strains from the ale strains (M2 and NCYC2592). The second component separates ale (M2) from lager (W34/70) and ale (NCYC2592). Considering the temperature of 37°C, which was used for differentiation via permissive growth, PCA separates the *Saccharomyces cerevisiae* strains from *Saccharomyces pastorianus* and as

expected, shows that *S. cerevisiae* (ale) strains grown better than *S. pastorianus* (lager) strain in all temperature studied, except at 15°C, which was previously described.

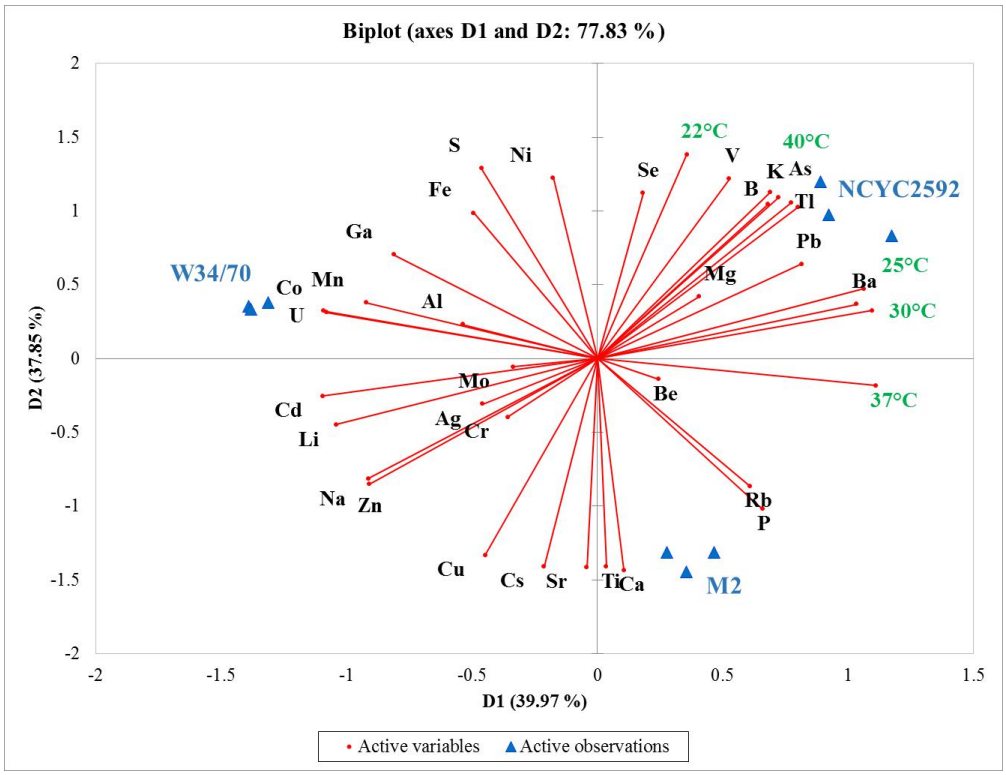

Fig. 3.5: Principle component analysis (Bio-Plot) to identify the correlation and similarities among all 37 variables including 32-mineral elements, 5-growing temperature and 3 yeast strains (NCYC2592, W34/70 and M2).

The first two components (PCA1 and PCA2) explains 77.83% of the separation. PCA1 and PCA2 are represented as D1 and D2, respectively. Fig. 3.5 represents the components of the global analysis as specific correlations to each yeast strain studied. From this, all yeasts evaluated were physiologically different considering permissive growth temperature, grow on different carbon source, mineral composition and consequently, these were confirmed by DNA fingerprint.

Observed all correlation and similarities illustrated in the PCA (Fig. 3.5) that yeast has a high presence of ions such as sodium, zinc, manganese, aluminium. has less presence of ions such as potassium, magnesium, barium. Likewise, strains that have a higher presence of elements such as calcium, phosphorus, copper. they have a lower concentration of elements such as iron, suffer, nickel, selenium. NCYC2592 and M2 strains have more phosphorus, potassium, magnesium than W34/70 strain. M2 strains have the highest concentration of calcium.

3.4. Discussion

Differentiation of brewing yeast is a important because supports their physiologic and genetic differences. Therefore, supports processing control and as a consequence, quality of product. Kopeck *et al.* (2016) compared more than 40 brewing yeasts *Saccharomyces* strains (ale and lager) concluded that *Saccharomyces pastorianus* cannot grow at 37°C and *Saccharomyces cerevisiae* grows (Kopeck *et al.* 2016, Walker and Stewart 2016, Spitaels *et al.* 2014, Boulton and Quain 2001). Fig. 3.1 shows all yeasts used in this study can grow at temperatures from 15 °C up to 30 °C. W34/70 strain can grow in low temperature at 15°C up to temperatures under 35°C and cannot grow at 37°C, which classifies this strain as *Saccharomyces pastorianus* (lager strain). On the other hand, NCYC2592 and M2 are ale strains, which can grow at 37°C. However, only NCYC2592 can grow at 40°C, which explains the fact of this strain has been used for bioethanol production due to their capacity of growth between 40–45 °C (Mohd Azhar *et al.* 2017).

The ability of yeast consume hexoses via glycolysis is well known thereby converting it into ethanol. Yeast grows several carbon sources, which includes monosaccharides and disaccharides. Brewing yeast strains are classified as lager and ale, which can grow on hexoses (monosaccharides). In addition, cell wall can transport disaccharides and are broken down in hexoses. All these carbohydrates are consumed via glycolysis. *Saccharomyces* strains contains an advanced system of hexose transport, which regulates the carbohydrate transportation and has a high affinity for glucose (Galdieri *et al.* 2014, van Maris *et al.*, 2006, Briggs *et al.* 2004). *S. cerevisiae* strains easily ferment glucose, mannose and fructose via glycolysis and galactose via combination of the Leloir pathway and glycolysis (van Maris *et al.*, 2006).

Although glucose is the preferred source of carbon and energy for the yeast *Saccharomyces* (Galdieri *et al.* 2014), fructose and mannose are the isomers of glucose that all yeast strains can grow on and are transported by the same hexose transporters. As expected, all three yeast strains used in this work grown on glucose and their isomers, mannose and fructose. In addition, *Saccharomyces cerevisiae* grown on galactose, which has the first uptake through the galactose permease (Gal2p) and then is converted into glucose-6-phosphate. Galactose is consumed via combination of the Leloir pathway and glycolysis (van Maris *et al.*, 2006). As expected, all three yeast strains used in this work can grow on galactoses; furthermore, all strains presented lower growth on galactose than on glucose.

Glucose and fructose are called “rapid fermentable sugars” or reduced sugars and rhamnose is more reduced than either of them. However, yeast *Saccharomyces cerevisiae* cannot grows on rhamnose as the sole carbon source

due to the inexistence of genes encoding rhamnose-metabolizing enzymes in *S. cerevisiae* genome (van Maris *et al.*, 2006). Therefore, as expected, NCYC2592 and M2 strains cannot grow on rhamnose as a carbon source (Table 3.2), which supports their classification as *Saccharomyces cerevisiae* strains. On the other hand, W34/70 can grow on rhamnose (Table 3.2) and maybe this strain has rhamnose-metabolizing enzymes in its genome.

Saccharomyces strains can ferment disaccharides maltose (genes encoded MAL1-4 and MAL6) and sucrose (invertase genes encoded SUC1-5 and SUC7), but they can use lactose and melizitose as a carbon source as well. As expected, all strains can grow on maltose, sucrose, lactose or melizitose as single carbon source (Table 3.2). However, using lactose or melizitose as a single carbon source, all strains presented a significant reduction on their growth when compared to maltose and sucrose as single carbon source. According to van Maris *et al.* (2006) yeast growth on lactose and melizitose as a single substrate is inconsistent, therefore, means OD600 up to 0.60 (Table 3.2).

Brewery yeast can use the majority of carbohydrates; however, ale (*Saccharomyces cerevisiae*) and lager (*Saccharomyces pastorianus*) strains can be differentiated due to their ability to assimilation or no assimilation melibiose (disaccharide). Therefore, ale strains cannot grow on melibiose, on the other hand, lager strains can. This occurs because lager strains have α-D-galactose activity, which hydrolyses mebibiose into galactose and glucose (Briggs *et al.* 2004, Barnett, 1981). Results show that all yeasts can grow on melibiose; however, *Saccharomyces cerevisiae* such as NCYC2592 and M2 grown on melibiose as a single substrate were inconsistent (OD lower than

because they have not α-D-galactose activity, therefore, cannot convert mebibiose into galactose and glucose (Briggs *et al.* 2004, Boulton and Quain 2001). On the other hand, W34/70 has a very more consistent growth on melibiose. This occurred because W34/70 is a *Saccharomyces pastorianus* (lager strains), which can converts mebibiose into galactose and glucose due to α-D-galactose activity.

During fermentation, independently of the kind of carbohydrate is transported through plasma membrane and catabolised via glycolysis. However, during anaerobic conditions, an essential redox balancing combines the activity of pyruvate decarboxylase and alcohol dehydrogenase. Then, the NADH produced by glyceraldehyde-3-phosphate dehydrogenase is reoxidized via alcoholic fermentation (van Maris *et al.* 2006, Briggs *et al.* 2004). W34/70 (*S. pastorianus*) grows significantly less than other two S. cerevisiae (NCYC2592 and M2, ale strains) on all carbohydrates used in this work, except rhamnose and melibiose. When W34/70 used rhamnose and melibiose as a single carbon source, they presented growth consistency. Therefore, the different growth behaviour of all strains used on different carbon source, it can support the thermal differentiation and confirms that NCYC2592 and M2 are ale strains (*Saccharomyces cerevisiae*) and W34/70 is a lager strain (*Saccharomyces pastorianus*).

DNA fingerprint is a technique associated with genetic engineering and contributes for verification of DNA compatibilities. In principle, DNA is a molecule finds up in cell's nucleus and has all genetics information of each being different from individual to individual. The standard of bands are unique for each individual and similarities proves genetic approaching. According to

Legras and Karst (2003) is possible to identify strains *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* using delta12-delta21 primers for DNA fingerprint (Chapter 2, section 2.2.6.).

The inorganic component of brewing yeast is composed of several minerals, which influence the properties and metabolic activities of yeast. The differences among yeasts are due to the differences in the type and abundance of such minerals due to their genetic and physiologic differences. Sugars are very important for fermentation and amino acids (nitrogen source) stimulates the carbon consumption and increases biomass production (Beltran *et al.* 2005). However, essential inorganic elements contribute for growth and consequently they can contribute for fermentation control and the quality of final product (flavours).

Three strains used in this study were genetically different. As expected, they differ in the quantities of inorganic composition (including Pi, K, Mg, Cu, Zn, Fe, Mn, Ag, Al, As, Ba, Be, Cd, Ca, Co, Cr, Cs, Li, Mo, Na, Ni, Pb, Rb, S, Se, Sr, Tl, U, V). During fermentation, yeast is exposed to different amount of nutrients, which includes essential inorganic elements that are present charged ions into wort (Barreto *et al.* 2012, Canadell *et al.* 2014, Sala 2015). As charged ions, inorganic elements might establish chemical gradients such as ATP synthesis into mitochondria by oxidative phosphorylation and/or secondary transport (Canadell *et al.* 2014). Different ions concentrations are accumulated into yeast cell at different role (Eide *et al.* 2005, Canadell *et al.* 2014).

Magnesium, potassium and phosphorus were identified in higher significant quantities in *Saccharomyces cerevisiae* strains (NCYC2592 and M2) than in

Saccharomyces pastorianus (W34/70) strain*s*. Considering that phosphorus, potassium and magnesium are charged ions. Additionally, they are responsible for the most of cytosolic activities, which includes enzymes activation in the glycolysis pathway, internal pH control and ATP formation. M2 has highest presence of calcium comparing to other two strains and it might be related to the flocculation characteristics of this strain (bottom-fermentation). In addition, this strain possesses more alkali metals such as rubidium and caesium than other two strain used in this study.

Lager strain (W34/70) possesses highest quantities of lithium and sodium than two other ale strains and it might be related to the natural capacity of replacement of potassium for yeast strains. Furthermore, *Saccharomyces cerevisiae* strains (NCYC2592 and M2) possess more potassium and less lithium and sodium than *Saccharomyces pastorianus* strain. In addition, yeasts *Saccharomyces cerevisiae* (NCYC2592 and M2) have more phosphorus, potassium, magnesium, calcium than *Saccharomyces pastorianus* (W34/70). On the other hand, W34/70 possesses more essential metal ions such as iron, zinc and manganese than NCYC2592 and M2.

All yeast strains used in this study have thirty-two mineral elements in their structure, which are fundamental for growth, health and surviving of yeast. However, mineral elements including ammonium-nitrogen, inorganic-phosphate, potassium, magnesium, zinc, iron, copper and manganese are the essential inorganic elements (Sala 2015, Canadell *et al.* 2014, Barreto *et al.* 2012), which influence on cell metabolic activities as co-factors and can support metabolic activities for growing and surviving, and as a consequence yeast health.

3.5. Conclusion

Two ale-type (NCYC2592 and M2) and one lager-type (W34/70) brewing yeast strains were characterized based on differences in permissive growth temperature and the presence of melibiose (α-galactosidase). DNA fingerprint confirmed their genetically differences. NCYC2592 and M2 have similar mineral composition. All yeast strains are genetically different and the characterisation of brewing yeast strains provides a preliminary understanding to conduct the thesis investigation entitled: ‘the impact of yeast nutrition on flavour formation during yeast fermentation’.

CHAPTER IV

FLAVOUR FORMATION DURING YEAST FERMENTATION

4. CHAPTER IV: FLAVOUR FORMATION DURING YEAST FERMENTATION

4.1. Introduction

Flavours are very important components of alcoholic beverages and during fermentation, a part of these flavours are produced by yeasts (Walker and Stewart 2016). Ethanol, glycerol, acetic acid, succinic acid and CO_2 are the highest concentration compounds produced during alcoholic fermentation (Stewart 2016, Walker and Stewart 2016). Also, beer flavours include short- and medium-chain fatty acids, higher alcohols and as a consequence, fatty acid esters and acetate esters (Yu *et al.* 2016, Krogerus and Gibson 2013, Saerens *et al.* 2010, Hazelwood *et al.* 2008, Verstrepen *et al.* 2003). All of these compounds are important for the quality of the end product; however, only fatty acid esters and acetate esters are flavour active (Saerens *et al.* 2010, Hazelwood *et al.* 2008, Verstrepen *et al.* 2003). All of the active-flavours have acetyl-CoA as a precursor as well as short-chain fatty acids and higher alcohols, which both compete via the Ehrlich pathway.

Several works have been published dealing with the analysis of brewing flavours (Yu *et al.* 2016, Krogerus and Gibson 2013, Saerens *et al.* 2010, Hazelwood *et al.* 2008, Verstrepen *et al.* 2003); however, none of them has reported the influence of sugars and ethanol presence on acetic acid formation and as a consequence ester formation nor the competition between higher alcohols and short/medium chain fatty acids formation. This chapter generates a more complete understanding of yeast flavour formation. In addition, the competition between the formation of higher alcohols and short/medium chain

fatty acid, which consequent effects on ester formation, including acetate esters and fatty acid esters.

4.2. Material/Method/Experimental design

Two *Saccharomyces cerevisiae* (NCYC2592 and M2) and one *Saccharomyces pastorianus* (W34/70) brewing yeast strains were used in this study (Section 2.1). Fermentations were conducted as described in section 2.1.3. A standard sweet wort (13°P) was obtained from the International Centre for Brewing Science (ICBS) at The University of Nottingham. The characterization involved specific gravity (13°P), pH (5.20), carbohydrates (chapter 2, table 2.1) and mineral composition (chapter 2, table 2.2).

4.3. Results

Fig. 4.1 illustrates all carbohydrates present in the wort including maltotetraose+dextrin (18.0 g/L), maltotriose (18.0 g/L), maltose (77.6g/L), sucrose (2.4 g/L), glucose (12.5 g/L) and fructose (1.6 g/L), which in all totalled 130.5 g/L (~13°P). NCYC2592 consumed 97.2 g of carbohydrates/L (from 130.5 to 33.3 g/L), W34/70 consumed 95.3 g of carbohydrates/L (from 130.5 to 35.2 g/L), and M2 consumed 97.0 g of carbohydrates (from 130.5 to 33.5 g/L).

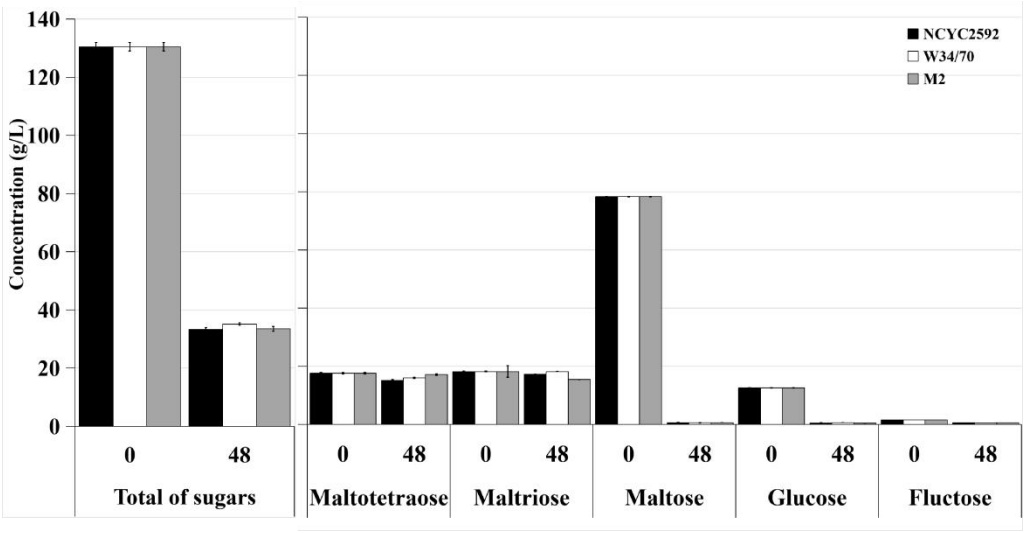

Fig.4.1: Illustrates the concentration of total of sugars, maltotetraose, maltotriose, maltose, glucose and fructose. These include initial concentration (wort concentrations) and final concentrations (48 hours). All fermentations were conducted for 48 h using yeasts NCYC2592 (■), W34/70 (□) and M2 (▣). All data is present in average of concentration (g/L) and standard deviation.

The final concentration of carbohydrates is a result of non-consumed sugars at 48 h, which includes maltotetraose, dextrin and maltotriose (Fig. 4.1). Under studied conditions, the sum of maltotriose and maltotetraose/dextrin represent the non consumed carbohydrates in wort. NCYC2592, W34/70 and M2 did not consume 34.8, 35.8 and 33.0 g of carbohydrate total/L, respectively. NCYC2592 and M2 consumed a little of maltrotriose, which were respectively 1.0 g/L and 2.8 g/L. W34/70 did not consumed maltrotriose (Fig. 4.1) by 48 h. By the end of fermentation, NCYC2592, W34/70 and M2 left, respectively, 17.2, 18.2 and 15.4 g of maltrotriose/L. As expected, all yeast strains applied consumed carbohydrates such as maltose, sucrose, glucose and fructose at 48 h of fermentation (Fig. 4.1).

4.3.1. Flavour formation during yeast alcoholic fermentation

Fig. 4.2 illustrates fermentation profile of *Saccharomyces* (NCYC2592, W34/70 and M2), which considers a well-known consume of sugars and ethanol formation. At 16 h, NCYC2592, W34/70 and M2 had fermented 85.8, 71.1 and 97.0 g of total sugars/L, which represent proximately 2/3 of total sugars and had generate 29.0, 36.9 and 28.9 g of ethanol/L. Furthermore, at 16 h, wort had 11.4, 34.8 and 26.3 g of sugars/L, which can be fermented. In conclusion, at 16 h, the ethanol concentration became higher than the concentration of total of sugars and at the same time, acetic acid stopped its accumulation and started being consumed (Fig. 4.3).

4.3.1.1. Glycerol and acetic acid (acetate) formation

Fig. 4.3 (A, B and C) illustrates glycerol and acetic acid formation (acetate). NCYC2592, W34/70 and M2 strains produced 1.07, 1.09 and 1.10 g of glycerol/L, and 0.47, 045 and 0.46 g of acetic acid/L, respectively. These were expected, because generally glycerol concentrations are found between 0.4-4.0 g/L and acetic acid up to 1 g/L (ASBC Methods of Analysis: Beer Flavour Database 2011). Acetic acid is accumulated from zero to 16 h. At 16 h, NCYC2592, W34/70 and M2 strains accumulated 0.47, 045 and 0.46 g of acetic acid/L, respectively. Afterwards, acetic acid started been consumed to a final concentration of 0.44, 0.41 and 0.36 g of acetic acid/L, respectively, NCYC2592, W34/70 and M2 (Fig. 4.3).

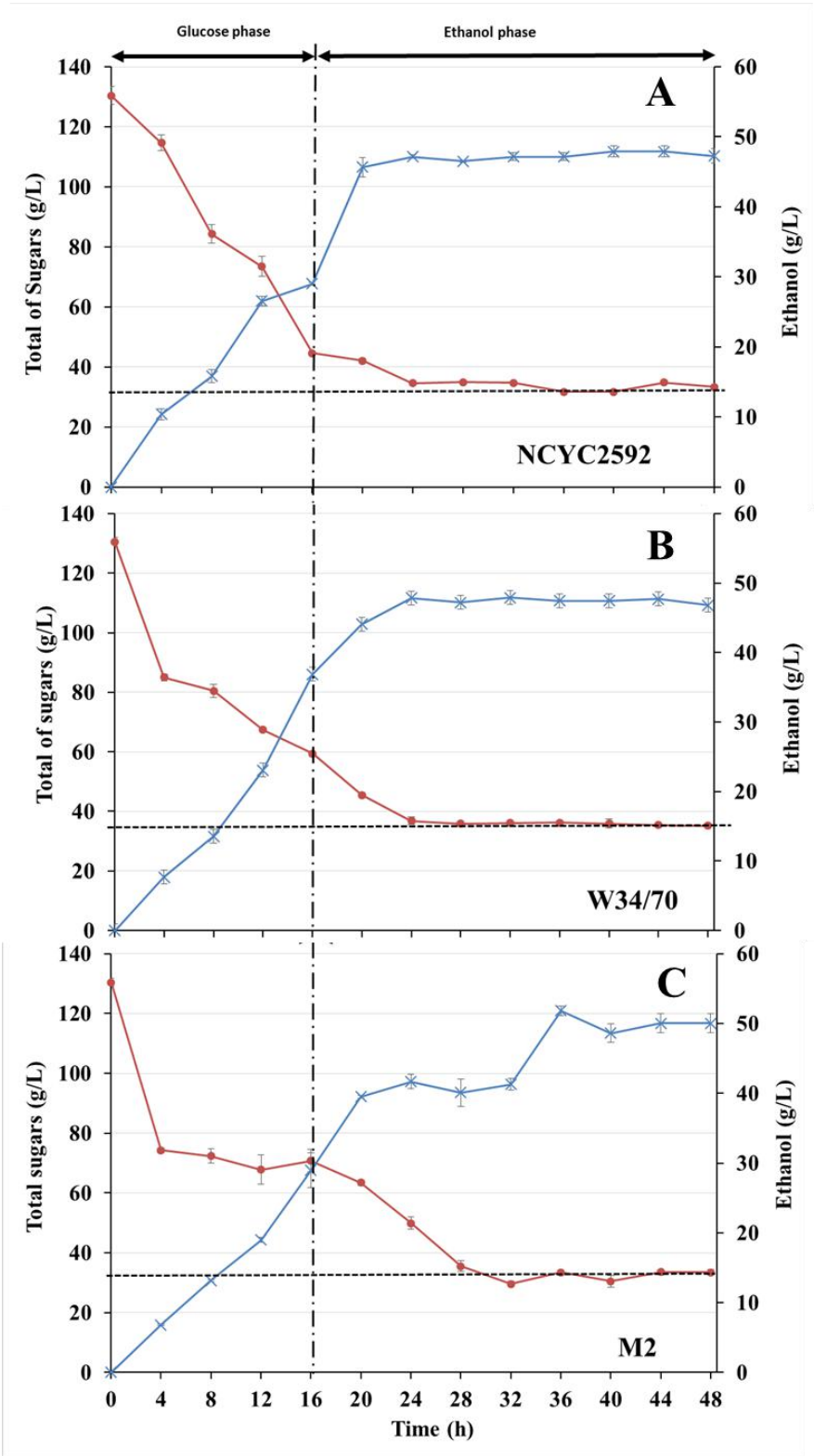

Fig. 4.2: Fermentation profile of *Saccharomyces* (NCYC2592, W34/70 and M2), which considers a well-known consume of sugars and ethanol formation: (A) NCYC2592, (B) W34/70 and (C) M2. Samples were collected at 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44 and 48 h and kept at -20℃. Total of sugars (—●—') and ethanol content (—✕—) were measured using HPLC. Horizon line represents the sum of maltotetraose/dextrin and maltotriose (total sugars g/L); and vertical line represents when ethanol content becomes higher than consumed carbohydrate content during fermentation.

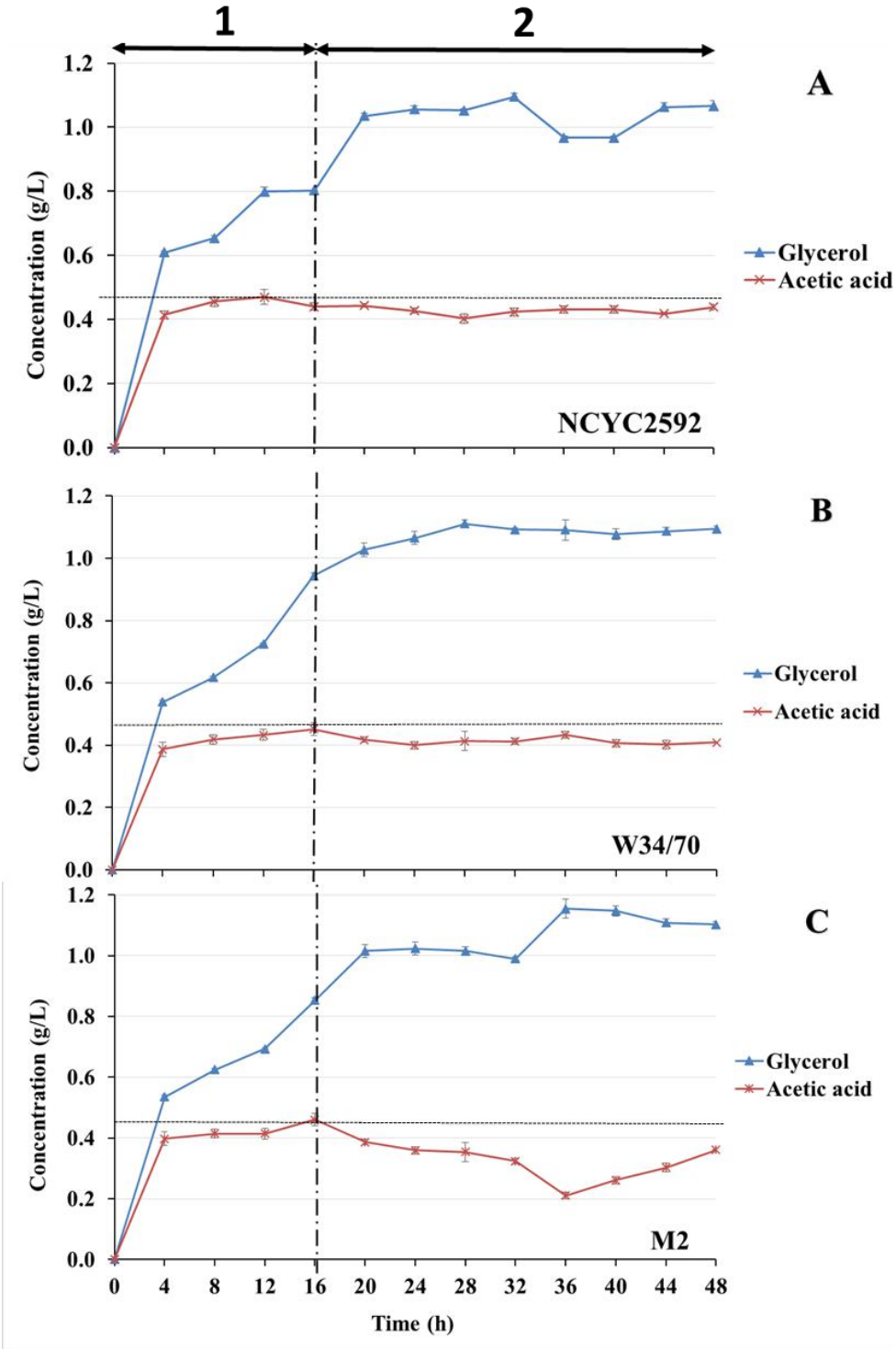

Fig. 4.3: The formation of ( ) glycerol and ( ) acetic acid were measured using HPLC: (A) NCYC2592, (B) W34/70 and (C) M2. The vertical dotted line represents the instant of time that 1) glucose domain finished and ethanol domain initiated.

4.3.2. Volatile-flavour formation under during yeast alcoholic fermentation

During yeast-aroma formation, acetyl-CoA is involved by reaction with higher alcohols or fatty acids generating acetate esters and/or fatty acid esters, respectively. In brewing fermentation, yeasts generate acetyl-CoA-cytosolic via PHD bypass, which is related to acetic acid/acetate formation (Pietrocola *et al.* 2015; Galdieri *et al.* 2014; Kocharin and Nielsen 2013; and Remize *et al.* 2000). Fig. 4.2 illustrates that acetic acid accumulation and consumption, which suggests the importance of glucose domain and ethanol domain classification. Furthermore, acetic acid formation is responsible for acetyl-CoA-cytosolic, which reacts with higher alcohols or medium chain fatty acids to generate, respectively, acetate esters and fatty acid esters.

4.3.2.1. Acetate Ester formation during yeast alcoholic fermentation

Fig. 4.4 illustrates the concentration of ethyl acetate, isobutyl acetate, isoamyl acetate, 2-Phenethyl acetate and total of acetate esters during fermentation. NCYC2592 and M2 generated 50% of total of acetate esters (2 mg/L and 8.5 mg/L, respectively) by 16 h (Fig. 4.4 E). W34/70 metabolised 70% of total of acetate esters (8.0 mg/L, respectively) by 16 h (Fig. 4.4 E). In all yeast strains, ethyl acetate (Fig.4.4 A) and isobutyl acetate (Fig. 4.4 B) was fully accumulated by 16 h. W34/70 and M2 metabolised 50% of isoamyl acetate (4 mg/L and 3.8 mg/L, respectively). On the other hand, NCYC2592 generated isoamyl acetate after 16 h, however, just at 48 h can be observed a significant concentration of isoamyl acetate (Fig. 4.4 C). W34/70 and NCYC2592

metabolised 50% (1.1 mg/L) and 20 % (0.4 mg/L) of 2-phenyl acetate by 16 h. On the other hand, M2 can generate 2-phenyl acetate after 16 h, however, the final concentration of this compounds 1.8 mg/L was similar to the final concentration observed in fermentation using NCYC2592 and W34/70. Therefore, confronting the data from Fig. 4.2, 4.3 and 4.4, was observed that during fermentation, acetate esters are influenced by carbohydrates, ethanol and acetic acid content.

4.3.2.2. Fatty acid ester formation during yeast alcoholic fermentation

Fig. 4.5 illustrates the concentration of ethyl butanoate (A), ethyl hexanoate (B), ethyl octonoate (C), ethyl decanoate (D) and the total of fatty acid esters (E) during fermentation. NCYC2592 and M2 produced 50% of fatty acid esters (12.5 and 14.2 µg/L, respectively), which occurred during the acetic acid accumulation (for the first 16 h) and other 50% were formed after 16h (Fig. 4.5E), which involved acetic acid consumption. At 16 h, W34/70 metabolised all individual fatty acid esters and as a consequence the total of fatty acid esters (20 µg/L). W34/70 and M2 strains generated ethyl octanoate (0.8 and 0.7 µg/L, respectively) by 16 h, on the other hand, NCYC2592 produced 25% of ethyl octanoate (0.1 µg/L) by 16 h. All strains produced 80% of ethyl decanoate during the first 16 h (Fig. 4.5 D). Although fatty acid esters were generated during acetic acid accumulation (first 16 h), they were generated after 16 h, which means the acetic acid consumed may be increase fatty acid ester production.

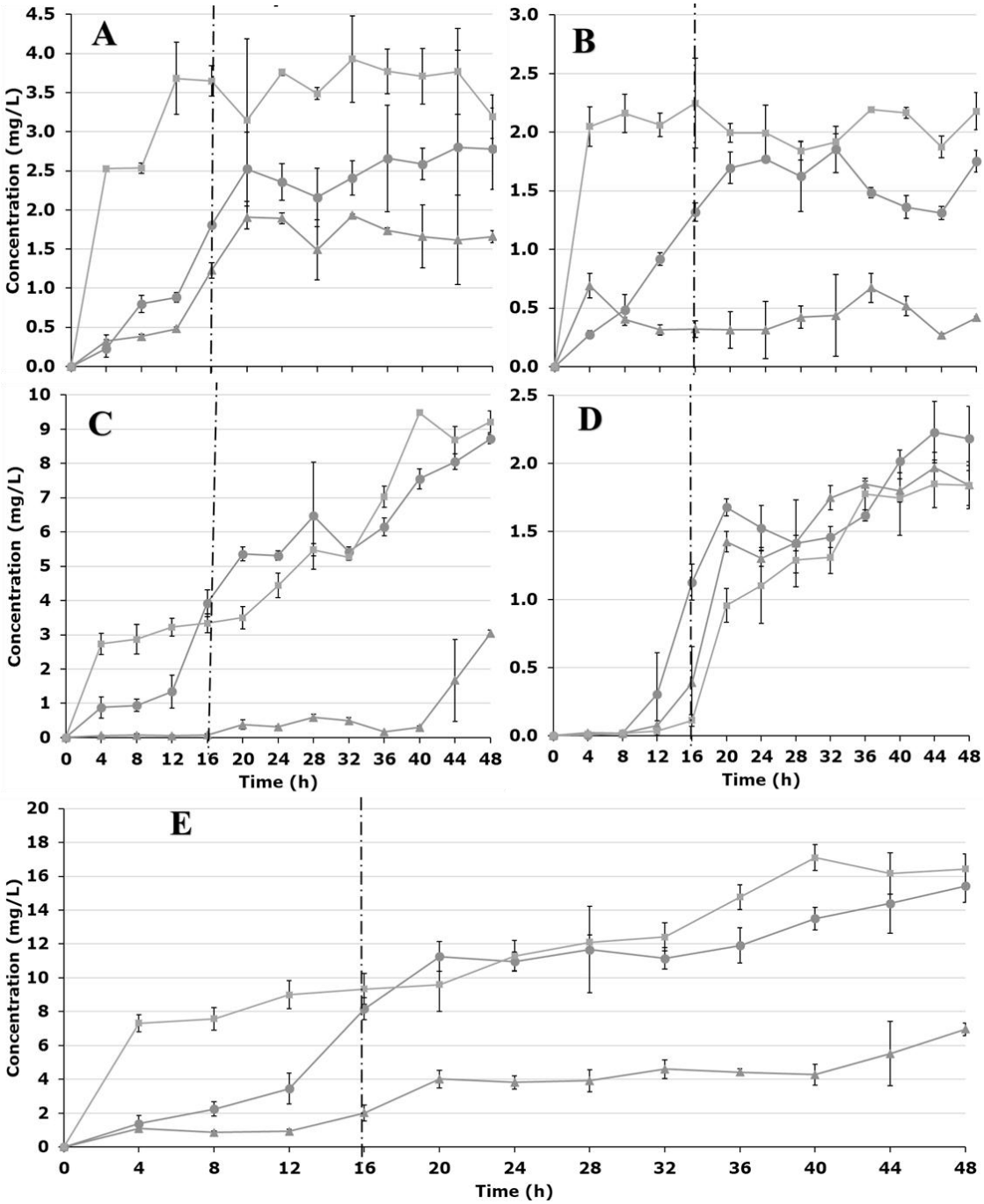

Fig. 4.4: The concentration of ethyl acetate (A), isobutyl acetate (B), isoamyl acetate (C), 2-Phenethyl acetate (D) and total of acetate esters (E) during fermentation. All fermentations were conducted for 48 h using yeasts NCYC2592 (—▲—), W34/70 (—●—) and M2 (—■—). Samples were collected in triplicate by destructive sampling at 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44 and 48 hours. The vertical dotted line represents the instant of time that glucose domain was finished and ethanol domain was initiated. All data is present in average of concentration (mg/L) and standard deviation.

4.3.2.3. Higher alcohols and fatty acid formation during yeast alcoholic fermentation

Higher alcohols and short-chain fatty acids are formed via the Ehrlich pathway (Yu *et al.* 2016, Hazelwood *et al.* 2008, Boulton and Quain, 2001). Furthermore, short-chain fatty acids are the precursor of medium and long chain fatty acids, which are driven via fatty acid pool (Saerens *et al.* 2010). Higher alcohol and fatty acid formation are based on yeast growth and consequently, all esters have yeast growth as a main key. Fig. 4.5 illustrates yeast growth by OD600 using Tecan and was observed that at 20 h, NCYC2592 (0.41 OD), W34/70 (0.42 OD) and M2 (0.48 OD), which represent the the end of exponential growth phase. Therefore, it suggests the yeast metabolism shifts from an aerobic to an anaerobic condition.

4.3.2.5. Higher alcohols formation as a precursor of acetate esters

Fig. 4.7 illustrates the concentration of propanol (A), isobutanol (B), 2-methyl butanol (C), isoamyl alcohol (D), 2-Phenyl-alcohol (E) and the total of higher alcohols (F) during fermentation. All yeast strains produced the total concentration of higher alcohols by 20 h (Fig. 4.6 F), except M2, which increased the accumulation of higher alcohols from 32 h to 48 h (from 340 mg/L to 518 mg/L).

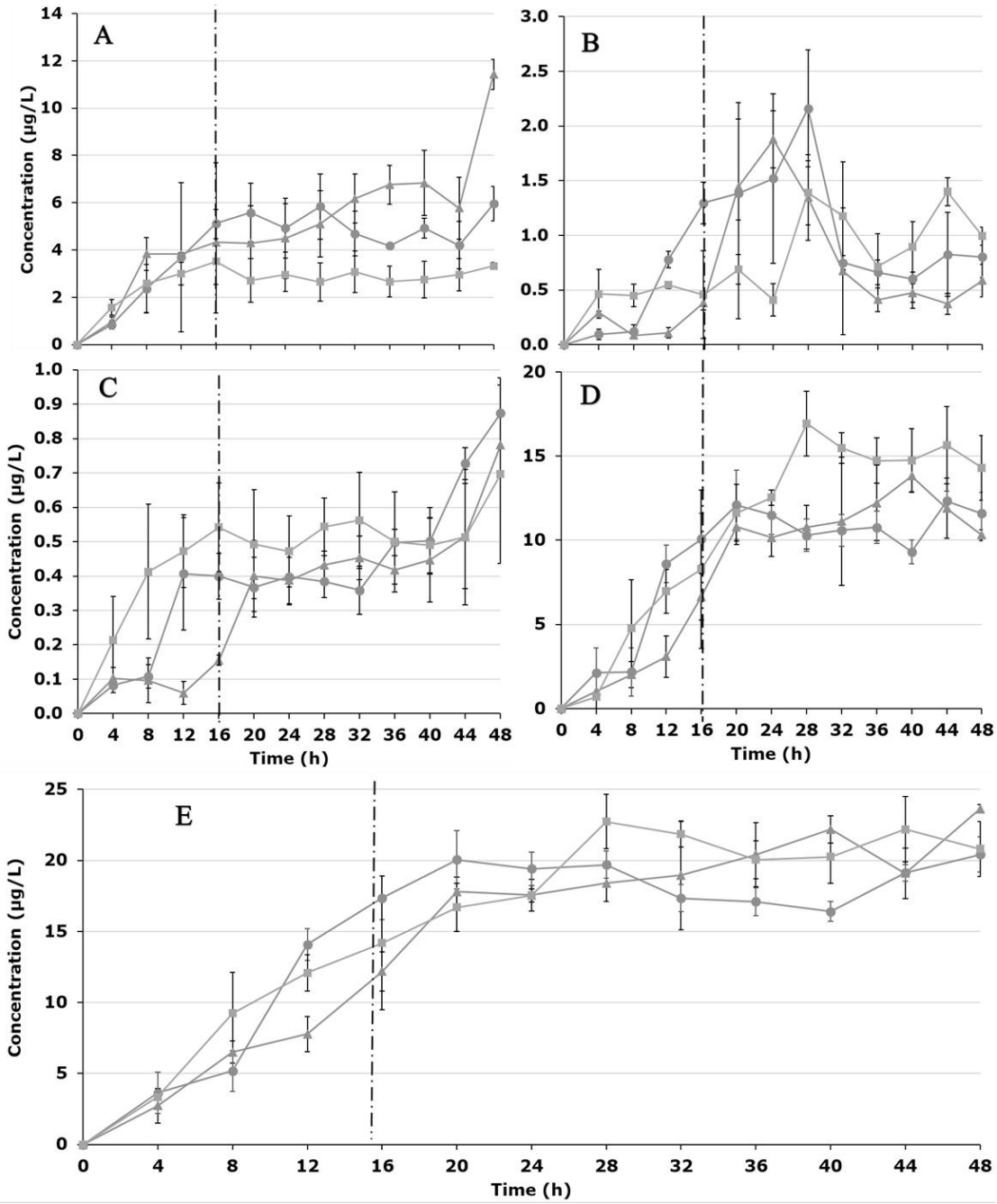

Fig. 4.5: The concentration of ethyl butanoate (A), ethyl hexanoate (B), ethyl octonoate (C), ethyl decanoate (D) and the total of fatty acid esters (E) during fermentation. All fermentations were conducted for 48 h using yeasts NCYC2592 (—▲—), W34/70 (—●—) and M2 (—■—). Samples were collected in triplicate by destructive sampling at 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44 and 48 hours. The vertical dotted line represents the instant of time that glucose domain was finished and ethanol domain was initiated. All data is present in average of concentration (mg/L) and standard deviation.

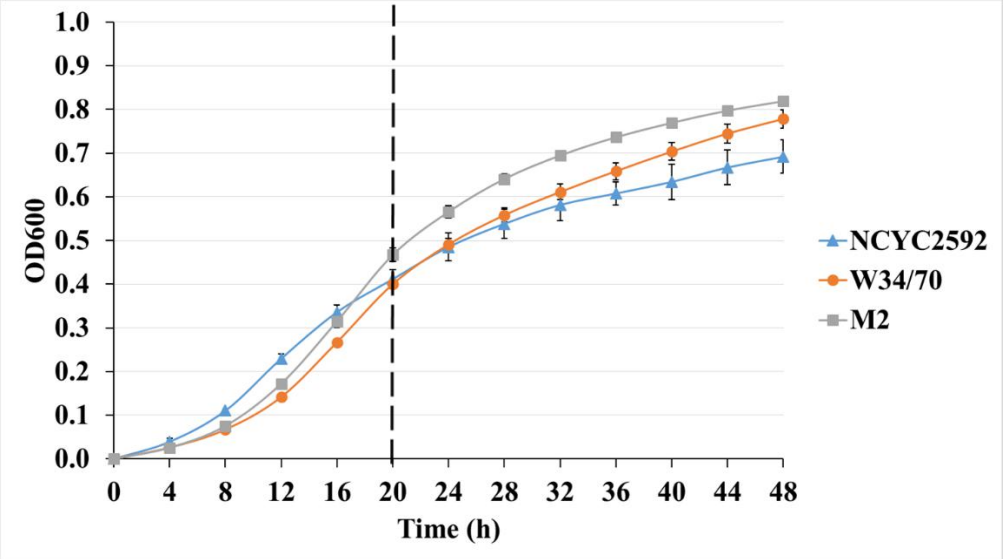

Fig. 4.6: NCYC2592, W34/70 and M2 growth curves. All growth curves measured using OD_{600} at 22°C for 48 hours. Results are the average of three replicates experiments with standard deviations indicated by error bars. The dotted line represents the ending of exponential phase

NCYC2592 and W34/70 produced the highest concentration of propanol, isobutanol, 2-methyl butanol, isoamyl alcohol, 2-Phenethyl-alcohol and the total of higher alcohols by 20 h of fermentation (Fig. 4.6). On the other hand, by 20 h, M2 produced 50% of individual higher alcohol such as isobutanol (52.5 mg/L), 2-methyl butanol (53.5 mg/L) and isoamyl alcohol (51.8 mg/L). Furthermore, M2 generated 72% of 2-phenethyl-alcohol by 20 h. Therefore, M2 produced all individual higher alcohols during all process; NCYC2592 and W34/70 produced all compounds, which compose higher alcohol groups by 20 h. Therefore, NCYC2592 and W34/70 stopped producing higher alcohols when yeast exponential phase finished (Fig. 4.5) at 20 h.

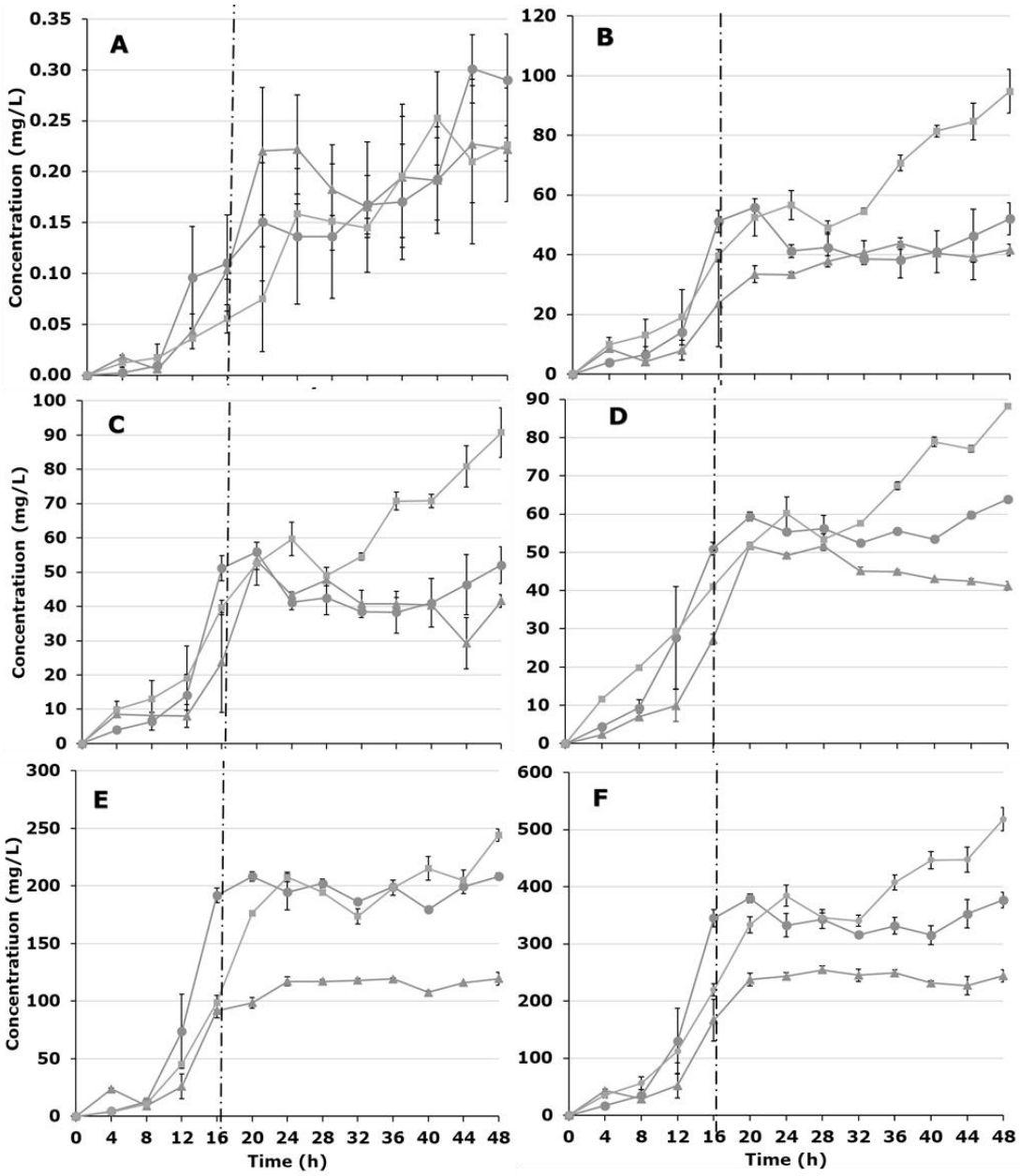

Fig. 4.7: The concentration of propanol (A), isobutanol (B), 2-methyl butanol (C), isoamyl alcohol (D), 2-Phenyl-alcohol (E) and the total of higher alcohols (F) during fermentation. All fermentations were conducted for 48 h using yeasts NCYC2592 (▲), W34/70 (●) and M2 (■). Samples were collected in triplicate by destructive sampling at 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44 and 48 hours. All data is present in average of concentration (mg/L) and standard deviation.

4.3.2.6. Short-and medium-chain fatty acid formation as a precursor of fatty acid esters

Fig. 4.8 illustrates the concentration of butanoic acid (A), isobutanoic acid (B), isovaleic acid (C), hexanoic acid (D), octanoic acid (E), decanoic acid (F) and the total of short-and medium-chain fatty acids (G) during fermentation. All strains used can produce short-chain fatty acids (butanoic acid, isobutanoic and isovaleic acid); and NCYC2592 and W34/70 can produce medium-chain fatty acids (hexanoic acid, octanoic acid and decanoic acid). Therefore, M2 strain accumulated very low concentration of hexanoic acid (0.02 mg/L), octanoic acid (0.02 mg/L) and decanoic acid (0.17 mg/L) (Fig. 4.7).

Fig. 4.7 G illustrates that M2 produced the highest concentration of total of fatty acids (7.9 mg/L) by 24 h, which are composed mostly for short-chain fatty acids. NCYC2592 generated a highest concentration of total fatty acids (20.6 mg/L) by 32 h. W34/70 accumulated a highest concentration of total fatty acids (12.7 mg/L) for 48 h. All fatty acids analysed were identified under the threshold levels (ASBC Methods of Analysis: Beer Flavour Database 2011).

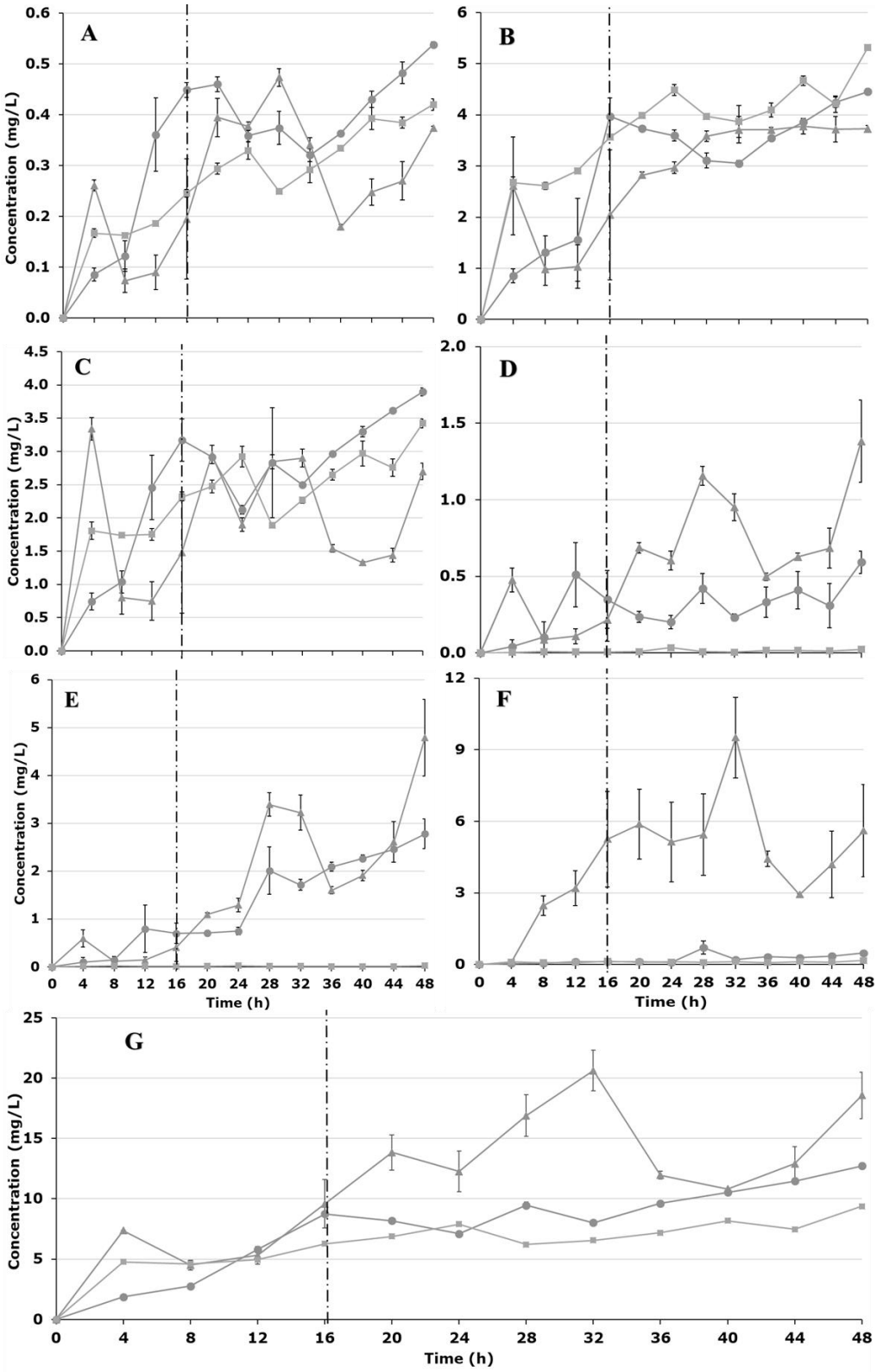

Fig. 4.8: The concentration of butanoic acid (A), isobutanoic acid (B), isovaleic acid (C), hexanoic acid (D), octanoic acid (E), decanoic acid (F) and the total of short-and medium-chain fatty acids (G) during fermentation. All fermentations were conducted for 48 h using yeasts NCYC2592 (▲), W34/70 (●) and M2 (■). Samples were collected in triplicate by destructive sampling at 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44 and 48 hours. All data is present in average of concentration (mg/L) and standard deviation.

4.3.2.7. Overall of yeast flavour formation

Fig. 4.8 suggests that acetyl-CoA is formed in both the glucose domain and/or ethanol domain; therefore, acetyl-CoA-cytosolic is generated from acetic acid/acetate and as a consequence is the main responsible for flavour-active compounds (ester groups). Competition between higher alcohols and fatty acid groups occurs during exponential phase. The instability of short-chain fatty acids during exponential phase is normal, which triggers to the formation of unsaturated fatty acids and as a consequence inhibits the formation of higher alcohols.

All flavour analysed where produced under growth conditions, which had finished at 20 h. By the first 20 h, higher alcohols are not accumulated because are quickly converted to acetate esters and also due to the competition with short-chain fatty acids via Ehrlich pathway (Fig. 4.8). Despite the competition between higher alcohol and fatty acid groups, fatty acid esters were formed and most of them were produced in the glucose phase. However, after consumption of more than two third of total wort sugars, acetic acid/acetate formation stopped and as a consequence, acetate concentration decreased in the ethanol phase to maintain the metabolic activities. Acetic acid is consumed to generate acetyl-CoA-citosolic and may occur due to the activation of both acetyl-CoA synthetases (Acsp1 and Acsp2) under ethanol presence (Pietrocola *et al.* 2015, Galdieri *et al.* 2014, Kocharin and Nielsen 2013). In conclusion, acetic acid formation/consumption is involved in the complex of flavour active formation (acetic acid/acetyl-CoA/esters complex) and during ethanol phase, which most of wort compounds are present in low concentrations, can be necessary addition of essential inorganic elements for supplementation of wort.

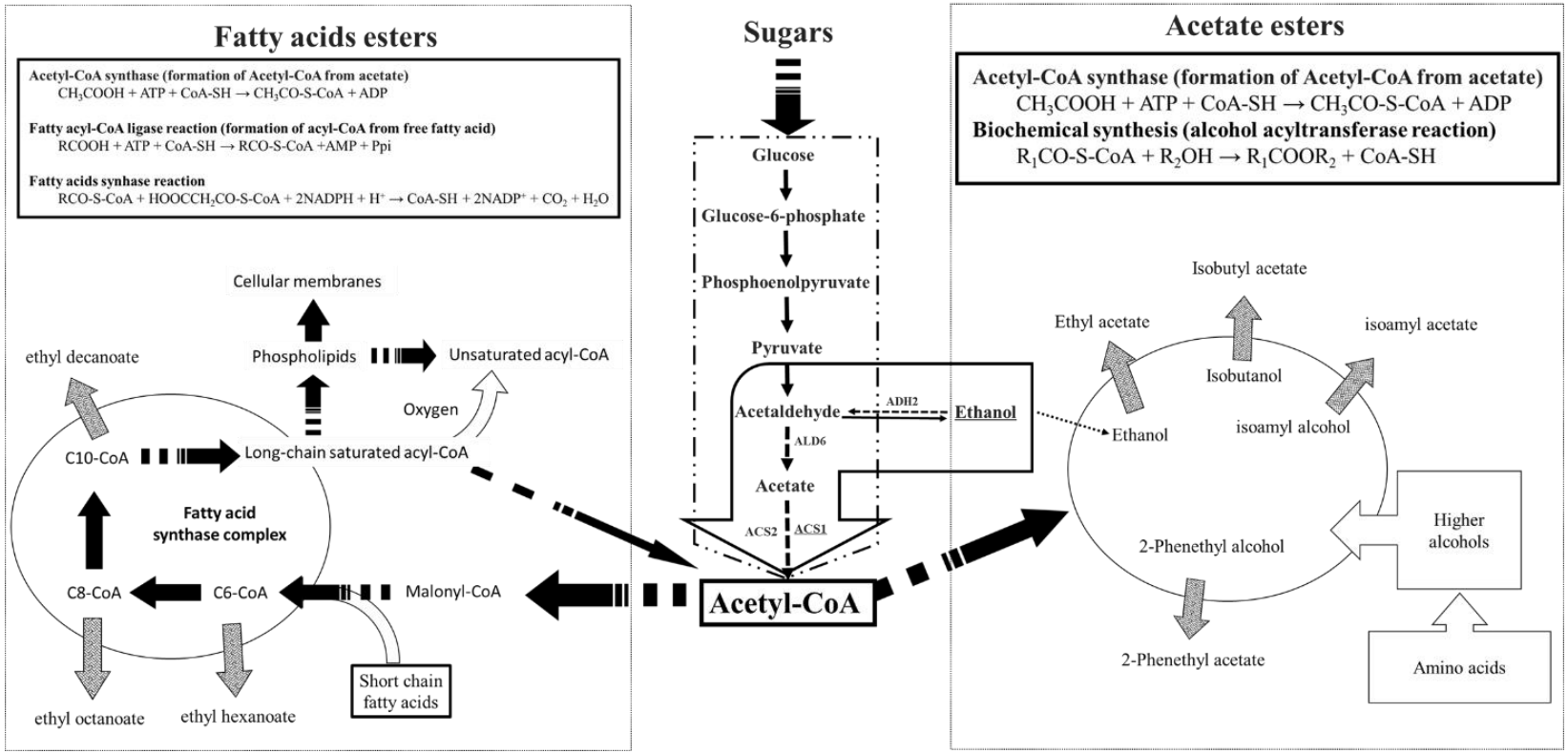

Fig.4.9: The formation of flavour during sugar domain and ethanol domain presented by a simple sugar consumption versus ethanol formation during fermentation

4.4. Discussion

Brewing yeasts cannot develop respiratory capability under fermentation conditions due to high concentration of carbohydrates (Pietrocola *et al.* 2015, Galdieri *et al.* 2014, Briggs *et al.* 2004). During fermentation, acetyl-CoA is mostly formed in the cytosol and is controlled by two acetyl-CoA synthetases, which are Acs1p or Acs2p (Kocharin and Nielsen 2013). These two acetyl-CoA synthetase differ due to their transcriptional regulation and kinetic properties (Van den Berg *et al.* 1996). Acetyl-CoA is a central and crucial intermediate metabolite (Pietrocola *et al.* 2015), and also responsible for regulates flavour generation including higher alcohols, short chain fatty acids, esters and fatty acids esters.

As explained above, ignoring non-fermentable such as maltotetraose/dextrins and a fermentable sugar such as maltotriose, which was not consumed under the studied conditions, the studied wort had 94g/L of consumed sugars. Fig. 4.2 illustrates the consumption of sugars and ethanol. The dotted line divides the fermentation into two phase: (1) glucose domain and (2) ethanol domain. Glucose domain was initiated when yeasts were inoculated (zero hour of fermentation). Ethanol phase was initiated when over two third (2/3) of the total sugars were consumed and as a result, the ethanol concentration (over 32 g/L) became higher than the total dissolved sugars (lower than 32g/L). The vertical dotted line represents the time that over two third (62 g) of the total sugars were consumed.

Kocharin and Nielsen (2013) had shown that acetate is a precursor of acetyl-CoA-cytosolic via ACS activation (including Acs1p and Acs2p). At high glucose concentration Acs2p is activated and Acs1p is repressed; on the other

hand, Acs1 is activated on ethanol presence (Kocharin and Nielsen 2013). These suggest that during alcoholic fermentation Acs1 can be activated during ethanol domain, mainly when observed that acetic acid accumulation stopped at 16 h and also started being consumed (Fig. 4.3), which advocates that acetic acid was converted acetyl-CoA and as a consequence of ester formation such as acetate esters and fatty acid esters using higher alcohols and fatty acids, respectively.

Acetate esters are formed by the reaction of acetyl-CoA with higher alcohols, which are related to amino acid consumption such as valine, leucine, isoleucine and phenylalanine are the precursor of isobutanol, isoamyl alcohol, 2-methylbutanol and 2-phenylethanol, respectively (Xu *et al.* 2017, Olaniran *et al.* 2017, Saerens, *et al.* 2010, Saerens *et al.* 2008, Yoshimoto *et al.* 1998, Verstrepen *et al.* 2003). Results show that 50% of acetate esters were generated acetate during the acetic acid accumulation (16h of fermentation), which is related to acetic acid accumulation period. Another 50% of acetate esters were formed after 16h, which is when the accumulation of acetic acid stopped and also started being consumed.

A second group of esters is composed by fatty acid ethyl esters (FAEEs), which are produced from yeast secondary metabolites. During alcoholic fermentation, volatile medium-chain fatty acid ethyl esters, which are produced from the reaction between medium-chain fatty acids and acetyl-co-A and generates desirable fruit aromas (Knight *et al.* 2014). The biochemical synthesis of medium-chain fatty acid esters is only partly understood and involve the enzyme acyl-CoA:ethanol O-acyltransferases (AEATases), encoded by EEB1 and EHT1 (Nancolas *et al.* 2017, Knight *et al.* 2014, Saerens

et al. 2008; Saerens *et al.* 2006; Dufour *et al.* 2003). NCYC2592 and M2 produced 50% of fatty acid esters during the acetic acid accumulation (16h of fermentation) and other 50% were formed after 16h, which the accumulation of acetic acid stopped, and as a consequence the acetate ester formation was supported by acetic acid consumption. In contrast, W34/70 strain produced the majority amount of fatty acid esters during acetic acid accumulation, except ethyl hexanoate.

All yeast showed an unstable formation of ethyl hexanoate, mainly, when acetic acid accumulation stopped. This occurred because ethyl hexanoate is percussed by hexanoic acid, which is the first medium-chain fatty acid generated in the fatty acid pool (Knight *et al.* 2014, Saerens *et al.* 2008). Furthermore, at 32 h was observed a reduction of ethyl hexanoate (Fig. 4.5B) and at 36 h ethyl octanoate increased (Fig. 4.5C). These trigger to the fact, hexanoic acid is a precursor of octanoic acid in fatty acid pool and these medium chain fatty acids are precursor of ethyl hexanoate and ethyl octanoate, respectively.

Higher alcohols and short-chain fatty acids are formed via the Ehrlich pathway (Dack *et al.* 2017, Yu *et al.* 2016, Hazelwood *et al.* 2008, Boulton and Quain, 2001). In addition, short-chain fatty acids are the precursor of medium and long chain fatty acids, which are driven via fatty acid pool (Zhuang *et al.* 2015, Saerens *et al.* 2010). Higher alcohol and fatty acid formation are based on yeast growth and consequently, all esters have yeast growth as a master key.

Only NCYC2592 accumulated a significant concentration of fatty acids after its exponential phase had finished. NCYC2592 used the fatty acid complex

synthesis because produced higher concentration of all medium chain fatty acid (Fig. 4.7F), which are present this fatty pool and were influenced by short chain fatty acids such as butanoic acid (Fig. 4.7A), isobutanoic acid (4.7B) isovaleric acid (4.7C) that were produced via Ehrlich pathway. Therefore, these trigger to medium chain fatty acid generation such as hexanoic acid (D), octanoic acid (E), decanoic acid via fatty acid complex synthesis. In contrast, W34/70 and M2 strains produced a similar concentration of fatty acids; however, lower concentration comparing to NCYC2592 strain. These occurred because W34/70 and M2 produced a low concentration of medium chain fatty acid, which includes hexanoic acid, octanoic acid and decanoic acid. Although W34/70 produced a similar concentration of hexanoic acid and octanoic acid comparing to NCYC2592, this strain lower concentration of decanoic acid, which is similar compared to M2. This suggests that W34/70 and M2 did not fully use the fatty acid complex synthesis and M2 used this complex driven small concentration of acetyl-CoA and short-chain fatty acids.

Moreover, NCYC2592 strain drove SCFA into the fatty acid synthesis complex and consequently formed medium chain fatty acids (MCFA) (Fig. 4.8). Similarly, W34/70 and M2 drove SCFA into the fatty acid synthesis complex (Fig. 4.8); however, considering the low concentration of decanoic acid formed (Fig. 4.7 F), suggests that these last two strains did not use the fatty acid synthesis complex fully (Fig. 4.8).

Finally, as expected, all SCFA and MCFA (isobutyric acid, butyric acid, hexanoic acid, octanoic acid and decanoic acid) were identified with values lower than the threshold levels (Xu *et al.* 2017, ASBC Methods of Analysis: Beer Flavour Database 2011). The fatty acid esters investigated during this

work were ethyl butanoate, ethyl hexanoate, ethyl octanoate and ethyl decanoate and they had butyric acid, hexanoic acid, octanoic acid and decanoic acid, respectively, as a fatty acid precursor.

4.5. Conclusions

All strains applied consumed low quantity of maltotriose by 48 h at 22°C. During the consumption of two third of fermented carbohydrates, acetic acid and ethanol are accumulated in parallel. At 16 h, ethanol concentration became higher than fermented carbohydrate concentration, acetic acid accumulation stopped and acetic acid concentrations started to reduce. Also, acetate esters and fatty acid esters are influenced by carbohydrates domain and ethanol domain. At 22°C, all strains grow similarly and the exponential phase finished at 20 h. NCYC2592 and W34/70 produced higher alcohols by 20 h (The end of exponential phase) and M2 produced 50% of higher alcohols at 20 h. All strains produced fatty acids (off-flavours) and were quantified with values under threshold levels.

CHAPTER V

INFLUENCE OF ESSENTIAL INORGANIC ELEMENTS IN FLAVOUR FORMATION DURING YEAST FERMENTATION

5. CHAPTER V: INFLUENCE OF ESSENTIAL INORGANIC ELEMENTS ON FLAVOUR FORMATION DURING YEAST FERMENTATION

5.1 Introduction

Essential inorganic elements are responsible for the activation of several enzymes and as a consequence are involved in yeast growth and carbohydrates consumption (Canadell *et al.* 2015, Barreto *et al.* 2012, Page and Di Cera, 2006, Walker 2004, Boubekeur *et al.* 2001). During fermentation, wort becomes a fermented wort that is a poor environment in term of carbohydrates and free-essential inorganic elements, which includes inorganic-phosphate, potassium, magnesium, iron, zinc, copper and manganese. However, fermented wort is a rich environment of alcohols, fatty acids and acetyl-CoA, which are ester precursors. These suggest that a supplementation of essential inorganic elements can increase the yeast active-flavour formation because they act as enzymatic co-factors performing signalling transport and structural roles (Cyert and Philpott 2013).

The main glycolytic pathway and pyruvate catabolism, which are both responsible for the main formation of ATP and acetyl-CoA, respectively, as a consequence, yeast-active flavour. Furthermore, it presents the dependence of essential inorganic for all metabolic reactions, which support ethanol, glycerol, acetate/acetic acid, acetyl-CoA and as a consequence, all yeast flavour formation. Several studies have been focused on flavour formation during fermentation, which includes flavour production (Fairbairn 2012); metal ions

influence on flavour stability (Zufall and Tyrell 2008); biosynthesis of higher alcohol (Vidal *et al.* 2014); volatile ester synthesis (Bilverstonem *et al.* 2015, Zhuang *et al.* 2015) and metabolic production of short chain fatty acids (Yu *et al.* 2016). Although all studies conducted, which generated knowledge about the importance of essential inorganic elements in yeast growth, metabolism and structure role, the role of essential inorganic elements on yeast-flavour production has not been published. Furthermore, gas chromatography-mass spectrometry (GC-MS; aroma analysis) with liquid injection and high performance liquid chromatography (HPLC; ethanol/glycerol analysis) were used to monitor the changes in volatile compounds produced during yeast fermentation and explained using Principal component analysis (PCA).

This chapter generates an more complete understanding of the role of the essential inorganic element on yeast-flavour formation during supplemented fermentations. Anticipating a more complete fundamental understanding of the role of essential inorganic elements on yeast flavour production and allowing brewers to have a more accurate control of yeast-flavour production.

5.2. Material/Method/Experimental design

Two *Saccharomyces cerevisiae* (NCYC2592 and M2) and one *Saccharomyces pastorianus* (W34/70) brewing yeast strains were used in this study (Section 2.1). Fermentations were conducted as described in section 2.1.3 using a standard sweet wort (13°P) (Chapter 2, table 2.1) and mineral composition (chapter 2, table 2.2). Initially, yeasts were propagated in 2%YPD media (Section 2.1.1.1.) at 25 °C for 48 h and were pitched $1.5x10^7$cell/mL into a

miniature fermentation vessel (180 mL). The fermentation vessel (FV) contained 100 ml aliquots of a sweet wort standard and magnetic stirrers for agitation at 200 rpm. All fermentations were conducted in triplicate for 72 h. The amount of each inorganic element supplemented (Chapter 2, section 2.3.2.) were added into 100 mL fermentation vessels using 100µl (pre-prepared solution, Chapter 2, section 2.1.3.) that did not disturb the fermentation system. Upon completing fermentation, they were measured in triplicate by destructive sampling for the high performance liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC-MS) analyses (Sections 2.5.1. and 2.5.2.).

5.3. Results

5.3.1. Essential inorganic elements influence yeast viability

Yeast viability is an important control analysis for brewers. Fig. 5.1 illustrates yeast viability as a demonstrative of fermentation quality/performance to support the overall understanding of influence of essential inorganic elements. NCYC2592 strain had 88.6% of viability when inoculated in sweet brewers wort (SBW); however, its viability decreased when inoculated in fermentation supplemented with ammonia-nitrogen (66.8%), potassium (66.1%), iron (70.5%), or manganese (63.2%). W34/70 had 84.7% of viability when inoculated in sweet brewers wort (SBW); however, its viability decreased when inoculated in fermentation supplemented with copper (59.4%) or CMN (61.9%). M2 strain had 73.1% of viability when inoculated in sweet brewers wort (SBW); however, its viability decreased when inoculated in fermentation supplemented with ammonia-nitrogen (66.5%) and copper (52.0%). In contrast,

only M2 had its viability positively affected during supplementation when inoculated in fermentation supplemented with magnesium (77.2%). All other treatments shows not significant change in the viability.

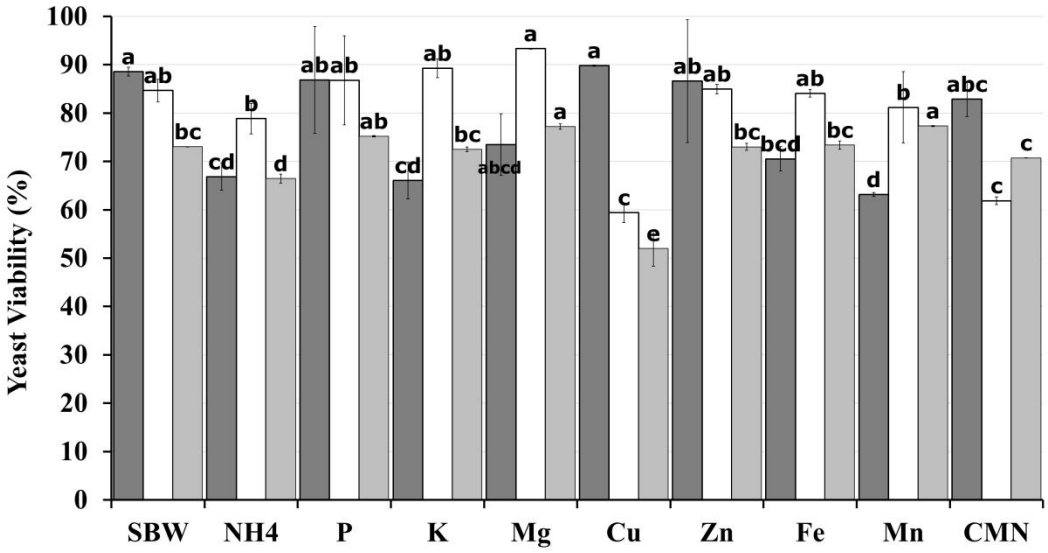

Fig. 5.1: Final yeast viability (%) during fermentations using yeast *Saccharomyces* NCYC2592 (■), W34/70 (□), M2 (▨), respectively. Comparison of 90 fermentation using three strains with 10 treatments, which are represented: SBW (standard brewers wort), NH^{4+} (ammonia-nitrogen), P (inorganic-Phosphate), K (potassium), Mg (magnesium), Cu (copper), Zn (zinc), Fe (iron), Mn (manganese) and CMN (a composite mixture of all nutrients).

5.3.2. Essential inorganic elements influence on ethanol and glycerol formation

Fig. 5.2 summarizes the data from table 5.1. Fig. 5.1 A and 5.1 B illustrate essential inorganic elements influence ethanol/glycerol formation. All treatment were compared to the control (standard brewers wort - SBW). When fermentations using M2 or W34/70 strains were supplemented with ammonia-nitrogen, inorganic phosphate, magnesium, zinc or iron, ethanol and glycerol production were not affected; however, when fermentation were supplemented with manganese or CMN (a composite mixture of all nutrients), W34/70

significantly decreased ethanol formation (Fig. 5.1 A, Table 5.1) from 76.3 g/L (control) to 65.5 g/L and 36.2 g/L, respectively. In contrast, when fermentation were supplemented with potassium using NCYC2592 strain ethanol formation increased (Fig. 3A/3B, Table 5.1) from 37.6 g/L to 66.1 g/L. M2 strain was not significant affected by supplementation used. Furthermore, glycerol formation was influenced by the same inorganic elements, which influenced ethanol formation. Therefore, glycerol production is response to ethanol toxicity (protective response) and redox balance (Zhang *et al.* 2013, Ansell *et al.* 1997, van Dijken and Scheffers 1986).

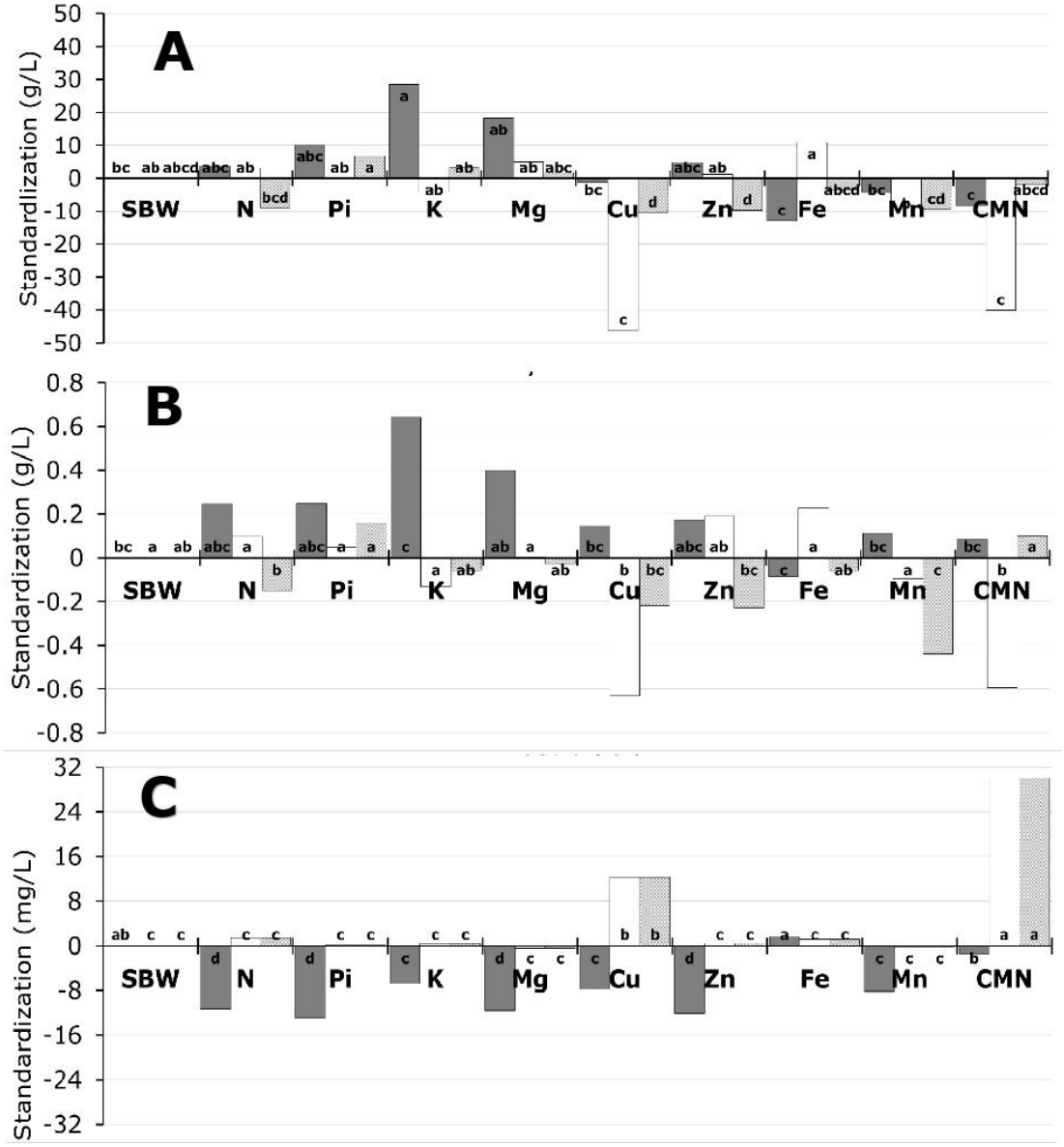

Figure 5.2.: Standardization for keys drives changes for compounds produced during the fermentations using yeast *Saccharomyces* NCYC2592 (■), W34/70 (□), M2 (▨), respectively. Comparison of 25 flavour compounds from 90 fermentation using three strains with 10 treatments, which are represented: SBW (standard brewers wort), NH^{4+} (ammonia-nitrogen), P (inorganic-Phosphate), K (potassium), Mg (magnesium), Cu (copper), Zn (zinc), Fe (iron), Mn (manganese) and CMN (a composite mixture of all nutrients). This overall picture are divided: A) Ethanol, B) glycerol and C) acetic acid.

5.3.3. Essential inorganic elements influence on acetic acid formation

Acetic acid is a precursor of acetate (anion) and acetyl-CoA cytosolic (Chen *et al.* 2013, Takahashi *et al.* 2006). Fig. 5.2 C illustrates the influence of essential inorganic elements on acetic acid formation. NCYC2592 reduced acetic acid

formation (control, 19.8 mg/L) when fermentations were supplemented with ammonia-nitrogen (8.5 mg/L), inorganic-phosphate (6.9 mg/L), potassium (13.1 mg/L), magnesium (8.3 mg/L), copper (12.2 mg/L)), zinc (7.7 mg/L) or manganese (11.7 mg/L) (Fig. 5.2 C/Table 5.1). In the Fig. 5.2 C, W34/70 and M2 strains increased acetic acid formation when fermentations were supplemented with copper or CNM (composite mixture of all nutrients). When fermentations were supplemented with copper W34/70 and M2 increased acetic acetic formation from 2.5 mg/L and 1.4 mg/L (control) to 13.8 mg/L and 13.7 mg/L, respectively. Also, When fermentations were supplemented with CNM, W34/70 and M2 increased acetic acid formation from control (2.5 mg/L and 1.4 mg/L) to 24.3 mg/L and 31.5 mg/L, respectively. Therefore, NCYC2592 responded positively to the most of supplementations for decreasing acetic acid accumulation. From the control treatment, W34/70 and M2 produced low concentration of acetic acid and were induced only by copper and CNM treatments to produce more acetic acid.

Table 5.1: Ethanol, glycerol and acetic acid produced by yeast during 10 different fermentations (including standard brewers wort, ammonia-nitrogen, inorganic phosphate, potassium, magnesium, copper, zinc, iron, manganese, and a composite mixture of all nutrients) using yeast *Saccharomyces* - NCYC2592, W34/70 and M2. All data is present: average of concentration, Tukey test* and standard deviation.

Treatments / Compounds	**NCYC2592**									
	standard brewers wort	NH^4	Pi	K	Mg	Cu	Zn	Fe	Mn	composite mixture of all nutrients
Ethanol (g/L)	37.60 ^{bc}±2.50	41.30 ^{abc}±13.60	47.70 ^{abc}±11.50	66.10 ^{a}± 6.60	55.90 ^{ab}±1.80	36.30 ^{bc}±9.70	42.30 ^{abc}±12.60	24.70 ^{c}±2.70	33.50 ^{bc}±4.70	29.40 ^{c}±6.60
Glycerol (g/L)	0.54 ^{bc}±0.03	0.80 ^{abc}±0.30	0.80 ^{abc}±0.20	1.20 ^{a}±0.10	0.94 ^{ab}±0.04	0.70 ^{bc}±0.20	0.72 ^{abc}±0.20	0.46 ^{c}±0.03	0.66 ^{bc}±0.08	0.63 ^{bc}±0.14
Acetic ac. (mg/L)	19.80 ^{ab}±0.90	8.50 ^{d}±0.20	6.92 ^{d}±0.12	13.10 ^{c}±0.50	8.25 ^{d}±0.24	12.20 ^{c}±0.08	7.73 ^{d}±0.26	21.56 ^{a}±1.08	11.70 ^{c}±1.70	18.40 ^{b}±0.26
Treatments / Compounds	**W34/70**									
	standard brewers wort	NH^4	Pi	K	Mg	Cu	Zn	Fe	Mn	composite mixture of all nutrients
Ethanol (g/L)	76.30 ^{ab}±5.20	79.30 ^{ab}±8.30	80.40 ^{ab}±2.20	72.20 ^{ab}±6.90	81.20 ^{ab}±4.90	30.70 ^{c}±1.60	77.50 ^{ab}±1.90	87.20 ^{a}±5.30	65.50 ^{b}±13.80	36.20 ^{c}±5.80
Glycerol (g/L)	1.20 ^{a}±0.08	1.33 ^{a}±0.14	1.28 ^{a}±0.05	1.10 ^{a}±0.10	1.20 ^{a}±0.10	0.61 ^{b}±0.04	1.42 ^{a}±0.16	1.46 ^{a}±0.13	1.15 ^{a}±0.25	0.64 ^{b}±0.08
Acetic ac. (mg/L)	2.50 ^{c}±0.05	3.30 ^{c}±0.10	2.27 ^{c}±0.13	2.38 ^{c}±0.26	3.43 ^{c}±2.90	13.80 ^{b}±0.50	1.77 ^{c}±0.06	2.62 ^{c}±0.12	0.80 ^{c}±0.01	24.30 ^{a}±0.06
Treatments / Compounds	**M2**									
	standard brewers wort	NH^4	Pi	K	Mg	Cu	Zn	Fe	Mn	composite mixture of all nutrients
Ethanol (g/L)	66.40 ^{abcd}±5.60	57.40 ^{bcd}±0.90	73.20 ^{a}±1.30	69.90 ^{ab}±2.90	68.10 ^{abc}±2.60	56.00 ^{d}±3.1	56.70 ^{d}±2.10	62.90 ^{abcd}±2.40	56.90 ^{cd}±7.30	64.60 ^{abcd}±1.90
Glycerol (g/L)	1.25 ^{ab}±0.14	1.10 ^{b}±0.04	1.40 ^{a}±0.06	1.22 ^{ab}±0.04	1.23 ^{ab}±0.08	1.04 ^{bc}±0.03	1.03 ^{bc}±0.03	1.20 ^{ab}±0.05	0.82 ^{c}±0.12	1.35 ^{a}±0.09
Acetic ac. (mg/L)	1.40 ^{c}±0.08	2.84 ^{c}±0.33	1.58 ^{c}±0.01	1.75 ^{c}±0.05	1.00 ^{c}±0.11	13.70 ^{b}±1.00	1.85 ^{c}±0.03	2.56 ^{c}±0.11	1.22 ^{c}±0.08	31.50 ^{a}±1.60

*Values not sharing the same letter are significantly different according to the Tukey test.

5.3.4. Essential inorganic elements influence on acetate ester formation

Acetate ester formation is dependent of alcohols, acetyl-CoA and enzymes responsible for the syntheses (Dack *et al.* 2017, Saerens *et al.* 2010, Saerens *et al.* 2008). Fig. 5.3 summarises the data from table table 5.2, which illustrates the influence of essential inorganic elements on acetate ester formation. NCYC2592 produced a low concentration of acetate esters (1.35 mg/L, control); however, in all supplementation treatments were observed that NCYC2592 increased the acetate ester formation (Fig. 5.3/Table 5.2). Furthermore, When inoculated in wort supplemented with ammonia nitrogen (12.8 mg/L) and inorganic phosphate (14.1 mg/L), NCYC2592 produced higher concentration of total acetate esters.

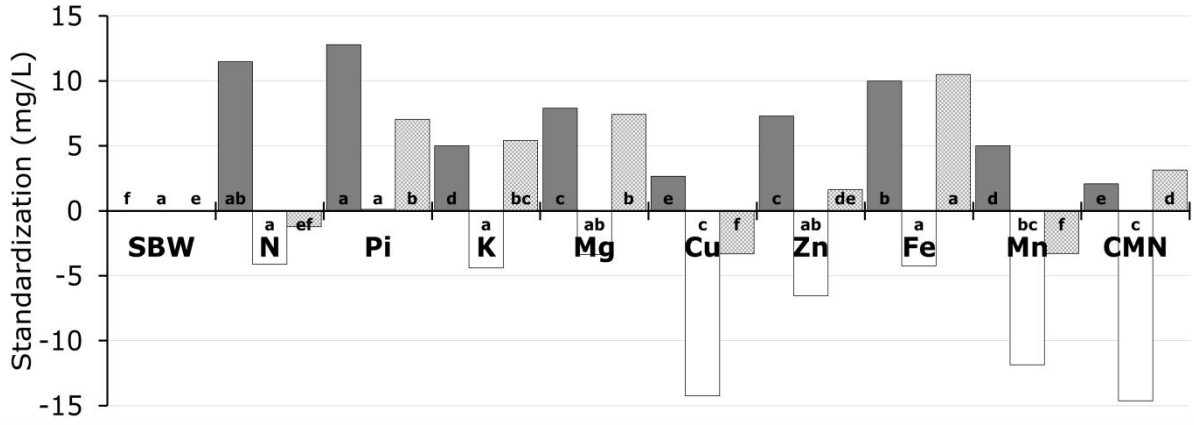

Figure 5.3: Standardization of acetate esters produced during the fermentations using yeast *Saccharomyces* NCYC2592 (■), W34/70 (□), M2 (▨), respectively. All analysed from 90 fermentation using three strains with 10 treatments, which are represented: SBW (standard brewers wort), NH^{4+} (ammonia-nitrogen), P (inorganic-Phosphate), K (potassium), Mg (magnesium), Cu (copper), Zn (zinc), Fe (iron), Mn (manganese) and CMN (a composite mixture of all nutrients).

Table 5.2: Acetate esters produced by yeast during 10 different fermentation treatments (including standard brewers wort, ammonia-nitrogen, inorganic phosphate, potassium, magnesium, copper, zinc, iron, manganese, and a composite mixture of all nutrients) using yeast *Saccharomyces s* - NCYC2592, W34/70 and M2. All data is present: average of concentration (mg/L), Tukey test* and standard deviation.

Treatments / Compounds	**NCYC2592**									
	standard brewers wort	NH^4	Pi	K	Mg	Cu	Zn	Fe	Mn	composite mixture of all nutrients
Isoamyl acetate	0.46 ^{g}±0.03	7.96 ^{ab}±0.24	8.42 ^{a}±0.98	3.15 ^{ef}±0.30	4.75 ^{cd}±0.17	2.00 ^{f}±0.20	5.22 ^{c}±0.06	6.88 ^{b}±0.30	3.63 ^{de}±0.30	2.11 ^{f}±0.30
Isobutyl acetate	0.60 ^{cd}±0.10	1.70 ^{ab}±0.04	1.58 ^{ab}±0.14	1.57 ^{ab}±0.22	2.20 ^{a}±0.65	1.00 ^{bcd}±0.06	1.02 ^{bcd}±0.05	1.28 ^{bc}±0.13	1.17 ^{bc}±0.15	0.37 ^{d}±0.11
2-Phenethyl acetate	0.28 ^{g}±0.04	3.15 ^{b}±0.24	4.03 ^{a}±0.54	1.62 ^{de}±0.08	2.30 ^{cd}±0.09	1.00 ^{ef}±0.10	2.40 ^{c}±0.11	3.18 ^{b}±0.16	1.53 ^{ef}±0.20	0.93 ^{fg}±0.00
Σ acetate esters	**1.35 ^{f}±0.07**	**12.80 ^{ab}±0.30**	**14.13 ^{a}±1.34**	**6.54 ^{d}±0.55**	**9.25 ^{c}±0.80**	**4.00 ^{e}±0.20**	**8.64 ^{c}±0.05**	**11.34 ^{b}±0.28**	**6.34 ^{d}±0.47**	**3.40 ^{e}±0.30**

Treatments / Compounds	**W34/70**									
	standard brewers wort	NH^4	Pi	K	Mg	Cu	Zn	Fe	Mn	composite mixture of all nutrients
Isoamyl acetate	9.80 ^{a}±1.05	7.60 ^{ab}±0.60	9.90 ^{a}±0.80	5.90 ^{abc}±4.40	6.50 ^{abc}±4.70	0.53 ^{bc}±0.01	5.45 ^{abc}±0.30	6.80 ^{abc}±0.40	2.00 ^{bc}±0.03	0.20 ^{c}±0.04
Isobutyl acetate	0.80 ^{a}±0.02	0.45 ^{c}±0.02	0.76 ^{ab}±0.03	0.65 ^{b}±0.02	0.65 ^{b}±0.04	0.42 ^{c}±0.07	0.45 ^{c}±0.01	0.48 ^{c}±0.01	0.23 ^{d}±0.03	0.41 ^{c}±0.07
2-Phenethyl acetate	4.70 ^{a}±0.10	3.22 ^{cd}±0.01	4.85 ^{a}±0.07	4.40 ^{ab}±0.50	4.80 ^{a}±0.50	0.13 ^{f}±0.00	2.90 ^{d}±0.04	3.80 ^{bc}±0.03	1.24 ^{e}±0.02	0.10 ^{f}±0.00
Σ acetate esters	**15.30 ^{a}±1.20**	**11.25 ^{a}±0.60**	**15.45 ^{a}±0.73**	**10.95 ^{a}±4.90**	**10.35 ^{ab}±5.25**	**1.08 ^{c}±0.08**	**8.80 ^{ab}±0.40**	**11.10 ^{a}±0.40**	**3.47 ^{bc}±0.06**	**0.70 ^{c}±0.10**

Treatments / Compounds	**M2**									
	standard brewers wort	NH^4	Pi	K	Mg	Cu	Zn	Fe	Mn	composite mixture of all nutrients
Isoamyl acetate	7.52 ^{de}±0.26	7.15 ^{de}±0.55	12.40 ^{ab}±0.50	11.25 ^{bc}±0.20	12.30 ^{b}±1.10	6.00 ^{e}±0.30	9.10 ^{cd}±0.60	14.70 ^{a}±1.20	5.80 ^{e}±0.80	10.60 ^{bc}±1.00
Isobutyl acetate	1.36 ^{cde}±0.09	1.20 ^{def}±0.20	1.90 ^{ab}±0.10	1.87 ^{abc}±0.12	1.80 ^{abc}±0.05	0.80 ^{f}±0.25	1.40 ^{bcd}±0.20	1.90 ^{a}±0.20	0.93 ^{ef}±0.04	1.23 ^{def}±0.11
2-Phenethyl acetate	3.65 ^{de}±0.21	2.90 ^{ef}±0.50	5.30 ^{bc}±0.09	4.76 ^{c}±0.04	5.85 ^{ab}±0.45	2.40 ^{f}±0.30	3.60 ^{de}±0.20	6.42 ^{a}±0.15	2.50 ^{f}±0.10	3.82 ^{d}±0.02
Σ acetate esters	**12.53 ^{e}±0.54**	**11.30 ^{ef}±1.20**	**19.60 ^{b}±0.60**	**17.90 ^{bc}±0.30**	**19.90 ^{b}±1.50**	**9.20 ^{f}±0.80**	**13.80 ^{de}±0.60**	**23.00 ^{a}±1.30**	**9.20 ^{f}±0.80**	**15.70 ^{cd}±1.10**

*Values not sharing the same letter are significantly different according to the Tukey test.

When inoculated in SBW (control), W34/70 produced 15.3 mg of total acetate esters/L. W34/70 decreased acetate ester formation when fermentations were supplemented with copper, manganese or CNM (composite mixture of all nutrients), which respectively, produced 1.08 mg/L, 3.47 mg/L and 0.7 mg/L. Similarly, M2 produced 12.5 mg acetate esters/L (control) and decreased acetate ester formation when fermentations were supplemented with copper (13.8 mg/L) or manganese (9.2 mg/L) (Fig 5.3/Table 5.2). When fermentations were supplemented with inorganic-phosphate, potassium, magnesium, iron or CNM, NCYC2592 and M2 behaved similarly increasing the acetate ester formation. Iron was the sole heavy metal, which increased acetate ester formation in all yeast strains applied although W34/70 did not present a significant effect under iron supplementation (Fig. 5.3/Table 5.2).

Specifying acetate ester compounds, NCYC2592 and M2 increased the formation of all acetate esters, which includes isoamyl acetate, isobutyl acetate and 2-phenethyl acetate, mainly, when fermentations were supplemented with inorganic phosphate, potassium and magnesium. NCYC2592 inoculated in SBW (control) produced 0.46, 0.60 and 0.28 mg/L, respectively, isoamyl acetate, isobutyl acetate and 2-phenethyl acetate. However, when this strain was inoculated in wort supplemented with inorganic phosphate the highest concentration of isoamyl acetate (8.4 mg/L), 2-phenethyl acetate (4.0 mg/L) and isobutyl acetate (1.58 mg/L) were generated. Although isbutyl acetate was also produced in high concentration under magnesium supplementation (2.2 mg/L). Similar behaviour was observed when M2 were inoculated in a wort supplemented with inorganic phosphate, which generated the quantity of

isoamyl acetate, isobutyl acetate and 2-phethyl acetate, respectively, 7.2 mg/L, 1.9 mg/L and 5.3 mg/L.

In contrast, W34/70 produced the highest concentration of all acetate esters when was inoculated in standard brewers wort (control), which were isoamyl acetate (9.8 mg/L), isobutyl acetate (0.8 mg/L) and 2-phenetyl acetate (4.7 mg/L). All strains produced low concentration of isobutyl acetate, but only isobutyl acetate produced from W34/70 was negatively effected by all wort supplementation (Table 5.3), except inorganic phosphate supplementation (0.76 mg/L). Therefore, wort supplementaion with inorganic phosphate, potassium and magnesium cause positive acetate ester formation using NCYC2592 and M2. Confronting the result of acetic acid (Table 5.1) and acetate ester (Table 5.2), when yeast accumulate high concentration of acetic acid occurs a reduction of acetate esters and vice-versa.

5.3.5. Essential inorganic elements influence on higher alcohol formation

Higher alcohol production is a consequence of amino acid consumption via Ehrlich pathway (Dack *et al.* 2017, Hazelwood *et al.*, 2008, Hazelwood *et al.* 2006). Higher alcohols are immediately secreted into the wort, or esterified with acetyl-CoA to synthesize their respective acetate esters because cannot be used as a carbon source for central metabolism (Vidal *et al.* 2014, Ashraf 2011, Hazelwood *et al.* 2008). Fig. 5.4 summarizes the data from Table 5.3, which illustrates the influence of essential inorganic elements on higher alcohol formation. NCYC2592 produced 210.1 mg of total higher alcohols/L when inoculated SBW (control) and was observed that wort supplemented with

inorganic phosphate or magnesium increased the concentration of higher alcohols to 318.5 mg/L and 319.8 mg/L, respectively. Although other supplemetnation treatments such as ammonia-nitrogen, potassium, iron, manganese or CNM (composite mixture of all nutrients) also increased higher alcohols formation using NCYC2592.

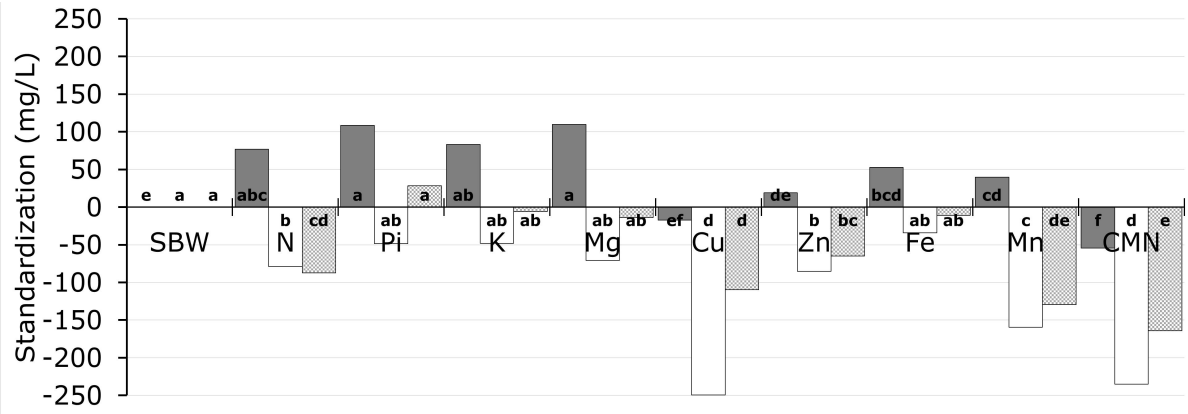

Figure 5.4: Standardization of higher alcohols produced during the fermentations using yeast *Saccharomyces* NCYC2592 (■), W34/70 (□), M2 (▨), respectively. All analysed from 90 fermentation using three strains with 10 treatments, which are represented: SBW (standard brewers wort), NH^{4+} (ammonia-nitrogen), P (inorganic-Phosphate), K (potassium), Mg (magnesium), Cu (copper), Zn (zinc), Fe (iron), Mn (manganese) and CMN (a composite mixture of all nutrients).

W34/70 and M2 strains produced highest concentration of higher alcohols when were inoculated SBW (control), respectively, 348.2 mg/L and 453.7 mg/L. In contrast, when inoculated in wort treatments with ammonia-nitrogen, copper, zinc, manganese or CNM (Fig. 5.4/Table 5.3), W34/70 and M2 decreased higher alcohol formation (Table 5.3). Compounds such as 2-methyl alcohol, isoalmyl alcohol and 2-phenethyl alcohol were the most affected by wort supplementations. These compounds increased under ammonia nitrogen, inorganic phosphate, potassium or magnesium supplementations when fermentation used NCYC2592, on the other hand, W34/70 and M2 did not present any significant effect under similar conditions. Furthermore, W34/70

and M2 reduced the production of higher alcohols such as 2-methyl alcohol, isoalmyl alcohol or 2-phenethyl alcohol when wort was supplemented with heavy metal supplementations (copper, manganese or CMN) (Table 5.3).

The lowest concentration of isoamyl alcohol and 2-phenethyl alcohol were observed when wort was supplemented with CMN, which had 35.9 mg/L, 27.1 mg/L and 57.6 mg/L, respectively, for NCYC2592, W34/70 and M2 (Table 5.3). Similarly, The lowest concentration of 2-phenethyl alcohol were observed when wort was supplemented with CMN, which had 98.2 mg/L, 72.6 mg/L and 152.4 mg/L for NCYC2592, W34/70 and M2, respectively (Table 5.3).

Table 5.3: Higher alcohols produced by yeast during 10 different fermentation treatments (including standard brewers wort, ammonia-nitrogen, inorganic phosphate, potassium, magnesium, copper, zinc, iron, manganese, and a composite mixture of all nutrients) using yeast *Saccharomyces s* - NCYC2592, W34/70 and M2. All data is present: average of concentration, Tukey test* and standard deviation.

Treatments / Compounds (m/L)	**NCYC2592**									
	standard brewers wort	NH^4	Pi	K	Mg	Cu	Zn	Fe	Mn	composite mixture of all nutrients
Propanol	0.53 ^{ab}±0.05	1.26 ^{a}±0.05	1.25 ^{a}±0.14	1.30 ^{a}±0.35	1.20 ^{a}±0.60	0.36 ^{b}±0.14	0.57 ^{ab}±0.16	0.60 ^{ab}±0.30	0.35 ^{b}±0.15	0.46 ^{ab}±0.02
Isobutanol	0.02 ^{a}±0.01	0.03 ^{a}±0.01	0.01 ^{a}±0.00	0.02 ^{a}±0.00	0.01 ^{a}±0.00	0.02 ^{a}±0.00	0.02 ^{a}±0.01	0.01 ^{a}±0.00	0.02 ^{a}±0.01	0.01 ^{a}±0.00
2-methyl butanol	23.9 ^{bc}±0.4	38.5 ^{a}±0.5	48.1 ^{a}±1.5	37.5 ^{ab}±2.6	47.9 ^{a}±0.5	22.6 ^{c}±1.0	38.1 ^{a}±0.6	39.7 ^{a}±2.5	37.9 ^{a}±13.1	21.3 ^{c}±0.8
Isoamyl alcohol	35.4 ^{e}±0.56	68.0 ^{ab}±1.50	67.3 ^{ab}±0.4	62.2 ^{bc}±1.9	73.1 ^{a}±3.1	39.9 ^{e}±0.8	51.9 ^{d}±1.7	51.9 ^{d}±0.5	59.1 ^{c}±4.5	35.90 ^{e}±0.3
2-Phenethyl alcohol	150.3 ^{bcd}±7.6	179.3 ^{ab}±8.3	203.1 ^{a}±8.5	192.4 ^{a}±5.8	197.6 ^{a}±21.1	130.2 ^{de}±3.6	138.4 ^{cd}±3.6	170.6 ^{abc}±9.6	152.7 ^{bcd}±15.8	98.2 ^{e}±0.2
Σ higher alcohols	**210.1 ^{e}±6.9**	**287.1 ^{abc}±8.5**	**318.5 ^{a}±7.9**	**294.4 ^{ab}±7.6**	**319.8 ^{a}±23.2**	**193.0 ^{ef}±2.0**	**229.0 ^{de}±5.8**	**262.8 ^{bcd}±7.8**	**250.0 ^{cd}±24.3**	**155.8 ^{f}±1.5**

Treatments / Compounds (m/L)	**W34/70**									
	standard brewers wort	NH^4	Pi	K	Mg	Cu	Zn	Fe	Mn	composite mixture of all nutrients
Propanol	0.30 ^{a}±0.10	0.25 ^{a}±0.02	0.16 ^{a}±0.05	0.21 ^{a}±0.03	0.47 ^{a}±0.34	0.22 ^{a}±0.03	0.25 ^{a}±0.09	0.33 ^{a}±0.09	0.11 ^{a}±0.03	0.16 ^{a}±0.04
Isobutanol	0.03 ^{a}±0.01	0.01 ^{a}±0.00	0.02 ^{a}±0.00	0.01 ^{a}±0.00	0.03 ^{a}±0.01	0.03 ^{a}±0.02	0.01 ^{a}±0.00	0.01 ^{a}±0.00	0.00 ^{a}±0.00	0.02 ^{a}±0.01
2-methyl butanol	64.7 ^{a}±1.3	38.4 ^{abc}±0.4	54.8 ^{ab}±0.7	35.0 ^{abc}±30.0	41.0 ^{abc}±1.2	12.4 ^{c}±0.4	47.4 ^{ab}±1.2	51.3 ^{ab}±1.6	27.9 ^{bc}±0.45	13.1 ^{c}±0.1
Isoamyl alcohol	65.5 ^{a}±3.5	59.5 ^{ab}±1.0	57.6 ^{ab}±0.6	69.7 ^{a}±19.7	39.1 ^{bcd}±12.0	32.3 ^{cd}±1.1	50.3 ^{abcd}±1.3	54.5 ^{abc}±1.7	33.4 ^{cd}±0.3	27.1 ^{d}±0.1
2-Phenethyl alcohol	217.7 ^{a}±2.1	171.4 ^{ab}±4.2	187.3 ^{ab}±3.4	195.3 ^{ab}±13.7	196.3 ^{ab}±84.7	54.6 ^{c}±0.9	165.1 ^{ab}±0.7	208.0 ^{ab}±5.2	127.2 ^{bc}±0.5	72.6 ^{c}±0.2
Σ higher alcohols	**348.2 ^{a}±6.7**	**269.5 ^{b}±5.5**	**299.9 ^{ab}±4.3**	**300.2 ^{ab}±24.2**	**302.6 ^{ab}±73.0**	**98.9 ^{d}±2.1**	**263.2 ^{b}±2.4**	**314.1 ^{ab}±8.4**	**188.7 ^{c}±1.3**	**112.9 ^{d}±0.3**

Treatments / Compounds (m/L)	**M2**									
	standard brewers wort	NH^4	Pi	K	Mg	Cu	Zn	Fe	Mn	composite mixture of all nutrients
Propanol	0.32 ^{bc}±0.03	0.55 ^{b}±0.08	0.13 ^{c}±0.02	0.56 ^{b}±0.06	0.25 ^{bc}±0.04	0.20 ^{c}±0.04	0.42 ^{bc}±0.04	0.56 ^{b}±0.07	0.38 ^{bc}±0.05	1.20 ^{a}±0.28
Isobutanol	0.02 ^{b}±0.00	0.02 ^{b}±0.00	0.02 ^{b}±0.00	0.04 ^{a}±0.01	0.02 ^{b}±0.00	0.01 ^{b}±0.01	0.02 ^{b}±0.01	0.01 ^{b}±0.00	0.03 ^{ab}±0.00	0.01 ^{b}±0.00
2-methyl butanol	102.9 ^{bc}±4.8	86.3 ^{cd}±4.2	128.3 ^{a}±3.2	128.5 ^{a}±5.1	107.8 b±6.3	78.5 ^{d}±2.7	97.3 ^{bc}±1.8	111.8 ^{ab}±8.8	78.6 ^{d}±8.7	78.4 ^{d}±7.9
Isoamyl alcohol	82.5 ^{ab}±4.1	75.4 ^{ab}±11.2	84.9 ^{a}±0.2	81.8 ^{ab}±0.3	77.7 ^{ab}±1.5	58.4 ^{cd}±0.8	71.0 ^{bc}±0.9	80.0 ^{ab}±3.2	56.4 ^{d}±3.0	57.6 ^{d}±1.3
2-Phenethyl alcohol	268.0 ^{a}±11.0	204.3 ^{cd}±6.4	268.8 ^{a}±3.8	231.1 ^{bc}±13.7	253.9 ^{ab}±13.0	206.8 ^{cd}±5.3	220.1 ^{c}±14.0	250.4 ^{ab}±7.8	188.6 ^{d}±6.2	152.4 ^{e}±6.1
Σ higher alcohols	**453.7 ^{a}±18.3**	**366.5 ^{cd}±21.7**	**482.1 ^{a}±2.1**	**447.8 ^{ab}±13.3**	**439.6 ^{ab}±20.1**	**343.9 ^{d}±5.8**	**398.2 ^{bc}±16.4**	**442.8 ^{ab}±17.6**	**324.0 ^{de}±15.7**	**289.7 ^{e}±10.7**

*Values not sharing the same letter are significantly different according to the Tukey test.

5.3.5. Essential inorganic elements influence fatty acid ester formation

Fatty acid esters (FAE) formation are dependent on the formation of short-and medium chain fatty acid, acetyl-CoA and enzymes responsible for the syntheses (Saerens *et al.* 2010). Fig. 5.5 summarises the data from table table 5.4, which illustrates the influence of essential inorganic elements in fatty acid ester formation. NCYC2592 produced a low concentration of acetate esters (8.8 mg/L, control); however, in all supplementation treatments were observed that NCYC2592 increased fatty acid ester formation, except CMN treatment (Fig. 5.3/Table 5.2). Furthermore, when inoculated in wort supplemented with magnesium, NCYC2592 produced higher concentration of total acetate esters (116.6 mg/L).

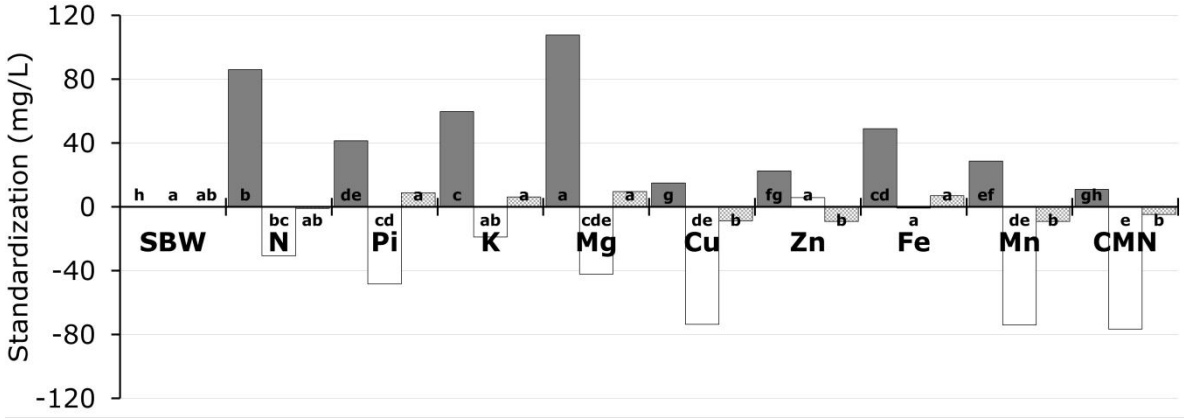

Figure 5.5: Standardization of fatty acid esters (FAE) produced during the fermentations using yeast *Saccharomyces* NCYC2592 (■), W34/70 (□), M2 (▨), respectively. All analysed from 90 fermentation using three strains with 10 treatments, which are represented: SBW (standard brewers wort), NH^{4+} (ammonia-nitrogen), P (inorganic-Phosphate), K (potassium), Mg (magnesium), Cu (copper), Zn (zinc), Fe (iron), Mn (manganese) and CMN (a composite mixture of all nutrients).

When inoculated in SBW (control), W34/70 produced 91.8 mg of total fatty acid esters/L, which was the highest concentration produced among all treatments (Fig. 5.5/Table 5.4). Therefore, fatty acid ester produced from W34/70 metabolism were mostly negatively affected by all supplementation

treatment. When fermentations were supplemented with CNM (composite mixture of all nutrients), W34/70 produced the lowest concentration of fatty acid esters (14.6 mg/L) (Table 5.4). Fatty acid ester produced using M2 were not affected by any supplementation treatment.

Specifying fatty acid ester compounds, NCYC2592 increased the formation of all fatty acid esters such as ethyl hexanoate, ethyl octanoate and ethyl decanoate, mainly, when fermentations were supplemented with ammonia-nitrogen, inorganic phosphate or magnesium. NCYC2592 inoculated in SBW (control) produced 0.34 mg/L, 0.40 mg/L and 7.9 mg/L, respectively, ethyl hexanoate, ethyl octanoate and ethyl decanoate (Table 5.4). However, when this strain was inoculated in wort supplemented with iron, ammonia-nitrogen and magnesium were generated the highest concentration of ethyl hexanoate (2.6 mg/L), ethyl octanoate (4.6 mg/L) and ethyl decanoate (111.5 mg/L) (Table 5.4). Fatty acid esters produced from W34/70 or M2 metabolism were effected negatively when inoculated in most of wort supplemented, except zinc supplementation, which had similar concentration to the control (Table 5.4).

Table 5.4: Fatty acid esters produced by yeast during 10 different fermentation treatments (including standard brewers wort, ammonia-nitrogen, inorganic phosphate, potassium, magnesium, copper, zinc, iron, manganese, and a composite mixture of all nutrients) using yeast *Saccharomyces s* - NCYC2592, W34/70 and M2. All data is present: average of concentration, Tukey test* and standard deviation.

Treatments / Compounds (mg/L)	**NCYC2592**									
	standard brewers wort	NH^4	Pi	K	Mg	Cu	Zn	Fe	Mn	composite mixture of all nutrients
Ethyl butanoate	0.18 ^{a}±0.08	0.40 ^{a}±0.25	0.52 ^{a}±0.20	0.50 ^{a}±0.05	0.57 ^{a}±0.10	0.38 ^{a}±0.05	0.20 ^{a}±0.05	0.29 ^{a}±0.02	0.46 ^{a}±0.16	0.29 ^{a}±0.09
Ethyl hexanoate	0.34 ^{e}±0.06	0.90 ^{d}±0.10	1.70 ^{b}±0.20	0.75 ^{de}±0.10	1.20 ^{bcd}±0.30	1.07 ^{cd}±0.05	1.40 ^{bc}±0.10	2.60 ^{a}±0.20	0.39 ^{e}±0.09	1.00 ^{cd}±0.09
Ethyl octanoate	0.40 ^{e}±0.15	4.64 ^{a}±0.18	2.85 ^{b}±0.18	1.72 ^{cd}±0.06	3.30 ^{b}±0.10	1.25 ^{d}±0.10	1.95 ^{c}±0.25	4.77 ^{a}±0.35	1.50 ^{cd}±0.30	1.60 ^{cd}±0.18
Ethyl decanoate	7.90 ^{h}±0.30	88.90 ^{b}±3.40	43.00 ^{de}±3.50	65.70 ^{c}±1.40	111.50 ^{a}±3.70	21.25 ^{g}±1.80	27.90 ^{fg}±3.70	50.25 ^{d}±4.50	35.00 ^{ef}±9.20	17.00 ^{gh}±2.00
Σ fatty acid esters	**8.80 ^{h}±0.60**	**94.90 ^{b}±3.50**	**58.70 ^{de}±3.30**	**68.70 ^{c}±1.40**	**116.60 ^{a}±4.40**	**24.00 ^{g}±2.00**	**31.50 ^{fg}±3.90**	**57.90 ^{cd}±4.70**	**37.40 ^{ef}±9.40**	**19.90 ^{gh}±1.60**
Treatments / Compounds (mg/L)	**W34/70**									
	standard brewers wort	NH^4	Pi	K	Mg	Cu	Zn	Fe	Mn	composite mixture of all nutrients
Ethyl butanoate	0.30 ^{a}±0.10	0.20 ^{a}±0.10	0.60 ^{a}±0.30	3.60 ^{a}±1.00	0.16 ^{a}±0.10	0.13 ^{a}±0.02	0.10 ^{a}±0.02	0.20 ^{a}±0.08	0.16 ^{a}±0.08	0.10 ^{a}±0.08
Ethyl hexanoate	2.35 ^{ab}±0.20	2.10 ^{ab}±0.10	2.00 ^{ab}±0.10	1.94 ^{b}±0.28	2.22 ^{ab}±0.12	0.74 ^{cd}±0.04	2.50 ^{a}±0.40	2.25 ^{ab}±0.18	1.00 ^{c}±0.10	0.421 ^{d}±0.04
Ethyl octanoate	3.70 ^{a}±0.30	3.80 ^{a}±0.15	3.25 ^{b}±0.03	3.75 ^{a}±0.18	3.53 ^{ab}±0.15	1.39 ^{d}±0.06	3.60 ^{ab}±0.05	3.75 ^{a}±0.09	1.60 ^{d}±0.14	2.24 ^{c}±0.05
Ethyl decanoate	85.45 ^{a}±10.80	54.45 ^{bc}±5.50	37.40 ^{cde}±0.40	63.00 ^{abc}±5.90	43.40 ^{cd}±25.90	15.20 ^{de}±0.80	91.30 ^{a}±2.50	84.20 ^{ab}±2.60	14.55 ^{de}±0.90	11.90 ^{e}±0.40
Σ fatty acid esters	**91.80 ^{a}±10.80**	**60.6 ^{bc}±5.6**	**42.8 ^{cd}±0.3**	**72.3 ^{ab}±11.4**	**49.30 ^{cde}±25.8**	**16.90 ^{de}±0.8**	**97.6 ^{a}±2.2**	**90.40 ^{a}±2.50**	**17.3 ^{de}±0.7**	**14.6 ^{e}±0.4**
Treatments / Compounds (mg/L)	**M2**									
	standard brewers wort	NH^4	Pi	K	Mg	Cu	Zn	Fe	Mn	composite mixture of all nutrients
Ethyl butanoate	0.24 ^{d}±0.01	0.25 ^{d}±0.08	0.33 ^{cd}±0.02	0.29 ^{cd}±0.02	0.70 ^{b}±0.10	0.72 ^{b}±0.03	0.62 ^{b}±0.06	0.70 ^{b}±0.10	0.54 ^{bc}±0.13	1.00 ^{a}±0.10
Ethyl hexanoate	0.77 ^{b}±0.06	1.38 ^{ab}±0.15	1.30 ^{ab}±0.30	1.40 ^{ab}±0.20	1.60 ^{a}±0.20	0.80 ^{b}±0.10	1.20 ^{ab}±0.10	1.60 ^{a}±0.30	1.58 ^{a}±0.24	1.10 ^{ab}±0.03
Ethyl octanoate	2.08 ^{ab}±0.05	1.88 ^{abc}±0.20	1.80 ^{abc}±0.20	1.90 ^{abc}±0.10	2.10 ^{a}±0.30	1.00 ^{d}±0.05	2.30 ^{a}±0.20	2.40 ^{a}±0.30	1.30 ^{cd}±0.20	1.50 ^{bcd}±0.10
Ethyl decanoate	36.40 ^{abc}±1.70	34.40 ^{abcd}±6.50	44.20 ^{a}±2.20	40.50 ^{ab}±2.30	43.60 ^{a}±4.50	27.50 ^{cd}±4.10	25.40 ^{d}±1.90	41.20 ^{a}±1.50	25.95 ^{d}±0.45	30.00 ^{bcd}±0.80
Σ fatty acid esters	**39.50 ^{ab}±1.70**	**37.00 ^{ab}±6.90**	**47.60 ^{a}±2.10**	**44.70 ^{a}±2.10**	**48.00 ^{a}±4.70**	**30.00 ^{b}±4.10**	**29.60 ^{b}±2.00**	**45.90 ^{a}±1.60**	**29.40 ^{b}±0.80**	**33.70 ^{b}±0.90**

*Values not sharing the same letter are significantly different according to the Tukey test.

5.3.6. Essential inorganic elements influence fatty acid formation

Fatty acids are undesired due to their unpleasant characteristics and can be classified as short-chain fatty acids and medium chain fatty acids. In beer, they are rarely found above threshold (Xu *et al.* 2017, ASBC Methods of Analysis: Beer Flavour Database 2011). However, medium chain fatty acids are an important precursor of fatty acid esters. Fig. 5.6 summarises the data from table table 5.5, which illustrates the influence of essential inorganic elements in fatty acid formation. NCYC2592 produced a low concentration of acetate esters (1.35 mg/L, control); however, in all supplementation treatments were observed that NCYC2592 increased the acetate ester formation (Fig. 5.3/Table 5.2). Furthermore, When inoculated in wort supplemented with ammonia nitrogen (12.8 mg/L) and inorganic phosphate (14.1 mg/L), NCYC2592 produced higher concentration of total acetate esters. When inoculated in SBW (control), NCYC2592 and M2 produced lower concentration of fatty acid (3.1 mg/L and 5.0 mg/L, respectively) than W34/70 (7.6 mg/L) (Fig. 5.6/Table 5.5).

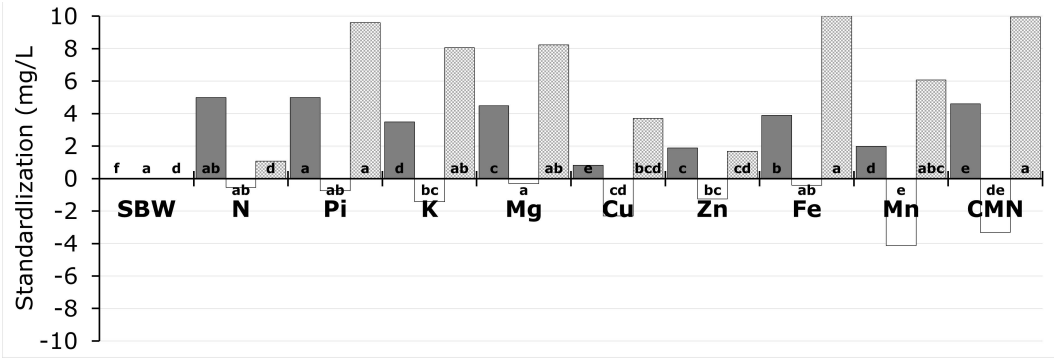

Figure 5.6: Standardization of fatty acid (FA) produced during the fermentations using yeast *Saccharomyces* NCYC2592 (■), W34/70 (□), M2 (▨), respectively. All analysed from 90 fermentation using three strains with 10 treatments, which are represented: SBW (standard brewers wort), NH^{4+} (ammonia-nitrogen), P (inorganic-Phosphate), K (potassium), Mg (magnesium), Cu (copper), Zn (zinc), Fe (iron), Mn (manganese) and CMN (a composite mixture of all nutrients).

NCYC2592 and M2 increased the amount of fatty acid produced when inoculated in most of wort supplemented with inorganic-phosphate, potassium, magnesium, iron, manganese or CMN (Fig. 5.6). However, in wort supplemented with inorganic phosphate, magnesium, iron or CMN, NCYC2592 increased the generation of fatty acids up to 8.1 mg/L and M2 also increased up to 15 mg/L the fatty acid formation (Fig. 5.6/Table 5.5). W34/70 decreased its fatty acid formation when inoculated in most of heavy metal treatments (Fig. 5.6).

Table 5.5: Fatty acids produced by yeast during 10 different fermentation treatments (including standard brewers wort, ammonia-nitrogen, inorganic phosphate, potassium, magnesium, copper, zinc, iron, manganese, and a composite mixture of all nutrients) using yeast *Saccharomyces* - NCYC2592, W34/70 and M2. All data is present: average of concentration, Tukey test* and standard deviation.

Treatments / Compounds (mg/L)	**NCYC2592**									
	standard brewers wort	NH^4	Pi	K	Mg	Cu	Zn	Fe	Mn	composite mixture of all nutrients
Isobutyric acid	0.80 ^{a}±0.02	1.48 ^{a}±0.06	1.23 ^{a}±0.03	1.10 ^{a}±0.01	1.43 ^{a}±0.03	0.95 ^{a}±0.03	1.13 ^{a}±0.01	1.15 ^{a}±0.01	1.68 ^{a}±1.09	0.74 ^{a}±0.02
Butanoic acid	0.21 ^{e}±0.03	0.56 ^{a}±0.02	0.58 ^{a}±0.05	0.42 ^{b}±0.02	0.52 ^{a}±0.02	0.28 ^{cde}±0.02	0.37 ^{bc}±0.03	0.52 ^{a}±0.02	0.35 ^{bcd}±0.05	0.26 ^{de}±0.00
Isovaleric acid**	0.76 ^{e}±0.06	1.26 ^{b}±0.05	1.27 ^{b}±0.06	1.19 ^{bc}±0.03	1.44 ^{a}±0.04	0.90 ^{d}±0.03	0.90 ^{d}±0.01	1.10 ^{c}±0.02	0.96 ^{d}±0.08	0.70 ^{e}±0.01
Hexanoic acid	0.46 ^{f}±0.02	1.60 ^{a}±0.04	1.52 ^{ab}±0.13	1.00 ^{c}±0.02	1.28 ^{b}±0.05	0.58 ^{ef}±0.04	0.88 ^{cd}±0.02	1.65 ^{a}±0.05	0.70 ^{de}±0.14	0.62 ^{ef}±0.02
Octanoic acid	0.79 ^{f}±0.08	2.86 ^{b}±0.10	3.32 ^{a}±0.10	2.42 ^{c}±0.06	2.53 ^{c}±0.05	1.13 ^{e}±0.06	1.60 ^{d}±0.03	2.38 ^{c}±0.07	1.19 ^{e}±0.12	1.05 ^{e}±0.02
Decanoic acid	0.13 ^{b}±0.02	0.37 ^{b}±0.01	0.30 ^{b}±0.02	0.50 ^{b}±0.02	0.43 ^{b}±0.14	0.13 ^{b}±0.00	0.16 ^{b}±0.02	0.22 ^{b}±0.02	0.26 ^{b}±0.04	4.38 ^{a}±0.80
Σ Fatty acids	**3.10 ^{d}±0.10**	**8.10 ^{a}±0.30**	**8.10 ^{a}±0.40**	**6.60 ^{b}±0.04**	**7.60 ^{ab}±0.20**	**4.00 ^{cd}±0.20**	**5.00 ^{c}±0.02**	**7.00 ^{ab}±0.20**	**5.10 ^{c}±0.90**	**7.80 ^{ab}±0.70**
Treatments / Compounds (mg/L)	**W34/70**									
	standard brewers wort	NH^4	Pi	K	Mg	Cu	Zn	Fe	Mn	composite mixture of all nutrients
Isobutyric acid	1.30 ^{a}±0.02	1.10 ^{abc}±0.02	1.08 ^{bcd}±0.01	0.90 ^{def}±0.10	0.85 ^{ef}±0.10	1.10 ^{abcd}±0.03	1.05 ^{cde}±0.03	1.25 ^{ab}±0.01	0.70 ^{f}±0.01	1.00 ^{cde}±0.01
Butanoic acid	0.48 ^{ab}±0.02	0.57 ^{a}±0.00	0.42 ^{abc}±0.00	0.30 ^{bc}±0.19	0.34 ^{bc}±0.07	0.43 ^{abc}±0.02	0.35 ^{bc}±0.01	0.44 ^{abc}±0.01	0.24 ^{c}±0.01	0.40 ^{abc}±0.00
Isovaleric acid**	1.60 ^{ab}±0.05	1.62 ^{ab}±0.03	1.65 ^{ab}±0.02	1.45 ^{ab}±0.00	2.05 ^{a}±1.06	1.89 ^{ab}±0.03	1.50 ^{ab}±0.03	1.64 ^{ab}±0.02	0.94 ^{b}±0.04	1.50 ^{ab}±0.01
Hexanoic acid	1.10 ^{a}±0.03	1.17 ^{a}±0.01	1.08 ^{ab}±0.01	1.04 ^{ab}±0.15	1.09 ^{a}±0.06	0.80 ^{cd}±0.02	0.91 ^{bc}±0.00	1.10 ^{a}±0.01	0.55 ^{e}±0.02	0.61 ^{de}±0.01
Octanoic acid	2.50 ^{a}±0.07	2.02 ^{cd}±0.03	2.13 ^{c}±0.01	1.94 ^{cd}±0.16	2.34 ^{ab}±0.07	0.91 ^{e}±0.06	1.89 ^{d}±0.03	2.15 ^{bc}±0.08	0.73 ^{ef}±0.02	0.65 ^{f}±0.02
Decanoic acid	0.60 ^{ab}±0.03	0.49 ^{bc}±0.01	0.47 ^{c}±0.02	0.52 ^{abc}±0.05	0.56 ^{abc}±0.05	0.20 ^{de}±0.03	0.61 ^{a}±0.01	0.56 ^{abc}±0.03	0.27 ^{d}±0.00	0.10 ^{e}±0.01
Σ Fatty acids	**7.60 ^{a}±0.20**	**7.00 ^{ab}±0.10**	**6.80 ^{ab}±0.05**	**6.10 ^{bc}±0.60**	**7.50 ^{a}±0.90**	**5.30 ^{cd}±0.20**	**6.30 ^{bc}±0.10**	**7.20 ^{ab}±0.10**	**3.40 ^{e}±0.10**	**4.20 ^{de}±0.06**
Treatments / Compounds (mg/L)	**M2**									
	standard brewers wort	NH^4	Pi	K	Mg	Cu	Zn	Fe	Mn	composite mixture of all nutrients
Isobutyric acid	1.60 ^{e}±0.10	2.40 ^{cd}±0.20	2.90 ^{b}±0.04	1.55 ^{e}±0.02	2.10 ^{d}±0.04	1.77 ^{e}±0.06	2.60 ^{bc}±0.16	2.32 ^{cd}±0.05	1.60 ^{e}±0.07	3.38 ^{a}±0.07
Butanoic_acid	0.39 ^{bc}±0.01	0.47 ^{bc}±0.09	0.50 ^{ab}±0.01	0.40 ^{bc}±0.02	0.45 ^{bc}±0.04	0.46 ^{bc}±0.05	0.40 ^{bc}±0.01	0.44 ^{bc}±0.02	0.36 ^{c}±0.03	0.60 ^{a}±0.01
Isovaleric acid**	1.10 ^{cd}±0.02	0.98 ^{d}±0.19	1.70 ^{a}±0.05	1.15 ^{cd}±0.03	1.46 ^{ab}±0.07	1.37 ^{bc}±0.14	1.46 ^{ab}±0.05	1.46 ^{ab}±0.03	1.05 ^{d}±0.05	1.71 ^{a}±0.03
Hexanoic acid	0.58 ^{d}±0.03	0.61 ^{cd}±0.04	0.74 ^{a}±0.01	0.71 ^{ab}±0.03	0.72 ^{ab}±0.01	0.45 ^{e}±0.01	0.59 ^{d}±0.02	0.70 ^{ab}±0.01	0.62 ^{cd}±0.03	0.67 ^{bc}±0.01
Octanoic acid	1.10 ^{bc}±0.10	1.33 ^{abc}±0.26	1.56 ^{a}±0.01	1.40 ^{abc}±0.03	1.44 ^{ab}±0.07	0.68 ^{d}±0.06	1.33 ^{abc}±0.04	1.34 ^{abc}±0.02	1.10 ^{c}±0.06	1.49 ^{a}±0.05
Decanoic acid	0.19 ^{b}±0.03	0.22 ^{b}±0.01	7.15 ^{a}±1.41	7.95 ^{a}±0.36	7.00 ^{a}±1.35	3.93 ^{b}±0.50	0.25 ^{b}±0.03	8.70 ^{a}±0.90	6.28 ^{a}±0.77	7.06 ^{a}±0.34
Σ Fatty acids	**5.00 ^{d}±0.30**	**6.02 ^{d}±0.81**	**14.54 ^{a}±1.40**	**13.01 ^{ab}±0.34**	**13.18 ^{ab}±1.21**	**8.65 ^{bcd}±4.18**	**6.70 ^{cd}±0.25**	**14.95 ^{a}±0.90**	**11.00 ^{abc}±0.60**	**14.90 ^{a}±0.40**

*Values not sharing the same letter are significantly different according to the Tukey test. **Isovaleric acid (3-methylbutanoic acid)

5.3.7. Overall of all fermented samples

Principal component analysis (PCA) was applied as a multivariate technique to summarise all linear correlations from a person table into a Bi-Plot (Fig. 5.7). PC1 and PC2 accounted for 34% and 18% of the variance, respectively. Overall all three yeasts behave in similar ways (grouped on PCA), ethanol and glycerol is positively correlated with PC1 and acetic acid formation is negatively correlated with PC1. Increased aroma formation is correlated with PC1. The relative profile of aroma compounds formed varies with species. PCA shows that all yeasts produced very different flavour profile when observed the standard brewers wort as reference 1 (NCYC2592), reference 2 (W34/70) and reference 3 (M2) (Fig. 5.7).

The first component separates the reference 1 (NCYC2592) from reference 2 (W34/70) and reference 3 (M2). The second component separates NCYC2592 and M2 strains from W34/70 strain. Therefore, PC1 shows the similarities between traditional brewing strains (W34/70 and M2) and PC2 shows the similarities between *Saccharomyces cerevisiae* strains (NCYC2592 and M2). This increased the simplicity of the interpretation presenting the similarities between NCYC2592 and M2 and similarities between W34/70 and M2 (Fig. 5.7).

The first two components (PCA1 and PCA2) represents the components of the global analysis as specific correlations to each yeast strain studied. Although NCYC2592 and W34/70 strains not process any initial similarities, fermentation supplemented with ammonia-nitrogen, inorganic-phosphate, potassium, magnesium and iron using NCYC2592 strain drove the metabolism of this strain to produce similar flavour profile as W34/70 (Fig. 4). Likewise,

fermentation supplemented with copper, manganese and a composite mixture of all nutrients using W34/70 strain drove the metabolism of this strain to produce similar flavour profile as NCYC2592 (reference 1). Therefore, supplementations using NCYC2592 strain were generally positively significant; on the other hand, supplementations using W34/70 strain had a negative significant affected.

M2 strain presented a particular behaviour when applied in all supplementation treatments (Fig. 5.7). Fermentation supplemented with ammonia-nitrogen, zinc and a composite mixture of all nutrients using M2 strain presented no significant difference in flavour profile. However, when M2 strain were used in fermentation supplemented with inorganic-phosphate, potassium, magnesium and iron were observed a positive increasing of all flavour concentration. Fermentations supplemented with copper and manganese using M2 presented more similarities to NCYC2592 fermentation; however, these similarities are from a reduction of flavour production.

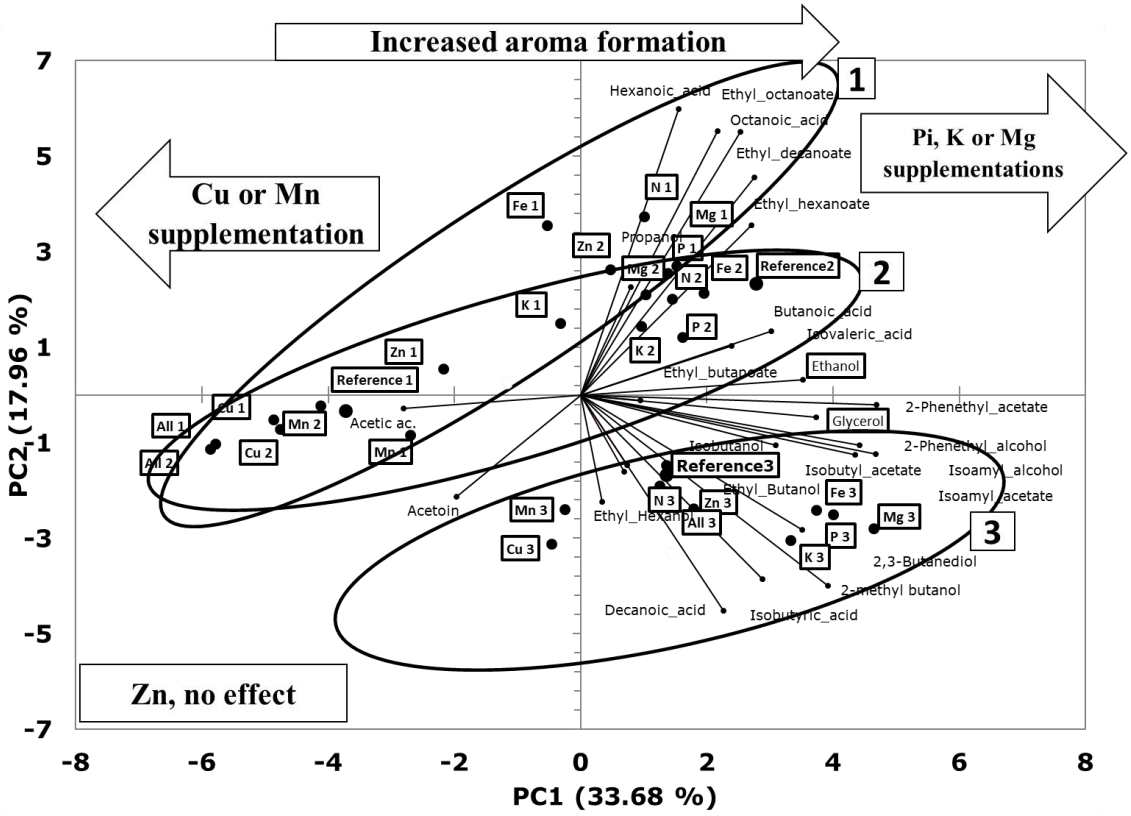

Fig. 5.7: Principle component analysis (Bi-Plot) of all 25-flavour compounds. The colours represent the yeast: 1) NCYC2592, 2) W34/70 and 3) M2. Comparison of 25 flavour compounds from 90 fermentation using three strains with 10 treatments (standard brewers wort - reference, nitrogen - N, phosphorus - P, potassium - K, magnesium - Mg, copper - Cu, zinc - Zn, iron - Fe, manganese - Mn, and a composite mixture of all nutrients - All). Ethanol and glycerol are highlighted on black box to understand better behave these two target-industrial compounds comparing to other compounds generated.

As expected, higher alcohol formation and acetate ester synthesis are highly correlated. These include isoamyl alcohol and 2-phenethyl alcohol and their respective acetate esters (isoamyl acetate and 2-Phenethyl acetate). Fatty acids and fatty acids esters have a linear positive correlation; except, decanoic acid and ethyl decanoate (Fig. 5.7). Other fatty acid esters such as ethyl hexanoate and ethyl octanoate have a linear positive correlation to their respective medium chain fatty acid, which are hexanoic acid and octanoic acid. Therefore, a linear positive correlation suggests that the compound formation in parallel or involves the same pathway (Fig. 5.7).

As expected, acetic acid and ester formation are inversely proportional (Fig. 5.7) because acetic acid is a precursor of acetate/Acetyl-CoA and as a consequence precursor of esters. Acetoin and 2,3-butanediol have no linear correlation because are diacetyl (2,3-butanedione) precursors and as a consequence compete by using the same pathway. NCYC2592 responded better to the most of treatments because this strain has in its composition lower concentration of all essential inorganic elements evaluated, except inorganic-phosphate, potassium and manganese, which had similar level comparing to M2 and W34/70 (Table .

5.4. Discussion

Yeast viability is an important control analysis for brewers because is a demonstrative of fermentation quality/performance (Layfield and Sheppard 2015, Luarasi *et al.* 2016, Marechal and Gervais 1994). Results evidenced the influence of essential inorganic elements on yeast-flavour formation, which starts with the effect of inorganic elements in yeast viability. Inorganic-phosphate, potassium and magnesium influenced positively on yeast viability because are involved in enzymatical activation in several pathway (including glycolysis, ethanol formation and acetyl-CoA) and ATP generation (Maguire and Cowan 2002, Boubekeur *et al.* 2001, Jacobson and Bernofsky 1974); and potassium presence controls yeast internal pH and consequently important factors such as growth, viability and yeast integrity (Rodriguez-Navarro, 2000; Arino *et al.* 2010). Heavy metals mostly affect negatively yeast viability, mainly, copper, which is related to electro-negativity and toxicity of heavy metal (transition metals) (De Freitas *et al.* 2003) although are involved in

ensuring metabolic activity of yeast during growth and fermentation (Wietstock *et al.* 2015, Cyert and Philpott 2013). All other heavy metals have less influence on yeast viability.

To understand yeast-flavour formation is indispensable evaluate ethanol, glycerol and acetic acid formation as the main taste compounds and industrial target. Ethanol and glycerol formation are positively correlated and generally were produced proportionally (Table 5.1) and are involved in redox balance (Zhang *et al.* 2013). Glycerol is synthesized as a physiological role in yeast metabolism, which includes maintaining a redox balance by converting surplus NADH to NAD^+ (Zhang *et al.* 2013, Ansell *et al.* 1997, van Dijken and Scheffers 1986) and by protecting yeast cells from osmotic stress (Zhang *et al.* 2013, Nevoigt and Stahl 1997, Blomberg 1992). When fermentations using M2 or W34/70 strains were supplemented with ammonia-nitrogen, inorganic phosphate, magnesium, zinc or iron, ethanol and glycerol production were not affected; however, when fermentation were supplemented with manganese or CMN (a composite mixture of all nutrients), W34/70 significantly decreased ethanol formation (Fig. 5.2 A, Table 5.1). In contrast, when fermentation were supplemented with potassium using NCYC2592 strain ethanol and glycerol formation increased (Fig. 5.2A/B, Table 5.1) because potassium is involved in maltose intakes (Boulton and Quain 2001, Priest and Stewart 2006) and as a consequence, increased ethanol production and, therefore, glycerol production as an ethanol toxic protective response and maintaining a redox balance (Zhang *et al.* 2013, Ansell *et al.* 1997, van Dijken and Scheffers 1986).

Acetic acid is a precursor of acetate (anion) and acetyl-CoA cytosolic (Chen *et al.* 2013, Takahashi *et al.* 2006). NCYC2592 reduced acetic acid formation

when fermentations were supplemented with ammonia-nitrogen, inorganic-phosphate, potassium, magnesium, copper, zinc or manganese (Fig. 5.2 C/Table 5.1). W34/70 and M2 strains increased acetic acid formation when fermentations were supplemented with copper or CNM (composite mixture of all nutrients). Furthermore, acetic acid accumulation occurred mostly when yeast viability decreased and as a consequence increasing yeast death. Therefore, during or after death, yeast cell breaks out and cannot consume the amount of acetic acid accumulated. As a consequence, a high final concentration of acetic acid and a reduction of acetate ester formation (Fig. 5.7). Acetic acid formation is very important for keeping yeast metabolic activities via Acetyl-CoA formation (Pietrocola *et al.* 2015, Galdieri *et al.* 2014) and as a consequence, regulates flavour generation including higher alcohols, short chain fatty acids, esters and fatty acids esters (Fig. 5.3).

Acetate ester formation is dependent of alcohols, acetyl-CoA and enzymes responsible for the syntheses (Knight *et al.* 2014, Saerens *et al.* 2010, Saerens *et al.* 2008). NCYC2592 increased acetate ester formation when inoculated in all treatments evaluated. W34/70 increased acetate ester formation when fermentations were supplemented with copper, manganese or CNM (composite mixture of all nutrients). M2 strains increased acetate ester formation when fermentations were supplemented with inorganic-phosphate, potassium, magnesium, iron or CNM. In contrast, M2 decreased acetate ester formation when fermentations were supplemented with copper or manganese (Fig 5.3/Table 5.2). All acetate ester formation was inversely proportional to acetic acid formation because which is related to an insufficient conversion of acetyl-CoA via acetic acid/acetate.

Therefore, amino acid consumption were positively affected through supplementations such as inorganic-phosphate, potassium or magnesium, which are involved in the enzymatical synthesis of acetate esters. Firstly acetaldehyde is converted to acetate via acetaldehyde dehydrogenases (ACDH) by potassium presence (K-ACDH) and can happen by magnesium presence (Mg-ACDH) using as coenzymes NAD1 and NADP1, which is related to redox balance (Boubekeur *et al.* 2001, Jacobson and Bernofsky 1974). Second, acetate is converted to acetyl-CoA by phosphorus and magnesium (Mg-ATP complex), which consumes ATP and magnesium (Maguire and Cowan 2002). Therefore, acetyl-CoA is involved in all metabolic activity necessaries for yeast growth/survival and ester formation.

Higher alcohol production is a consequence of amino acid consumption via Ehrlich pathway (Dack *et al.* 2017, Hazelwood *et al.*, 2008, Hazelwood *et al.* 2006). Higher alcohols are immediately secreted into the wort, or esterified with acetyl-CoA to synthesize their respective esters because cannot be used as a carbon source for central metabolism (Vidal *et al.* 2014, Ashraf 2011, Hazelwood *et al.* 2008). NCYC2592 increased higher alcohol formation when fermentations were supplemented with ammonia-nitrogen, inorganic-phosphate, potassium, magnesium, iron, manganese or CNM (composite mixture of all nutrients). W34/70 and M2 strains decreased higher alcohol formation when fermentations were supplemented with ammonia-nitrogen, copper, zinc, manganese or CNM (Table 5.4). Higher alcohols formation by alcohol dehydrogenase and zinc-dependence are well known. However, Zinc is responsible for the activation of ADH4, therefore, ADH1, ADH3 and ADH5 may be activated by magnesium and also, potassium secretion of hydrogen ion

and the cytosolic enzyme requirements such as $NADP^+$, which is activated by magnesium (Boubekeur *et al.* 2001, Remize *et al.* 2000).

Fatty acid esters (FAEs) formation are dependent on the formation of short-and medium chain fatty acid, acetyl-CoA and enzymes responsible for the syntheses (Zhuang *et al.* 2015, Saerens *et al.* 2010). In alcoholic beverages, FAEs generate desirable fruit aromas (Knight *et al.* 2014). NCYC2592 increased fatty acid ester formation when fermentations were supplemented with ammonia-nitrogen, inorganic-phosphate, potassium, magnesium, copper, iron or manganese. W34/70 strain decreased fatty acid ester formation when fermentations were supplemented with ammonia-nitrogen, inorganic-phosphate, magnesium, copper, maganese or CNM (Table S1.4). M2 strains decreased fatty acid ester formation when fermentations were supplemented with copper, zinc, manganese or CNM.

In the study conditions, NCYC2592 and M2 increased the formation of all FAEs, which includes ethyl hexanoate, ethyl octanoate and ethyl decanoate, when fermentations were supplemented with inorganic phosphate and magnesium. On the other hand, W34/70 produced the highest concentration of all fatty acid esters when was inoculated in standard brewers wort (control) and when fermentations were supplemented with inorganic phosphate, potassium and magnesium, W34/70 strain produced a similar concentration of FAEs comparing to the control (SBW). FAEs are important active flavour, but they are generated from off-flavour (fatty acids); therefore, to fully understand the essential inorganic elements influence on fatty acid esters is vital to evaluate fatty acid formation.

Yeasts are able to produce short- and medium-chain fatty acids (SMFAs) (Zhuang *et al.* 2015, Yu *et al.* 2016, Hazelwood *et al.* 2008). SMFAs are often considered undesirable off-flavours (Boulton and Quain, 2001). NCYC2592 increased fatty acid formation when fermentations were supplemented with ammonia-nitrogen, inorganic-phosphate, potassium, magnesium, zinc, iron, manganese or CMN (composite mixture of nutrients). M2 strains increased fatty acid formation when fermentations were supplemented with inorganic-phosphate, potassium, magnesium, iron, manganese or CNM. W34/70 strain decreased fatty acid formation when fermentations were supplemented with potassium, zinc, manganese or CNM. Fatty acids are undesired due to their unpleasant characteristics and can be classified as short-chain fatty acids and medium chain fatty acids. In beer, fatty acids are unpleasant flavours and important precursor of fatty acid esters, but are rarely found above threshold values. Although essential inorganic elements can increase formation of fatty acids, none of them were found above threshold (Xu *et al.* 2017, ASBC Methods of Analysis: Beer Flavour Database 2011). Therefore, in all treatments, fatty acids were produced as precursor of fatty acid esters. In conclusion, the supplementation with inorganic-phosphate and magnesium were significantly increased fatty acid production, therefore, these elements are involved in the completion between higher alcohols and short-chain fatty acids, which occurs via Ehrlich pathway (Yu *et al.* 2016).

Inorganic-phosphate, potassium and magnesium supplementations influenced positively all strains, which occurred because: Firstly, inorganic-phosphate is involved in cellular biosyntheses such as nucleic acids, nucleoproteins, phospholipids, ATP and several metabolic pathways (Walker and Stewart 2016,

Canadell *et al.* 2015). Second, potassium is involved in the export of H^+ (Internal pH control), Na^+ and toxic cations like lithium and phosphorus uptake (Canadell *et al.* 2015, Barreto *et al.* 2012). Third, magnesium is vital for yeast division/growth, metabolic activities, respiro-fermentative metabolism, mitochondrial structure/function, response to environmental stress, fermentation performance and ethanol production (Udeh and Kgatla 2013, Udeh *et al.* 2014, Walker *et al.* 2006, Walker 2004). Finally, magnesium can be transported into cells via potassium and phosphorus transport systems, two plasma membrane transporters, ALR1 and ALR2 (Knoop *et al.* 2005) and Mg-ATP complex, which consumes ATP and magnesium acts as an enzymatic co-factor (Pisat *et al.* 2009, Maguire and Cowan 2002, Conway and Beary 1962).

5.5. Conclusion

This study illustrated for the first time how flavour profiles is impacted when singular or a complex mix of eight different essential inorganic elements were added during fermentation. Ammonia-nitrogen, inorganic phosphate, potassium and magnesium significantly increased the production of target industrial compounds (ethanol and glycerol) and decreased acetic acid; furthermore increased the formation of higher alcohols and esters. Copper, iron, manganese or a composite mixture of all nutrient supplementations influenced negatively flavour formation. Zinc had less impact on flavour formation.

CHAPTER 6

SYNTHETIC SWEET WORT: COMPOSITION, YEAST GROWTH, CARBOHYDRATES CONSUMPTION AND FLAVOUR PRODUCTION

6. CHAPTER VI: SYNTHETIC SWEET WORT: COMPOSITION, YEAST GROWTH, CARBOHYDRATES CONSUMPTION AND FLAVOUR PRODUCTION

6.1. Introduction

Chapter 5 presented a more complete understanding of the role of essential inorganic elements on yeast flavour production. Also, concludes that inorganic elements such as phosphorus, potassium and magnesium were the most important elements, which can affect positively the yeast-favour formation. Therefore, it suggests that quantity of each element in wort composition may affect yeast growth and consequently yeast-flavour formation.

Wort is produced using biological materials, so it is naturally difficult to control variations during the physical-chemical extraction process, which brings all the components from malt into wort (Mathias *et al.* 2017, He *et al.* 2014, Keukeleire 2000). To understand the role of several components present in wort a defined medium which can 'mimic' wort composition is required for comparative yeast fermentation. Some researchers have developed a formulation of defined/semi-defined media (Taidi *et al.* 2003, Kennedy *et al.* 1997); however, any 'artificial wort' recipe published have not demonstrated enough similarity to natural wort composition, which includes key components: macronutrients (carbohydrates and free amino acids), essential inorganic elements (including potassium, phosphorous, magnesium, calcium, iron, zinc, copper, manganese), vitamins and lipids (Yu *et al.* . 2018, Briggs *et al.* 2004, Boulton and Quain 2001). This chapter create a more complete synthetic sweet wort and compare to beer sweet wort during yeast fermentation.

6.2. Material/Method/Experimental design

This chapter presents: (6.3.1) the synthetic wort preparation and influence of individual components on pH, (6.3.2) Comparison of yeast growth using a beer sweet wort, a synthetic sweet wort and a CN medium (carbon and nitrogen based medium), (6.3.3) Importance of essential inorganic elements in yeast growth, (6.3.4) CO_2 loss accumulation during fermentation and (7.3.5) Comparison of flavour composition at the end of fermentation in a final beer sweet wort and a synthetic sweet wort. All synthetic sweet wort constituents used in this chapter, which includes carbohydrates and amino acids, essential inorganic elements (micro inorganic nutrients and metal ions), vitamins and lipids, were added to the medium according to the composition described in the table 2.3 (Chapter 2, Section 2.1.5), The fully defined synthetic wort (SW) was tested by performing parallel fermentations using two *Saccharomyces cerevisiae* (NCYC2592 and M2) and one *Saccharomyces pastorianus* (W34/70) brewing yeast strains (Chapter 2, Section 2.1).

6.3. Results

6.3.1. Synthetic wort preparation and influence of individual components addition on pH

Synthetic sweet wort (SSW) developed in this study had similar pH to a beer sweet wort (BSW) (Table 6.1). The final pH of synthetic wort (5.41±0.02) has no significant difference compared to the sweet beer wort (pH=5.42±0.01). Before the autoclaving process, the base of synthetic sweet wort was prepared using a group of carbohydrates (including maltose, sucrose, glucose, and fructose), lipids and calcium (Chapter 2, Section 2.1.5, Table 2.3). Other

substances, which compose synthetic wort (including amino acids, vitamins and essential inorganic elements), were added in after sterilization process. Amino acids and vitamins were responsible for buffering capacity because their addition increased the pH to 6.8 and 7.11, respectively, which represent an increase of 2.5 and 2.8, respectively. In contrast, essential inorganic elements were responsible for decreasing the pH of wort from 7.1 to 5.4 (Table 6.1).

Table 6.1: Influence of individual components addition in water during synthetic wort preparation on pH of synthetic wort

Composition	Quantity	Unit/L	pH
Carbohydrates*	134	g	4.30±0.02**
Lipids*	0.3	mL	
$CaSO_2.2H_2O$ (Calcium)*	550 (156)	mg (mg of ion)	
Amino acids	3.50	g	6.80±0.03
Vitamins	0.21	g	7.11±0.02
Essential inorganic elements*			
$Na_2PO_4.H_2O$ (Phosphate)	1300 (253.35)	mg (mg of ion)	5.51±0.01
KCl (Potassium)	1000 (524.50)		5.48±0.04
$MgCl_2$ (Magnesium)	300 (76.57)		5.38±0.01
Copper (Cu) by $CuSO_4.5H_2O$	0.20 (0.05)		5.38±0.02
Iron (Fe) by $FeSO_4.7H_2O$	0.20 (0.04)		5.37±0.02
Zinc (Zn) by $ZnSO_4.7H_2O$	0.70 (0.15)		5.42±0.01
Manganese (Mn) by $MnSO_2.4H_2O$	0.20 (0.06)		5.41±0.02
Final pH			**5.41±0.02**

*Carbohydrates (including maltose, sucrose, glucose, and fructose), lipids and calcium ($CaSO_2.2H_2O$) were added in solution before autoclaving process.
**pH value were measured after autoclaving process.

6.3.2. Comparison of yeast growth using a beer sweet wort, a synthetic sweet wort and a CN medium (Carbon and nitrogen based medium)

Fig. 6.1 shows the yeast growth comparison of a beer sweet wort (BW), a beer sweet wort synthetic (SW) and a CN medium (a solution of carbohydrates and amino acid). Two *Saccharomyces cerevisiae* (NCYC2592 and M2) and one *Saccharomyces pastorianus* (W34/70) brewing yeast strains were used in this study (Chapter 2, Section 2.1). CN medium, which was composed of carbohydrates (including maltose, sucrose, glucose and fructose) and amino acids (including 19 essential amino acids) was utilised as a comparison to a beer sweet wort (BW) and a beer sweet wort synthetic (SW), which are both more complete. Results show that in BW and SW, yeast can grow up to 0.6 OD (Optical density). In contrast, when inoculated in CN medium, all yeast strains grow up to 0.40 OD (Fig. 6.1). These significant growth differences occurred because CN medium has only carbohydrates and amino acids in its composition and lacks essential inorganic elements, vitamins and lipids. Therefore, together with carbon source and nitrogen source, inorganic elements, vitamins and lipids are essential for yeast growth.

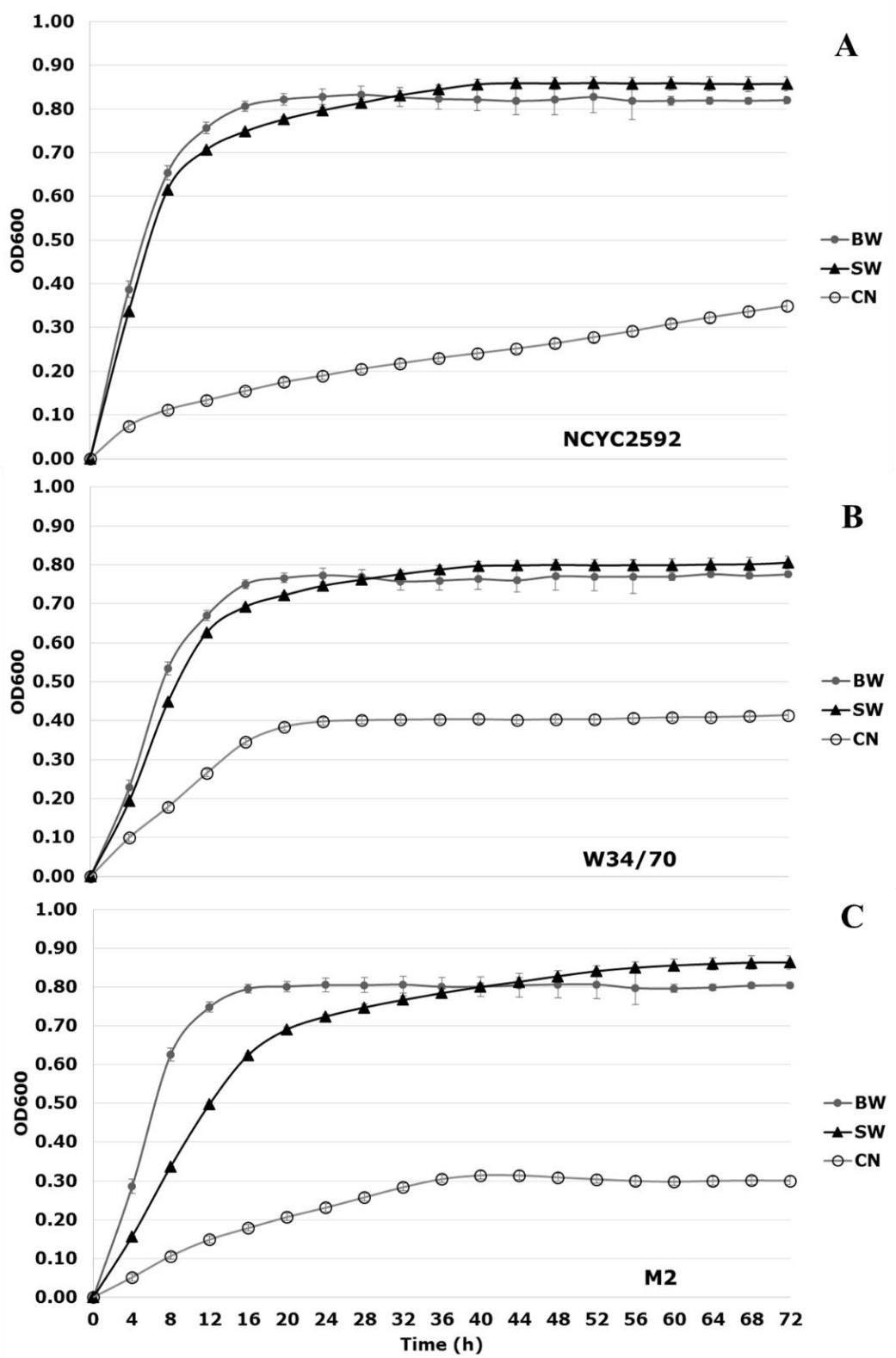

Fig. 6.1: Comparing of yeast growth during fermentation of a beer sweet wort (—●—), a synthetic sweet wort (—▲—) and CN medium (—○—) using yeasts *Saccharomyces* yeast strains A) NCYC2592, B) W34/70 and C) M2. Each value is the mean of 3 replicates and error bars shown by standard deviation.

6.3.3. Importance of essential inorganic elements in yeast growth

Fig. 6.2 and Fig 6.3 display metabolic profiles generated using three *Saccharomyces* strains (NCYC2592, W34/70 and M2) when the concentration of essential elements such as ammonia-nitrogen, phosphorus, potassium and magnesium was varied. Ammonia-nitrogen was added at 0, 75, 150 and 300 mg/L, which were graphically represented by N0, N1, N2 and N3, respectively. The value 150 mg/L (N2) represented 10% of the total amino acid present in the beer wort. Fig. 6.2A, Fig. 6.2B and Fig. 6.2C illustrate that all the ammonia-nitrogen concentrations studied did not affect the yeast growth because they were able to use amino acids as a nitrogen source. In fact, yeast growth were not affected when inoculated in wort with absence, deficiency or supplemented with ammonia-nitrogen.

Inorganic phosphorus is important for yeast growth. Fig. 6.2D, Fig. 6.2E and Fig. 6.2F illustrate that the absence of inorganic-phosphate in the medium significantly affected growth. Inorganic-phosphate (Pi) was added at 0, 126, 253and 506 mg/L, which were graphically represented by P0, P1, P2 and P3, respectively. The value 253.00 mg/L (P2) represented the concentration of Pi present in natural beer wort (Chapter 2, section 2.1.5). As expected Pi absence, (treatment P0 - Fig. 6.2D, Fig. 6.2E and Fig. 6.2F) affected yeast growth. All other treatments, including 126.00 mg/L (P1), 253.00 mg/L (P2) and 506.00 mg/L (P3), did not affect the yeast growth. Considering that natural beer wort Pi quantities were represented by treatment P2, it suggests that treatment P1 (126.00 mg/L) did not affect growth but yeast quality can affect. The treatment P3 (506.00 mg/L), which represents the double amount of Pi added into the wort, did not affect growth.

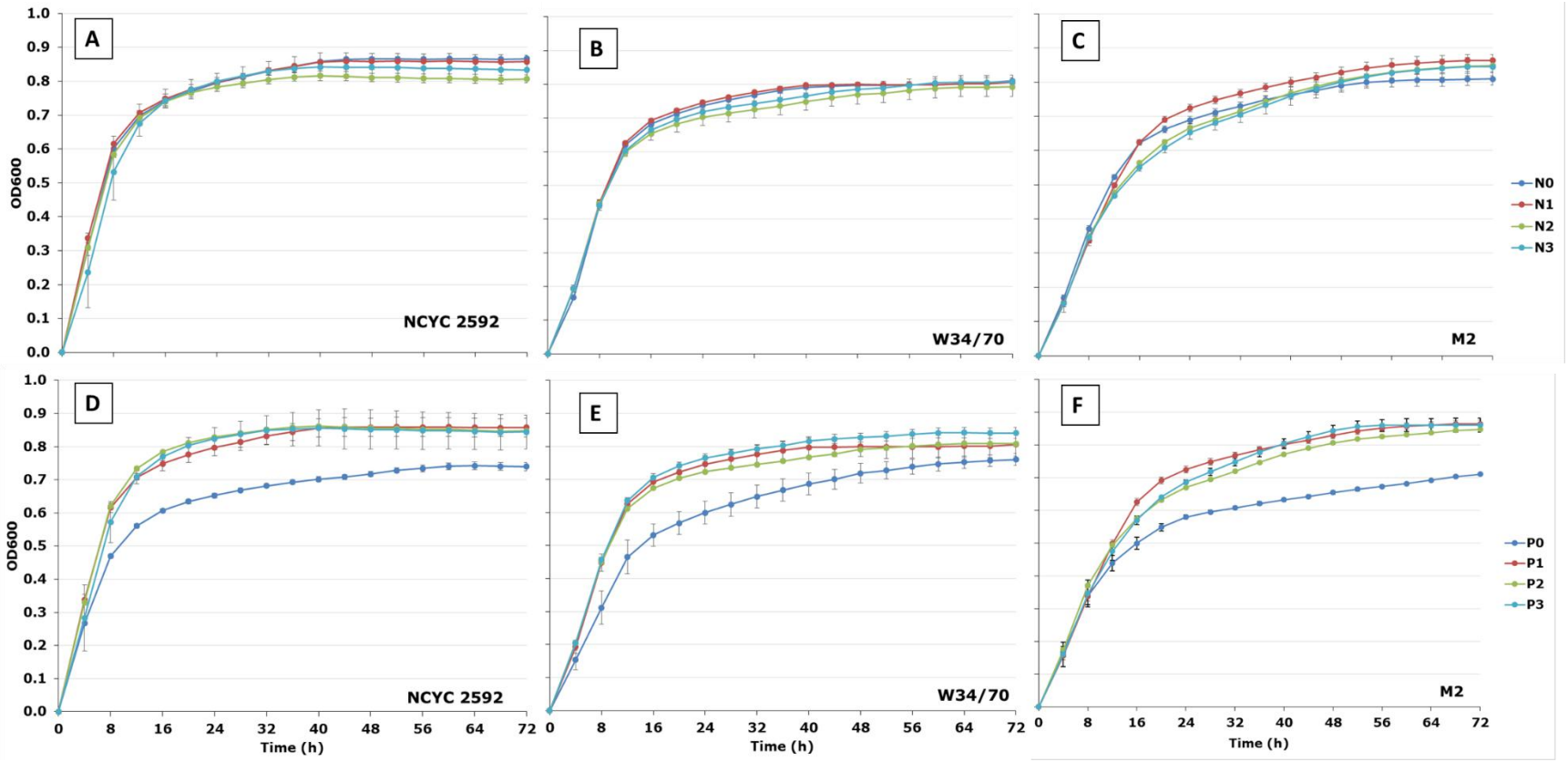

Fig. 6.2: Growth curves of *Saccharomyces* yeast strains NCYC2592, W34/70 and M2. Variables are ammonium-nitrogen (A, B and C) and inorganic phosphate (D, E and F). Ammonium-nitrogen is graphically represented by N0 (ammonium-nitrogen absence), N1 (75 mg/L), N2 (150 mg/L) and N3 (300 mg/L) and inorganic-phosphate is graphically represented by P0 (inorganic-phosphate absence), P1 (126 mg/L), P2 (253 mg/L) and P3 (506 mg/L).All results are the average of three replicate experiments with standard deviations indicated by error bars.

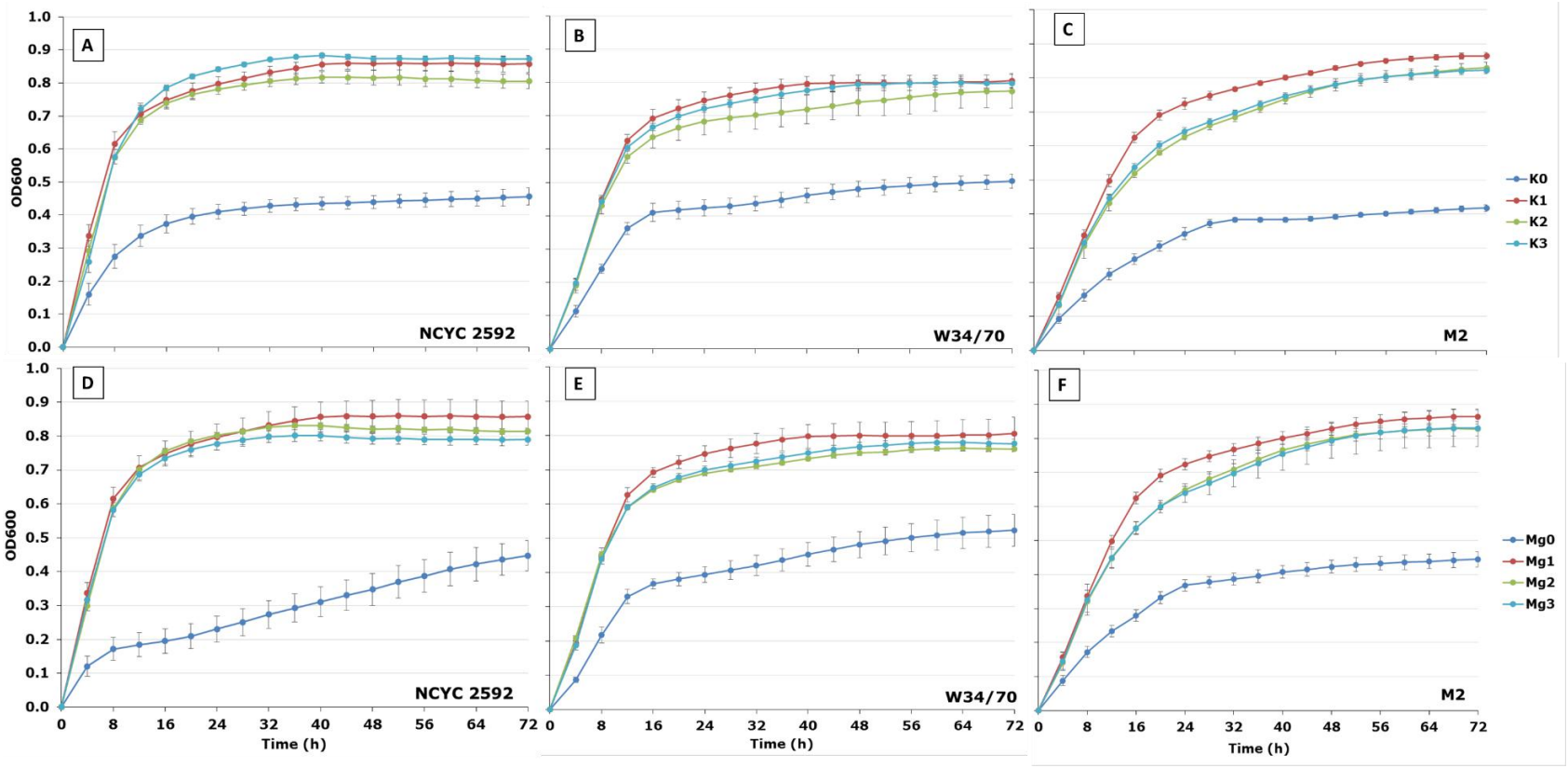

Fig 6.3: Growth curve of *Saccharomyces* yeast strains NCYC2592, W34/70 and M2, respectively. Variables are potassium (A, B and C) and magnesium (D, E and F). Potassium is graphically represented by K0 (potassium absence), K1 (262.25 mg/L), K2 (524.50 mg/L) and K3 (1049mg/L) and magnesium is graphically represented by Mg0 (magnesium absence), Mg1 (38 mg/L), Mg2 (76 mg/L) and Mg3 (152mg/L). All results are the average of three replicates experiments with standard deviations indicated by error bars.

As expected, the absence of potassium, (treatment K0 Fig. 6.3A, Fig. 6.3B and Fig. 6.3C) significantly reduced the growth of all the brewing yeast strains utilized. All other treatments, including 262.25 mg/L (K1), 524.50 mg/L (K2) and 1049.00 mg/L (K3), did not affect the yeast growth. Treatment K1 (262.25 mg/L) contained lower potassium concentration than the control (524.50 mg/L) but did not affect the growth. The treatment K3 (1049.00 mg/L), which represents the double amount of potassium added into the wort, did not affect the growth. The individual absence of either inorganic-phosphate or potassium did reduce yeast growth.

Magnesium is the most necessary essential inorganic elements because is found lower amounts in yeast cells than phosphorus and potassium and it is a co-factor of many enzymes in the glycolysis pathway. Environmental magnesium abundance is essential for cell growth and proliferation because it serves as co-factor of several cellular enzymes (Cyert and Philpott 2013, Wolf and Trapani 2008). Evaluating the influence of magnesium in yeast growth, the quantity of magnesium utilised were 0, 38, 76 and 152mg/L, which were graphically repented by Mg0, Mg1, Mg2 and Mg3, respectively (Fig. 6.3 D, E and F). The value 76mg/L (treatment Mg2) represented the concentration of magnesium present in the beer wort (chapter 2, section 2.1.5.). The treatment Mg0 (Fig. 6.3) significantly affected growth for all the brewing yeast studied. All other treatments, including 38.00 mg/L (Mg1), 76.00 mg/L (Mg2) and 152.00 mg/L (Mg3), had no significant differences. Therefore, an absence of magnesium (Mg0) in the wort can negatively effect the yeast growth.

The individual absence of inorganic-phosphate, potassium and magnesium reduced growth in all brewing yeast used in this study (Fig. 6.2 and Fig. 6.3).

However, all of the other treatments demonstrated no significant effect (Fig. 6.2 and Fig. 6.3). Therefore, inorganic elements, including inorganic-phosphate, potassium and magnesium are vital for yeast growth and may influence on yeast-flavour formation.

Fig. 6.4 and Fig. 6.5 illustrate growth curve of *Saccharomyces* yeast strains NCYC2592, W34 and M2, considering variations in concentration of heavy metal ions such as copper (Cu), iron (Fe), zinc (Zn) and manganese (Mn). These ions are important for yeast metabolic activities (De Freitas *et al.* 2003); however, all treatment from absence to supplementation did not affect the yeast growth (Fig. 6.4 and Fig. 6.5) .

The synthetic sweet wort developed in this study showed a greater similarity with a beer sweet wort and provides the importance of inorganic elements for yeast growth because SW presented a better growth than a CN medium (a solution of carbohydrates and amino acid). It was demonstrated that essential inorganic elements (phosphorus, potassium and magnesium) are vital for yeast growth and also have their responsibility on pH of wort. Therefore, synthetic wort developed is a great tool for yeast growth studies.

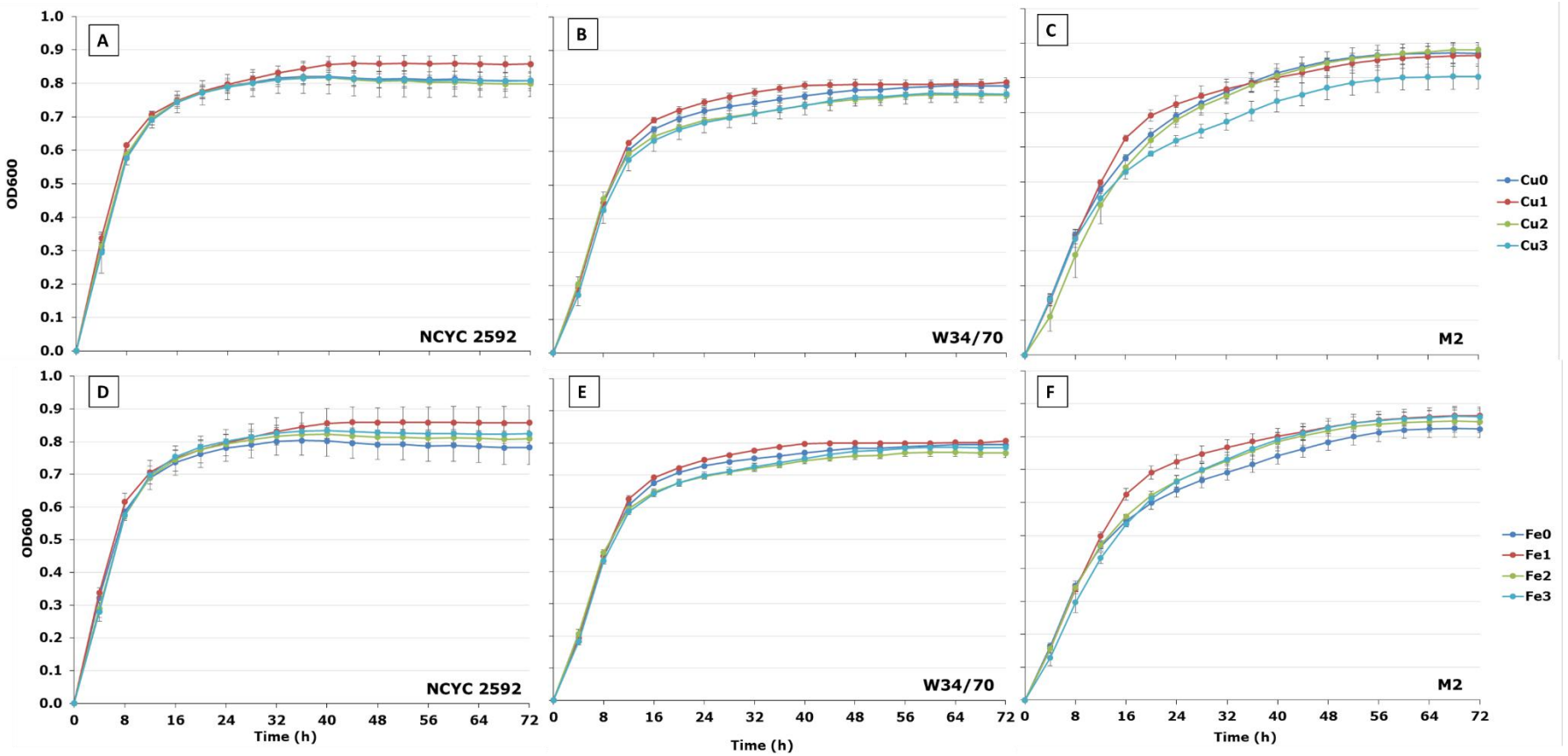

Fig. 6.4: Growth curve of *Saccharomyces* yeast strains NCYC2592, W34 and M2. Variables are copper, iron, zinc and manganese. Copper is graphically represented by Cu0 (copper absence), Cu1 (0.05 mg/L), Cu2 (0.10 mg/L) and Cu3 (0.15 mg/L); and iron is graphically represented by Fe0 (iron absence), Fe1 (0.04 mg/L), Fe2 (0.08 mg/L) and Fe3 (0.12 mg/L). All results are the average of three replicates experiments with standard deviations indicated by error bars.

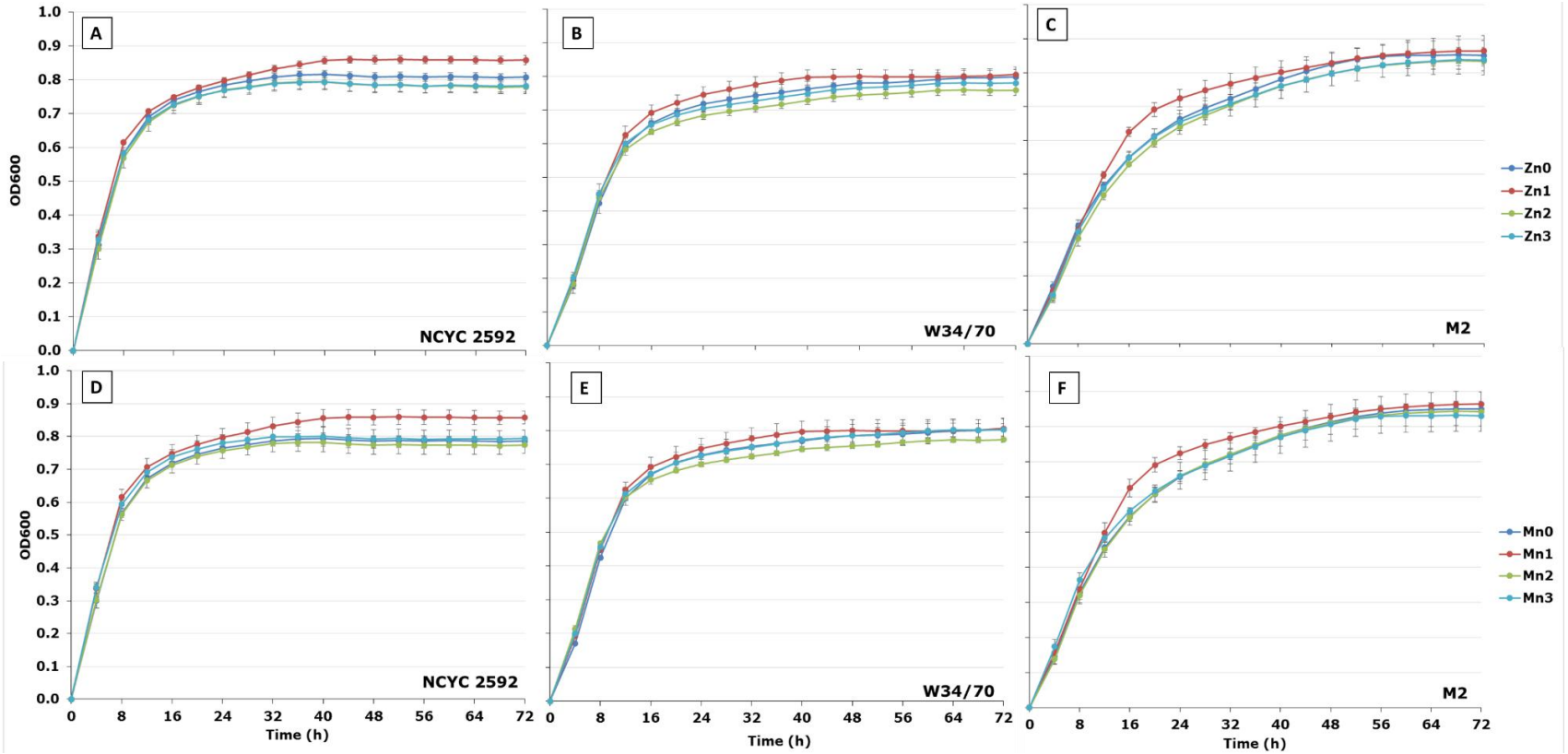

Fig. 6.5: Growth curve of *Saccharomyces* yeast strains NCYC2592, W34 and M2. Variables are zinc and manganese. Zinc is graphically represented by Zn0 (zinc absence), Zn1 (0.15 mg/L), Zn2 (0.30 mg/L) and Zn3 (0.45 mg/L); and Manganese is graphically represented by Mn0 (manganese absence), Mn1 (0.06 mg/L), Mn2 (0.12 mg/L) and Mn3 (0.18 mg/L). All results are the average of three replicates experiments with standard deviations indicated by error bars.

6.3.4. CO_2 loss accumulation during fermentation

Fig. 6.6 illustrates the cumulative average of CO_2 loss (g) during the fermentation of a beer sweet wort and a defined synthetic wort using NCYC2592, W34/70 and M2 strains. All strains produced a similar amount of CO_2 and they produced a maximum amount of CO_2 of (5g/100mL), as expected. NCYC2592 strain produced a similar cumulative pattern of CO_2 loss throughout fermentation when inoculated in BSW and SW (Fig. 6.6 A). W34/70 strain presented a different cumulative pattern of CO_2 loss during the first eight hours of fermentation, but the final amount of CO_2 produced was similar (Fig. 6.6 B). M2 strain showed a different cumulative pattern of CO_2 loss between 20 and 44-fermentation hours (Fig. 6.6 C). In conclusion, all strains consumed similar quantity of carbohydrates from both worts.

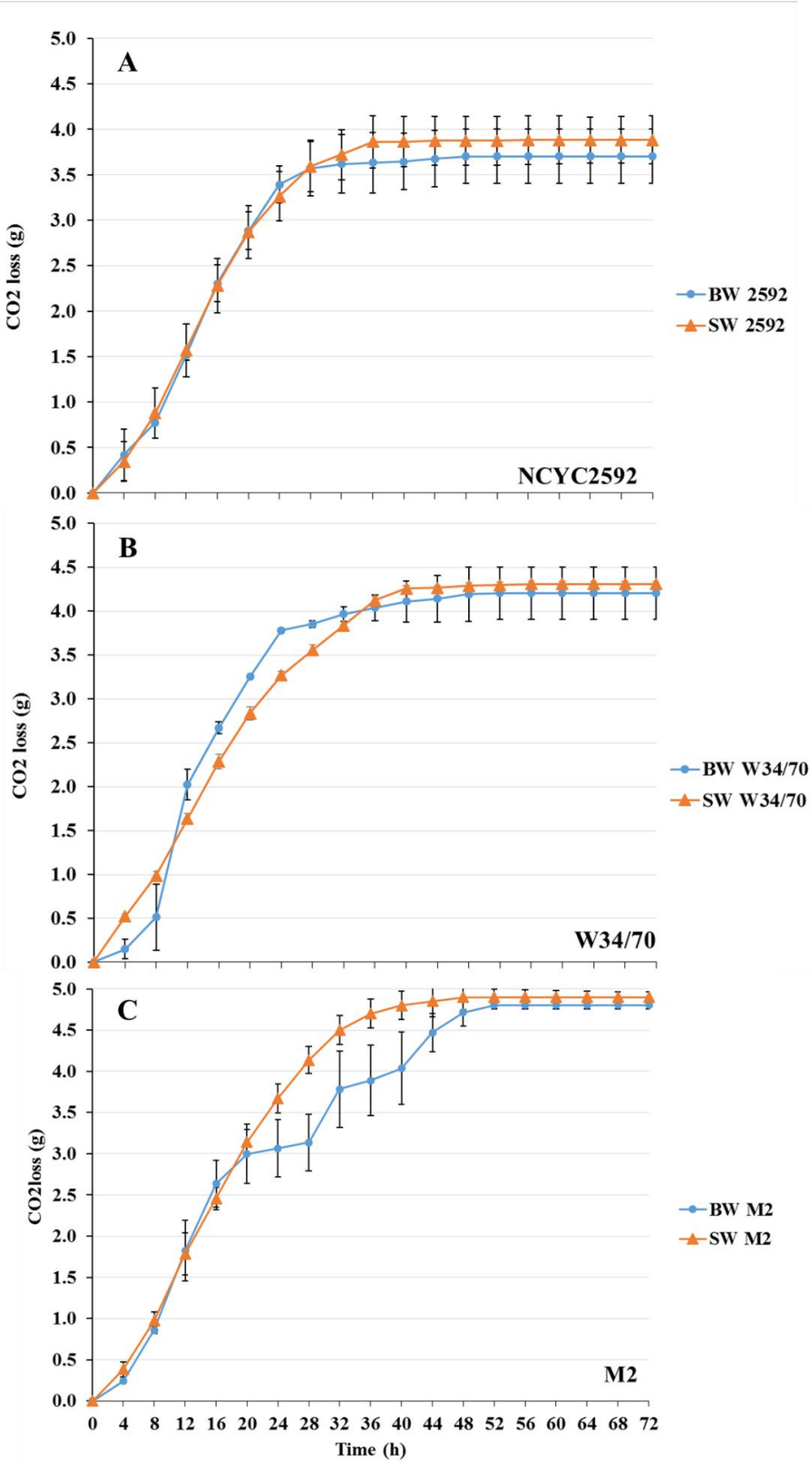

Fig. 6.6: Fermentation comparison of a beer sweet wort ( ) and a synthetic wort ( ) by cumulative average of CO_2 loss (g) using yeasts *Saccharomyces* strains A) NCYC2592, B) W34/70 and C) M2. Each value evaluated on 3 replicates and error bar shown by standard deviation.

6.3.5. Flavour compounds in a final beer sweet wort and a synthetic sweet wort

Table 6.2 shows the concentrations of flavour compounds in a BSW (beer sweet wort) and a SW (synthetic wort) using NCYC2592, W34/70 and M2. BSW and SW produced all compounds measured. Ethanol and glycerol concentrations did differ significantly in both products. The quantity of acetic acid measured in FB and FSB were in the expected range lower than the typical threshold (1 g/L) and also in the range found in beer between 0-2.8 g/L (ASBC Methods of Analysis: Beer Flavour Database, 2011).

BSW fermentation generates more active-flavours compounds than in SW. For example, NCYC2592 produced more 2-Phenethyl acetate in BSW (1.8 mg/L) than in a SW (0.99 mg/L). Also isoamyl acetate, which was measured in BSW (3.0 mg/L) and a SW (1.0 mg/L). In contrast, NCYC2592 produced more 2-Phenethyl alcohol in SW (292.3 mg/L) than in a BSW (119.6 mg/L). Also isoamyl alcohol, which was measured in SW (74.2 mg/L) and a BSW (41.1 mg/L). Furthermore, a similar behaviour were observed when used W34/70 and M2. Active flavour such as fatty acid esters were obeserved in higher quantity in SW than in a BSW (For example, ethyl hexanoate and ethyl decanoate). W34/70 and M2 produced double amount of ethyl hexanoate in a SW (1.8 mg/L and 1.8 mg/L, respectively) comparing to BSW (0.8 mg/L and 0.9 mg/L).

BSW produced more off-flavours (fatty acids) than SW. Fatty acids were produced in higher concentrations in a BSW than SW. NCYC2592 produced higher concentration of decanoic acid in a BSW (5.6 mg/L) than in a SW (0.3

mg/L). W34/70 produced higher concentration of isovaleic acid in a BSW (3.9 mg/L) than in a SW (0.6 mg/L). M2 produced higher concentration of isobutyric acid in a BSW (5.2 mg/L) than in a SW (2.4 mg/L). Data from Table 6.2 was simplified in a PCA Bi-Plot (Fig. 6.6) to illustrate the overall change of all treatments and their similarities .

Table 6.2: Concentrations of flavour compounds in a final beer (BW) and a final synthetic beer (SW) derived from different fermentations using NCYC2592, W34/70 and M2, assessed using GC-MS analysis. Data value represent the mean of triplicate samples ± standard deviation.

Compounds	NCYC2592		W34/70		M2	
	SW	BW	SW	BW	SW	BW
Glycerol (g/L)	1.27 ± 0.02	1.07 ± 0.02	1.28 ± 0.02	1.09 ± 0.01	1.31 ± 0.05	1.10 ± 0.01
Acetic acid (g/L)	0.35 ± 0.03	0.44 ± 0.01	0.36 ± 0.02	0.41 ± 0.00	0.21 ± 0.00	0.62 ± 0.00
Ethanol (g/L)	53.27 ± 1.00	47.27 ± 1.04	52.80 ± 0.58	46.80 ± 0.42	56.74 ± 1.27	50.03 ± 1.36
Esters (mg/L)						
Ethyl_acetate	33.26 ± 8.42	6.61 ± 0.76	30.20 ± 1.14	27.81 ± 5.22	27.51 ± 3.40	31.93 ± 2.80
Isobutyl_acetate	14.09 ± 2.41	4.19 ± 0.14	13.11 ± 1.52	17.52 ± 0.92	11.56 ± 2.04	21.79 ± 1.58
Isoamyl_acetate	1.02 ± 0.51	3.04 ± 0.10	1.27 ± 0.18	8.72 ± 0.14	0.73 ± 0.17	9.22 ± 0.32
2-Phenethyl_acetate	0.99 ± 0.05	1.84 ± 0.17	0.93 ± 0.07	2.18 ± 0.24	1.08 ± 0.03	1.44 ± 0.55
Higher alcohols (mg/L)						
1-Propanol	0.35 ± 0.27	0.22 ± 0.01	0.38 ± 0.24	0.19 ± 0.05	0.44 ± 0.08	0.23 ± 0.06
Isobutanol	9.54 ± 7.35	0.10 ± 0.01	9.34 ± 8.00	0.15 ± 0.01	12.12 ± 1.97	0.24 ± 0.03
2-methyl butanol	53.38 ± 20.98	41.61 ± 1.89	40.37 ± 14.05	52.07 ± 5.36	49.40 ± 33.46	94.80 ± 7.29
Isoamyl_alcohol	74.15 ± 4.52	41.14 ± 1.18	66.85 ± 0.72	63.95 ± 0.25	75.32 ± 0.81	88.22 ± 0.25
2-Phenethyl alcohol	292.27 ± 20.76	119.56 ± 5.69	261.57 ± 6.33	208.40 ± 2.87	258.42 ± 3.39	244.26 ± 5.33
Fatty acids (mg/L)						
Isobutyric_acid	1.50 ± 0.04	3.73 ± 0.06	1.31 ± 0.02	4.46 ± 0.06	2.39 ± 0.11	5.32 ± 0.06
Butanoic_acid	0.35 ± 0.05	0.37 ± 0.00	0.29 ± 0.01	0.54 ± 0.01	0.33 ± 0.01	0.42 ± 0.01
Isovaleric_acid	0.67 ± 0.02	2.70 ± 0.12	0.62 ± 0.01	3.90 ± 0.06	1.47 ± 0.06	3.42 ± 0.07
Hexanoic_acid	0.12 ± 0.05	1.38 ± 0.27	0.14 ± 0.04	0.59 ± 0.07	0.15 ± 0.05	0.02 ± 0.01
Octanoic_acid	0.80 ± 0.32	4.79 ± 0.80	1.10 ± 0.12	2.78 ± 0.31	0.92 ± 0.09	0.02 ± 0.01
Decanoic_acid	0.29 ± 0.04	5.60 ± 1.93	0.55 ± 0.06	0.48 ± 0.03	0.33 ± 0.03	0.17 ± 0.01
Fatty acids ester (µg/L)						
Ethyl_butanoate	10.46 ± 4.12	11.43 ± 0.64	0.47 ± 0.22	1.96 ± 0.73	7.04 ± 0.14	3.33 ± 0.16
Ethyl_hexanoate	1.53 ± 0.24	0.59 ± 0.15	1.78 ± 0.82	0.80 ± 0.19	1.80 ± 0.46	0.99 ± 0.08
Ethyl_Butanol	3.18 ± 0.34	1.03 ± 0.52	1.72 ± 0.19	1.19 ± 0.34	1.81 ± 0.81	1.48 ± 0.35
Ethyl_octanoate	1.12 ± 0.64	0.78 ± 0.08	1.10 ± 0.38	0.87 ± 0.10	1.32 ± 0.40	0.70 ± 0.26
Ethyl_Hexanol	0.79 ± 0.73	0.48 ± 0.34	1.46 ± 0.28	0.68 ± 0.04	1.07 ± 0.63	1.30 ± 0.08
Ethyl_decanoate	31.70 ± 19.39	10.30 ± 0.31	44.50 ± 14.11	11.59 ± 7.24	45.35 ± 29.97	14.30 ± 4.93
VDK (mg/L)						
Acetoin	0.97 ± 0.04	0.26 ± 0.01	0.87 ± 0.05	0.12 ± 0.01	0.73 ± 0.02	0.19 ± 0.01
2,3-Butanediol	1.86 ± 0.23	0.76 ± 0.10	1.37 ± 0.07	0.29 ± 0.04	1.24 ± 0.15	0.41 ± 0.07

6.3.6. Overall of all samples

Fig. 6.7 illustrates a principal component analysis (PCA), which shows that all yeasts used in this study exhibited different flavour compound production. The blue dotes represent six different fermentation, which are B1 (NCYC2592 strain inoculated in BW), B2 (W34/70 strain inoculated in BW), B3 (M2 strain inoculated in BW), S1 (NCYC2592 strain inoculated in SW), S2 (W34/70 strain inoculated in SW), S3 (M2 strain inoculated in SW). The first two components (F1 and F2) explain 84.70% of the separation. Fermentations conducted in a SW all yeast strains showed similar flavour production. All strains produced all the flavour compounds illustrated in the Fig. 6.7 and quantified in table 6.2.

First component (F1) separates the BW from the SW. The second component (F2) separates NCYC2592 inoculated in a BW and NCYC2592, W34/70 and M2 inoculated in a SW from W34/70 and M2 inoculated in a BW. Therefore, when NCYC2592 was used in fermentation using a BW or SW, this strain showed lower difference on its flavour formation than other two strains. Furthermore, carbohydrates (maltotetraose, dextrin and maltotriose) and and peptides (polypeptide/small peptides) were not a part of SW composition and when inoculated in this wort yeasts produced more active-flavour such as esters and higher alcohols (Fig. 6.7). The type of carbohydrates and peptides may influence on the formation of yeast active-flavours.

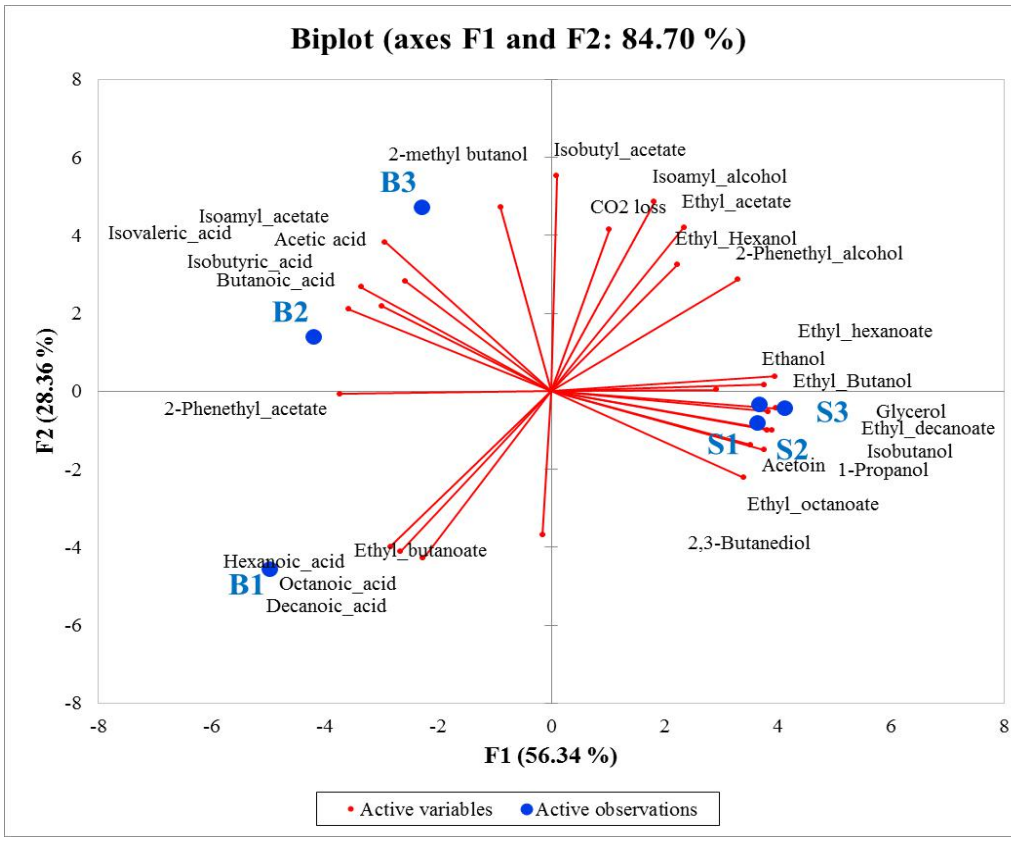

Fig. 6.7: Principle component analysis (Bio-Plot) to identify the correlation and similarities among all 27 variables including ethanol, glycerol, acetic acid, higher alcohols, fatty acid, acetate esters and fatty acid ethyl esters. Fermentation were conducted in a beer sweet wort (BW) and a synthetic sweet wort (SW) using three-yeast strains (NCYC2592, W34/70 and M2). The blue dotes represent six different fermentation, which are B1 (NCYC2592 strain inoculated in BW), B2 (W34/70 strain inoculated in BW), B3 (M2 strain inoculated in BW), S1 (NCYC2592 strain inoculated in SW), S2 (W34/70 strain inoculated in SW), S3 (M2 strain inoculated in SW).

6.4. Discussion

Hydrogen-ionic potential (pH) is an important factor on fermentation control (Li *et al.* 2016, Li *et al.* 2015). Wort is a very complex matrix and constituent such as amino acids and peptides are responsible for wort buffering potential (Peyer, *et al.* 2017, Li *et al.* 2016, Li *et al.* 2015, Bamforth 2001). Although the wort buffering capacity involves small peptides, amino acids, the this work brings the influence of individual compounds such as inorganic elements,

amino acids, carbohydrates and vitamins in wort on pH and as a consequence contributes for the comprehension of wort characteristics. Furthermore, a medium, which possesses only carbohydrates and amino acids, and lacks essential inorganic elements, vitamins and lipids, cannot support yeast growth because are important for guarantee wort characteristics and as a consequence yeast metabolic activities.

When observed the influence of inorganic element influence such as ammonia-nitrogen, inorganic-phosphate, potassium, magnesium, zinc copper, iron and manganese from absence to supplementation levels, only inorganic phosphate, potassium and magnesium have influence on yeast growth. Inorganic-phosphate consumption and transportation are related to potassium and the lack of potassium has a greater effect on yeast growth than the absence of Pi because the lack of potassium inhibits phosphate intake (Canadell *et al.* 2014, Barreto *et al.* 2012). Therefore, the absence of inorganic-phosphate in the medium significantly decreases yeast growth because is regulated by potassium presence and consequently ADP and ATP formation (Barreto *et al.* 2012 and Sala 2015). Furthermore, potassium is important for yeast internal pH control, which is essential for cytosolic activities. In cells, potassium plays several important metabolic roles being required for activation of metabolic process, including protein translation and pyruvate synthesis (Cyert and Philpott 2013, Page and Di Cera 2006). Potassium is important for balancing charge across the plasma membrane, and contributes to intercellular pH control (Cyert and Philpott 2013).

Magnesium is a divalent cation responsible for most of glycolytic reaction activation and can also substitutes other cations activities due to its

electronegativity (Udeh and Kgatla 2013, Cyert and Philpott 2013, Birch and Walker 2000). Magnesium is the most necessary essential inorganic elements because is essential for cell growth and proliferation and serves as co-factor of several cellular enzymes (Udeh *et al.* 2014, Cyert and Philpott 2013, Wolf and Trapani 2008). As expected the absence of magnesium, observed via treatment Mg0 (Fig. 6.3) significantly affected growth for all the brewing yeast utilized, which is similar to phosphorus and potassium absence treatments. These occurred because phosphorus, potassium and magnesium are involved in several important metabolic activities and also they help each other during transportation from wort into cell (Lim *et al.* 2011, Pisat *et al.* 2009, White *et al.* 2003, Maguire and Cowan 2002, Walker *et al.* 2000). These inorganic elements are important for yeast growth and fermentation, therefore, they can influences on yeasts-flavour formation by supplementation and may influence the yeast-flavour formation without altering the yeast growth.

All copper, iron, zinc and manganese concentrations added in the wort did not affect the yeast growth, which includes their absence. These metal ions are toxic for yeast cells when wort has high concentration of them (De Freitas *et al.* 2003, Sturtz *et al.* 2001); however, results showed that all concentration of copper, iron, zinc and manganese tested in this were not toxic for the yeast and as a consequence did not affect the growth (Fig. 6.4 and Fig. 6.5). Therefore, all heavy metals are important for yeast metabolic activities (De Freitas *et al.* 2003) and have a little contribution on pH of wort; however, among the inorganic elements, only inorganic-phosphate, potassium and magnesium are vital for yeast growth and have a bigger influence on wort pH.

As expected, all strains produced a similar amount of CO_2 (5g/100mL) and suggests a similar carbohydrate assimilation (Priest and Stewart 2006, Boulton and Quain 2001, Stassi *et al.* 1987). As a consequence, ethanol and glycerol formation were similar, however, active-flavour formation were significantly different. The fact of beer sweet wort contains maltotetraose/dextrin and maltotriose; and synthetic wort was prepared without these carbohydrates in its composition, suggest that carbohydrate can influence on flavour formation because when they are broken down to glucose each carbohydrate requires a different energetic requires although maltotriose uptakes occurs using maltose transporters (Magalhães *et al.* 2016). Synthetic wort produced more active flavours (esters) than beer sweet wort. It may occurs because BSW contains maltotriose, which can be consumed by brewing yeast and SW was not prepared with this carbohydrate in its composition. Additionally, This work presents for the first time a more complete comparison between a beer wort and a synthetic wort.

6.5. Conclusions

The synthetic sweet wort is defined and imitates typical brewer's wort in terms of carbohydrates, amino acids, essential inorganic elements (micro inorganic nutrients and metal ions), vitamins and lipids concentrations. Amino acids and vitamins are the buffer compounds and essential inorganic elements increase the free hydrogen in wort. Yeast growth requires a minimal quantity of essential inorganic elements as a guarantee of growth. Phosphorus, potassium and magnesium are vital for yeast growth. During fermentations, inorganic

elements influence yeast growth and the flavour formation. Synthetic wort can support fermentation studies of the yeast growth, carbohydrate consumption, essential inorganic elements assimilation, and flavour formation (including ethanol, glycerol, acetic acid, higher alcohols, short chain fatty acids, esters and fatty acids esters). SW is an important/useful tool for fermentation studies.

CHAPTER VII

EVALUATION OF WORT MINERAL COMPOSITION FOR YEAST-FLAVOUR PRODUCTION DURING ALCOHOLIC FERMENTATION USING A RESPONSE SURFACE METHOD

7. CHAPTER VII: EVALUATION OF WORT MINERAL COMPOSITION ON YEAST-FLAVOUR FORMATION DURING ALCOHOLIC FERMENTATION

7.1. Introduction

Essential inorganic elements such as inorganic-phosphate, potassium and magnesium were identified as important co-factors, which can affect positively active-flavour formation during yeast alcoholic fermentation (Chapter 5). Several studies have evaluated the influence of some inorganic elements such as ammonia-nitrogen, inorganic-phosphorus, potassium, magnesium and heavy metals for fermentation performance and ethanol production (Wietstock *et al.* 2015, Silva *et al.* 2008, Silva *et al.* 2006, De Freitas *et al.* 2003, Blackwell *et al.* 1998, Szczypka *et al.* 1997, Crapo *et al.* 1992, Turrens and Boveris 1980). However, the role of inorganic-phosphate, potassium and magnesium on yeast-flavour formation has not been published. To describe process phenomena and their interactions, multivariate method such as 1) Response Surface Methodology (RSM) for experimental design and 2) Principal Component Analysis (PCA) have been successfully used for describing bioprocess (Deepak *et al.* 2008, Myers & Montgomery 2002, Anjum e al. 1997). A response surface method (RSM) is applied in order to evaluate the interactive effects among the variables and, consequently, executes a more complete multivariable combinations with a simultaneously variation of all studied factors. RSM has been successfully applied to model and optimize biochemical and biotechnological processes related to food systems (Myers & Montgomery 2002, Anjum e al. 1997).

This chapter generates a new knowledge of the role of inorganic-phosphate, potassium, magnesium as independent variables (input) on flavour formation as dependent variables (output) during alcoholic fermentation using RSM, which considers significance, prediction, optimization and mathematical model for fermentation control. As a result, the optimization of experiment explains the interactions and role of inorganic phosphate, potassium, magnesium on yeast-flavour formation.

7.2. Material/Method/Experimental design

Response surface method aims to find a mathematical model, which can predict the process in study, evaluating the influence of input variables (inorganic-phosphate, potassium and magnesium) on output variables (ethanol, yeast growth and aromas). For this purpose, a synthetic wort was used (Chapter 2, section 2.1.5), which is more controllable medium; and yeast (NCYC2592), which shows a more similar behaviour when inoculated in beer sweet wort and in a synthetic wort (includes yeast growth, CO_2 weight loss and flavour formation). A response surface method (Chapter 2, Section 2.4.1) and analysis of model (Chapter2, section 2.7.3) were applied. Upon of completing fermentations were measured in triplicate by destructive sampling for the high performance liquid chromatography (HPLC) (Chapter 2, Sections 2.5.1.) and gas chromatography/mass spectrum (GCMS) analyses (Chapter 2, Section 2.5.2).

7.3. Results

7.3.1. Evaluation of the effect and model verification

All response variables (output) were evaluated by analysis of variance from D-Optimal design and observed that all responses fitted in a quadratic model. Therefore, models for ethanol, yeast growth and aroma groups were all significant, when observed p-values lower than 0.05 (Table 1 to Table 6). All insignificant terms were excluded from the mathematical models to improve the model prediction. A lack of fit, which showed p-values greater than 0.05, were no significant and consequently indicated a good fit to the model. On the other hand, models that were significant and possessed a significant lack of fits were added the standard deviation values to the equation model. In conclusion, after exclusion of all insignificant terms from each individual model, equations are summarized in the table 7.7.

Table 7.1: Analysis of variance for the response surface quadratic model for Total of yeast cell

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	$2.839E^{+15}$	4	3.154E+14	6.08	0.00
B-Potassium	$8.288E^{+14}$	1	8.288E+14	15.98	0.00
C-Magnesium	$8.854E^{+14}$	1	8.854E+14	17.07	0.00
B^2	$2.447E^{+14}$	1	2.447E+14	4.72	0.04
C^2	$4.139E^{+14}$	1	4.139E+14	7.98	0.01
Residual	$9.336E^{+14}$	14	5.187E+13		
Lack of Fit	$9.232E^{+14}$	12	5.770E+13	11.09	0.08
Pure Error	$1.040E^{+13}$	2	5.202E+12		
Sum Total	$3.772E^{+15}$	18			

Table 7.2: Analysis of variance for the response surface quadratic model for ethanol production.

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	2674.30	2	1337.15	11.92	0.00
C-Magnesium	1618.54	1	1618.54	14.43	0.00
C^2	1382.53	1	1382.53	12.33	0.00
Residual	2804.24	25	112.17		
Lack of Fit	2801.90	23	121.82	104.16	0.01
Pure Error	2.34	2	1.17		
Sum Total	5478.55	27			

Table 7.3: Analysis of variance for the response surface quadratic model for acetate ester formation

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	20.91	4	5.23	8.82	0.00
A-Phosphorus	3.97	1	3.97	6.71	0.02
B-Potassium	6.46	1	6.46	10.90	0.00
C-Magnesium	3.66	1	3.66	6.18	0.02
B^2	6.48	1	6.48	10.94	0.00
Residual	13.63	23	0.5927		
Lack of Fit	13.51	21	0.6433	10.55	0.09
Pure Error	0.1219	2	0.0610		
Total model	34.54	27			

Table 7.4: Analysis of variance for the response surface quadratic model for higher alcohol production

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	$1.817E^{+05}$	5	36344.96	10.63	0.00
A-Phosphorus	69257.32	1	69257.32	20.26	0.00
B-Potassium	31104.50	1	31104.50	9.10	0.01
C-Magnesium	24934.60	1	24934.60	7.29	0.01
B^2	19170.11	1	19170.11	5.61	0.03
C^2	22191.61	1	22191.61	6.49	0.02
Residual	75201.81	22	3418.26		
Lack of Fit	74988.97	20	3749.45	35.23	0.03
Pure Error	212.83	2	106.42		
Sum Total	$2.569E^{+05}$	27			

Table 7.5: Analysis of variance for the response surface quadratic model for fatty acid ester formation

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	34.84	2	17.42	6.89	0.00
C-Magnesium	11.34	1	11.34	4.48	0.04
A^2	24.53	1	24.53	9.70	0.01
Residual	63.24	25	2.53		
Lack of Fit	61.18	23	2.66	2.58	0.32
Pure Error	2.06	2	1.03		
Sum Total	98.08	27			

Table 7.6: Analysis of variance for the response surface quadratic model for fatty acid formation

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	20.78	2	10.39	10.23	0.00
C-Magnesium	5.06	1	5.06	4.98	0.03
A^2	15.14	1	15.14	14.91	0.00
Residual	25.39	25	1.02		
Lack of Fit	23.56	23	1.02	1.12	0.58
Pure Error	1.83	2	0.91		
Sum Total	46.17	27			

7.3.2. Equations definition

All equations response variables are summarized in table 7.1, which includes their respective statistical coefficients. All models were generated from non-linear regressions (quadratic models) using Design Expert software. The analysis of variance results shows that all models were significant although the models for ethanol and higher alcohols presented a significant lack of fit, which affects the prediction power of the model. However, all models developed can be used for making industrial decisions, mainly when considered the active-flavour production (esters) and reduction of off-flavour

production (fatty acids). Therefore, all models are summarised in table 7.7, which results in six simple equations (7.1-7.6).

Table 7.7: Mathematical model defined for higher alcohols, acetate esters, fatty acids, fatty acid esters, total yeast cell and ethanol.

N°	Equation*	p-value
(7.1)	Higher alcohols=+12.24 + 0.25 (P) +0.38 (K) +0.42 (Mg) $-2.94x10^{-4}$ $(K)^2$	0.00
(7.2)	Acetate Ester = -0.31 $+1.9x10^{-3}$ (P) $+5.6x10^{-3}$ (K) $+5.7x10^{-2}$ (Mg) - $4.4x10^{-6}$ (K^2)	0.00
(7.3)	Fatty acids= +6.33 -0.02 (Mg) $-2.40x10^{-5}$ $(P)^2$+0.98	0.02
(7.4)	Fatty acids esters =+1.50+0.04(Mg) $-3x10^{-5}$ $(P)^2$	0.04
(7.5)	Total yeast cell = $+2.55x10^{+07}$+$4.30x10^{+4}$(K)+$3.29x10^{+05}$(Mg)-29.18(K^2) $-1.5x10^{+3}$(Mg^2)	0.00
(7.6)	Ethanol =+27.93 +0.48 (Mg) $-2.59x10^{-3}$(Mg^2)	0.03

P – Inorganic phosphate (mg/L), K- potassium (mg/L), and Mg - magnesium (mg/L)

Table 7.7 shows all mathematical model defined by D-Optimal design for higher alcohols, acetate esters, fatty acids, fatty acid esters, the yeast growth (number of yeast cell/mL) and ethanol. Equation 7.1 represents higher alcohol formation, which possesses linear effect of inorganic-phosphate, potassium and magnesium; and quadratic term of potassium and magnesium. Equation 7.2 represents acetate ester formation, which possesses linear effect of inorganic-phosphate, potassium and magnesium; and quadratic effect of potassium. This equation has r-square 0.7539, a p-value lower than 0.05 and consequently is a significant model. Equations 7.3 and 7.4, which, respectively, represent fatty acids and fatty acid esters, possess linear effect of magnesium; however, their signal are opposite and respectively fatty acids (-0.02Mg) and fatty acid esters (+0.04Mg). Therefore, fatty acid and fatty acid esters groups are magnesium dependent because fatty acids decreases on magnesium presence. In contrast, magnesium was responsible for increasing the formation of fatty acid esters. Equation 7.5 represents the yeast growth (number of yeast cell/mL), which

possesses linear and quadratic effect of potassium and magnesium. Equation 6 represents ethanol production, which possesses linear and quadratic effect of magnesium and suggests ethanol formation is magnesium dependent. All equations were applied for generating the contour plot of a response surface (acetate esters, higher alcohols, fatty acid esters, fatty acids and yeast growth) and one factor plot (ethanol). Additionally, linear terms (main effects) are responsible for mostly of significant influences in the equations; on the other hand, quadratic terms are responsible for model curvature, which represents the surface-curvature effects (Table 7.7).

7.4. Evaluation of the design variables in flavour formation

7.4.1. Evaluation of the design variables in acetate ester formation

Acetate esters are the most important active flavours, which are found above threshold values. Acetate ester synthesis involves alcohol acetyl transferases including AATase I, AATase II and Lg-AATase I, which are encoded by the genes ATF1, ATF2 and Lg-ATF1, respectively (Saerens *et al.* 2010). In yeast cell, reaction involving higher alcohols (amino acid consumption) and acetyl-CoA (carbohydrates consumption) form acetate esters. Fig. 7.1 illustrates contour plot for acetate esters (A) and higher alcohols (B). Both plots show inorganic phosphate versus potassium to an average of magnesium content at 150 mg/L. Inorganic-phosphate and magnesium impacted significantly on acetate ester formation; however, mostly inorganic-phosphate and magnesium impact were better observed in supplementation level of inorganic-phosphate (250- 500 mg/L), magnesium (80-160 mg/L). For these changes be observed,

potassium should be present in the wort minimally from 350 mg/L. To obtain higher concentration of acetate esters, inorganic-phosphate, potassium and magnesium should be at 490, 600 and 150 mg/L (Fig. 1A), respectively. As a result of optimal conditions, the total content of acetate esters increased from 2.3 mg/L to 3.2 mg/L, which represents 40% of increasing.

7.4.2. Evaluation of the design variables in higher alcohol formation

As important acetate ester precursor, higher alcohol formation occurs via Ehrlich pathway (Hazelwood *et al.* 2008, Hazelwood *et al.* 2006). The consumption of amino acids for higher alcohols formation is known; however, results shows essential inorganic elements such as inorganic phosphate, potassium and magnesium are involved in higher alcohol formation (Fig. 7.1B) and as a consequence, amino acid consumption and acetate esters (Fig. 7.1A). Fig. 7.1B illustrates contour plot for higher alcohols, which is similarly to acetate esters was plotted showing inorganic phosphate versus potassium to an average of magnesium content at 150 mg/L. Magnesium impacted significantly on higher alcohol formation; however, mostly magnesium impact was better observed in supplementation levels of magnesium (80 to 160 mg/L). To obtain higher concentration of higher alcohols inorganic-phosphate, potassium and magnesium should be at 490, 600 and 150 mg/L, respectively (Fig. 1B). As a consequence, total content of higher alcohols increased from 224.8 mg/L to 322.4 mg/L, which represents an increasing of 43.4%. As expected, these optimal quantity of inorganic elements for higher alcohol formation are similar to the values found for acetate ester formation, which are alcohol dependent.

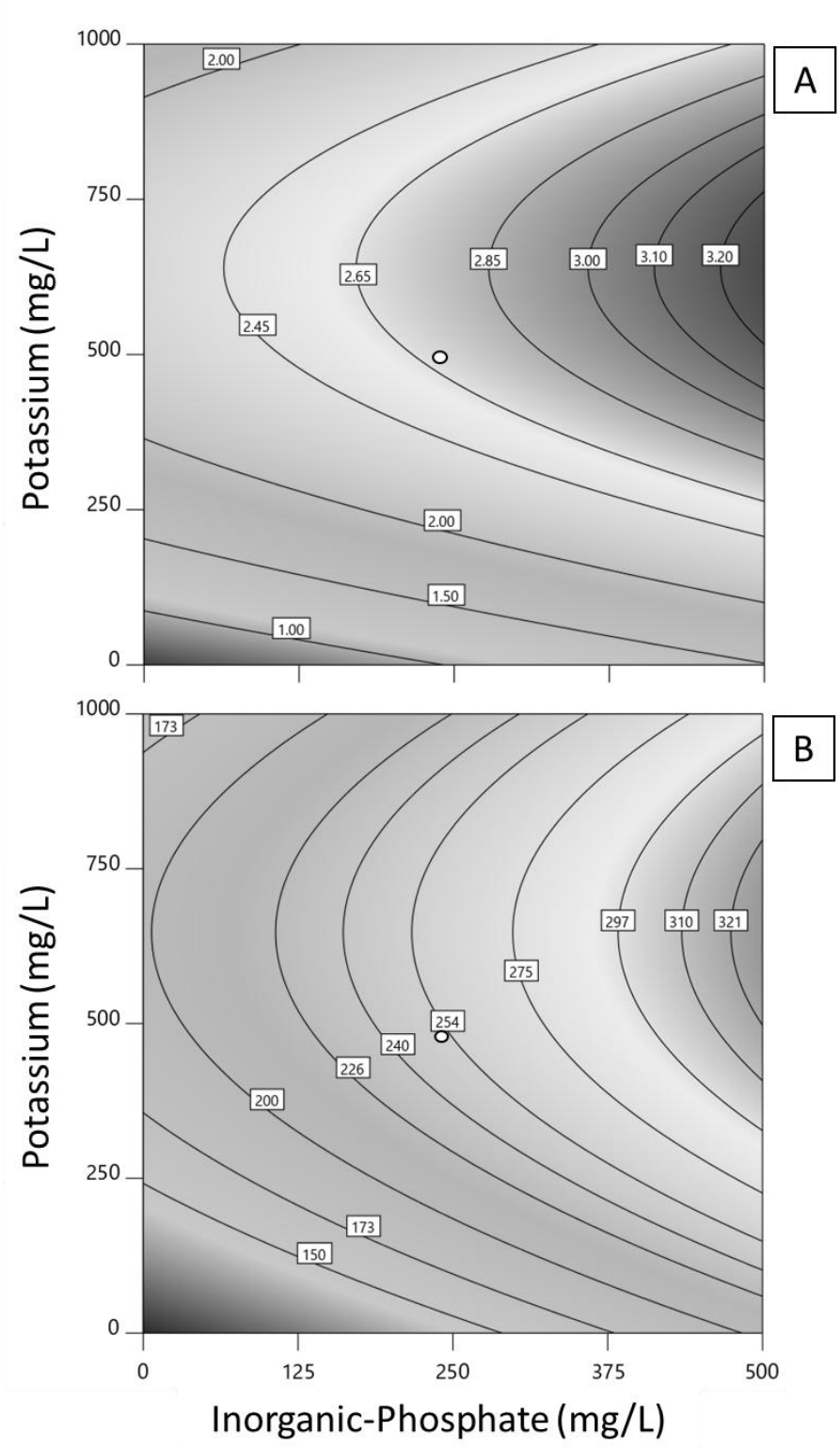

Fig. 7.1: Contour plot for inorganic phosphate vs potassium having magnesium content fixed at 150 mg/L on (A) acetate esters (mg/L) and (B) higher alcohols (mg/L).

7.4.3. Evaluation of the design variables in fatty acid esters

Fatty acid esters are important active flavours. Fatty acid ester formation involves short- and medium-chain fatty acids, ethanol, acetyl-coA and their enzymes (EEB1 and EHT1) (Zhuang *et al.* 2015, Verstrepen *et al.* 2003). Results shows essential inorganic elements such as magnesium and inorganic-phosphate are involved in fatty acid ester formation (Fig. 2A). However, potassium has not a significant effect on fatty acid ester formation. Fig. 2A illustrates contour plot for fatty acid esters was plotted showing inorganic phosphate versus magnesium to an average of potassium content at 500 mg/L. Inorganic-phosphate and magnesium impacted significantly on fatty acid ester formation; however, when inorganic-phosphate was found with concentration levels under 100 mg/L, magnesium has small influence on fatty acid ester formation. When inorganic phosphate was in concentrations higher than 200 mg/L, observed higher influence of magnesium on FAE formation. To generate higher concentration of fatty acid esters inorganic-phosphate, potassium and magnesium should be at 300, 500 and 155 mg/L, respectively (Fig. 2A). As a consequence, total content of fatty acid esters increased from 2.8 mg/L to 5.0 mg/L, which represents an increasing of 77%.

7.4.4. Evaluation of the design variables in fatty acids

Fatty acids are an important precursor of fatty acid esters (Knight *et al.* 2014, Saerens *et al.* 2010, Saerens *et al.* 2006). Furthermore, fatty acids and higher alcohols compete via Ehrlich pathway to be formed (Yu *et al.* 2016, Hazelwood *et al.* 2008). Results shows essential inorganic elements such as magnesium and inorganic-phosphate are involved in fatty acid formation (Fig.

7.2B) similarly as fatty acid ester formation (Fig. 7.2A). Potassium has no influence on fatty acid formation. Magnesium has a domain of generation and reduction of fatty acids; however, inorganic-phosphate has an influence as a quadratic term. Fig. 2B illustrates contour plot for fatty acids was plotted showing inorganic phosphate vs magnesium to an average of potassium content at 500 mg/L. Inorganic-phosphate and magnesium impacted significantly on fatty acid formation. When magnesium was present in the medium in levels under 50 mg/L, inorganic-phosphate drove fatty acid formation and as a consequence, fatty acid formation increased, which is an undesirable because are off-flavours. However, when inorganic-phosphate was found within concentration levels under 100 mg/L, magnesium has higher influence on reducing of fatty acid formation. Furthermore, when inorganic-phosphate concentrations were observed above 250 mg/L and magnesium was added in supplementation levels (above 80 mg/L) an higher reduction of fatty acid formation was observed, which behaved similarly to the formation of fatty acid esters (Fig. 7.2B). To reduce the concentration of fatty acid inorganic-phosphate, potassium and magnesium should be at 300, 500 and 155 mg/L, respectively (Fig. 7.2B). As a consequence, total content of fatty acids decreased from 4.2 mg/L to 2.0 mg/L, which represents a reduction of 48.7%. As expected, this optimal condition is similar to the optimal conditions to increase the formation of fatty acid esters, which occurred because fatty acids are precursor of fatty acid esters and consequently their formation are inversely proportional.

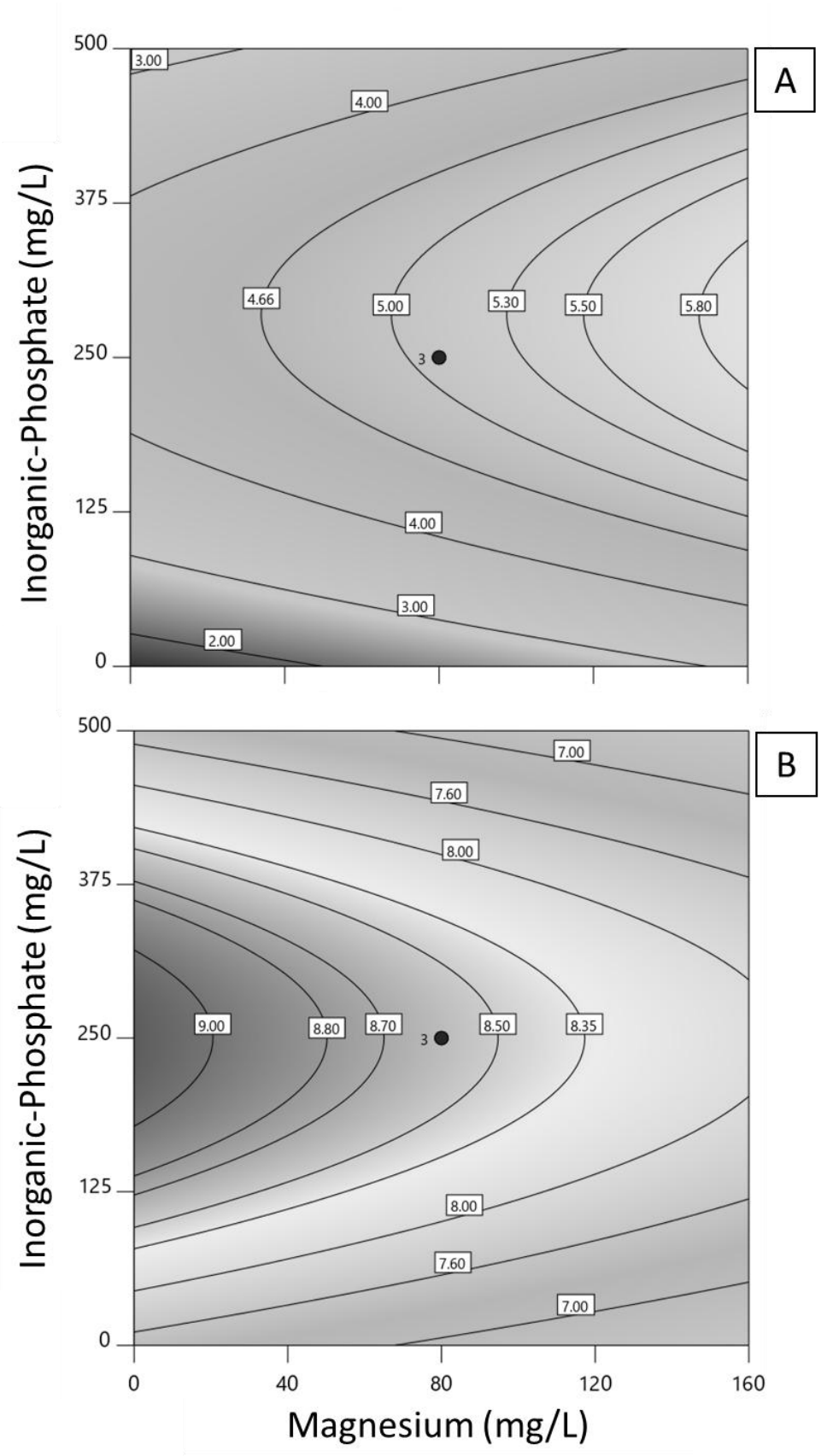

Fig. 7.2: Contour plot for inorganic phosphate vs magnesium having potassium content fixed at 500 mg/L on (A) fatty acid esters (µg/L) and (B) fatty acids (mg/L)

7.4.5. Evaluation of the design variables in yeast growth

It is known that yeast-flavours are produced during yeast growth. However, data shows that under supplementation conditions yeast stopped growing (Fig. 7.3A) and increased aroma formation (Fig. 7.1 and Fig. 7.2). Inorganic-phosphate did not affect yeast growth; in contrast, potassium and magnesium are significantly important for yeast growth. Fig. 3A illustrates contour plot for yeast growth (Total cell number) was plotted showing potassium versus magnesium to an average of inorganic-phosphate content at 250 mg/L. To guarantee and yeast-growth and fermentation performance; potassium and magnesium should be present in a minimal concentration of 500 mg/L and 70 mg/L, respectively. When wort was supplemented with potassium and magnesium, respectively, 550 mg/L and 100mg/L, yeast growth did not significantly increase from $5.7x10^7$ (central point) to $5.8x10^7$ (Supplementation) (Fig. 3A). However, all desirable aromas were generated under supplementation level of essential inorganic elements, which suggests under supplementation conditions yeast increases aroma formation without affecting the growth.

7.4.6. Evaluation of the design variables in ethanol formation

Ethanol formation was driven by magnesium presence (Fig. 7.3B). Results show that ethanol formation is magnesium dependent (Fig. 7.3B). To guarantee the ethanol formation, the wort should contain minimally 70 mg of Mg/L because when magnesium concentration are lower than 70 mg/L occurred a reduction of ethanol formation (Fig. 7.3B). In contrast, when magnesium is

present between 70-110 mg/L, ethanol concentrations increased 5 g of ethanol/L (Fig. 7.3B).

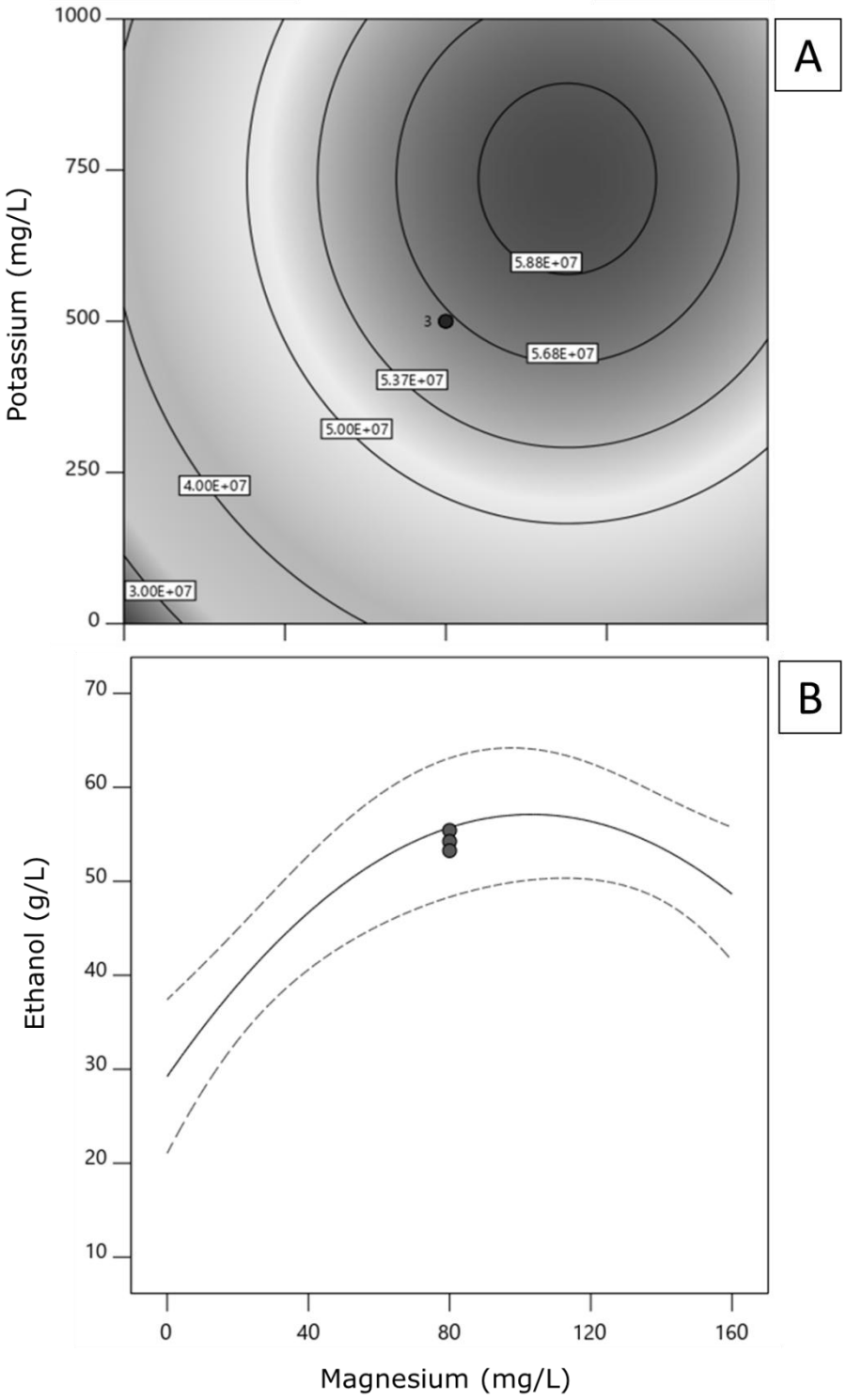

Fig. 7.3: A) Contour plot for potassium vs magnesium having inorganic phosphate fixed at 250 mg/L on yeast growth; and b) One factor plot for the influence of magnesium (mg/L) with potassium content at 500 mg/L and inorganic phosphate at 250 mg/L on ethanol (g/L) formation.

7.4.7. Overall of all fermentation treatments

Data was simplified in a PCA Bi-Plot to illustrate the overall change of all individual compounds and their similarities (Fig. 7.4). The first two components (PCA1 and PCA2) explain 60.01% of the variability. PC1 and PC2 represent individually a variance of (46.08%) and PC2 (13.03%), respectively (Table S7). Additionally, Correlation matrix (Pearson (n)) was evaluated to support that Fig. 7 is a real representation of the data. The first component separates groups 3 and 4 (the absence or deficiency of essential inorganic elements), which produced higher quantity of acetic acid, decanoic acid, acetoin and glycerol; from groups 1 and 2 (supplementation treatments and reference - T26, T27 and T28), which produced higher quantity of ethanol, glycerol, higher alcohols and acetate esters and represent. Second component separates groups 2 and 4, which produced similar concentration of alcohols; from groups 1 and 3, which produced similar quantity of off-flavour (fatty acids), fatty acid esters and acetic acid.

Group 4 represents the absence or deficiency of phosphorus and/or potassium (mostly lower than 250 and 500 mg/L, respectively). Group 1 had mostly a quantity of phosphorus and/or potassium similar as a control (250 and 500 mg/L, respectively) but supplementation levels of magnesium (mostly higher than 80 mg/L). Therefore, phosphorus and potassium concentration determine the differences between these two groups, which is represented by PC1. In contrast, Group 3 represents the absence or deficiency of magnesium (mostly lower than 80 mg/L); and Group 2 represents all magnesium supplementation levels, except T4 (500 mg of phosphorus/L, 600 mg of potassium/L, and 32 mg of magnesium/L). Although T4 had a deficient concentration of magnesium,

which triggered to lower formation of ethanol in the group 2. Hence, suggests that magnesium is very important mineral for alcohol formation (including ethanol and higher alcohols).

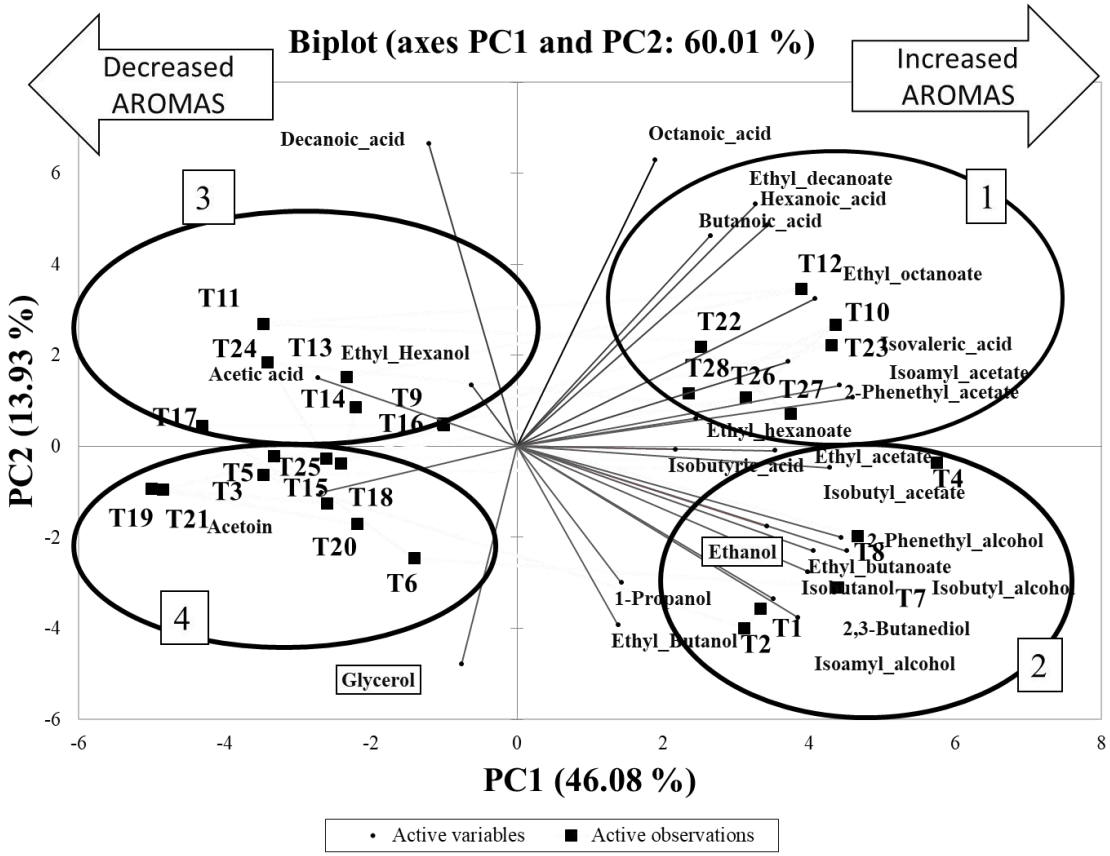

Fig. 7.4: Principle component analysis (Bi-Plot) to identify the correlation and similarities among all 27 variables including ethanol, glycerol, acetic acid, higher alcohols, fatty acid, acetate esters and fatty acid ethyl esters. Fermentation were conducted in a synthetic sweet wort (SW) using a *Saccharomyces cerevisiae* strain (NCYC2592). All treatments applied by a response surface method: T1 (500, 180 and 116.8 mg/L); T2 (500, 1000 and 160 mg/L); T3 (500, 0 and 0 mg/L); T4 (500, 650 and 32 mg/L); T5 (500, 1000 and 0 mg/L); T6 (500, 0 and 160 mg/L); T7 (500, 1000 and 103.7 mg/L); T8 (500, 515 and 160 mg/L); T9 (500, 0 and 55.2 mg/L); T10 (157.5, 1000 and 160 mg/L); T11 (157.5, 750 and 0 mg/L); T12 (157.5, 250 and 160 mg/L); T13 (157.5, 0 and 21.6 mg/L); T14 (0, 600 and 160 mg/L); T15 (0, 1000 and 84.8 mg/L); T16 (0, 1000 and 0 mg/L); T17 (0, 0 and 0 mg/L); T18 (0, 415 and 24 mg/L); T19 (0, 0 and 160 mg/L); T20 (0, 1000 and 160 mg/L); T21 (0, 0 and 96.8 mg/L); T22 (350, 755 and 132 mg/L); T23 (350, 1000 and 36 mg/L); T24 (350, 300 and 0 mg/L); T25 (350, 0 and 160 mg/L); T26 (250, 500 and 80 mg/L); T27 (250, 500 and 80 mg/L); and T28 (250, 500 and 80 mg/L). Groups 1 and 2 increased aromas formation when fermentations were conducted at control conditions (T26, T27 and T28) or under supplementation levels. Groups 3 and 4 decreased aromas formation when fermentations were conducted with essential inorganic element concentrations below control conditions (deficiency conditions).

Therefore, PCA shows an absence or deficiency of one factor affected negatively flavour production. Furthermore, treatments such as T3, T5, T16, T17 and T24, which were conducted with absence of magnesium, produced the lowest concentration of alcohols and acetate esters, which supports the data presented in Figs. 7.1 (A – acetate esters, B – higher alcohols) and 7.3B (ethanol). Furthermore, magnesium is involved in fatty acid ester formation and fatty acid (off-flavours) reduction; however, level of inorganic-phosphate and potassium (250 and 500 mg/L, respectively) are essential.

7.5. Discussion

Ethanol formation is Mg-dependent. In contrast, inorganic-phosphate and potassium did not affect ethanol formation. However, magnesium is transported into yeast cell by potassium and phosphorus transport systems (Knoop *et al.*, 2005, Walker 2004). Therefore, magnesium cannot drive ethanol formation itself because the minimal quantity of potassium (500 mg/L) for yeast growth and inorganic-phosphate (250 mg/L) for ATP generation/driving magnesium into yeast cell via MgATP complex (Udeh *et al.* 2014, Maguire and Cowan 2002, Birch and Walker 2000). Furthermore, magnesium may activates enzymes, which are responsible to flavour formation because of higher alcohols and ethanol use a similar genes during their formation (De Smidt *et al.* 2008, Briggs *et al.* 2004, Hazelwood *et al.* 2008). As a consequence, magnesium can influence acetate ester and fatty acid ester (active-flavour).

Alcohol formation (including ethanol and higher alcohols) are generated by alcohol dehydrogenase (ADH), which includes ADH1, ADH2, ADH3, ADH4 and ADH5; and is known that, in *Saccharomyces cerevisiae*, only ADH4 is zinc dependent (De Smidt *et al.* 2008, Knoop *et al.*, 2005, Briggs *et al.* 2004, Walker 2004). Therefore, magnesium may activate one of other ADHs because is a co-factor of several enzymes and also has been reported as a vital fermentation element (Birch *et al.* 2003, Knoop *et al.*, 2005, Walker 2004, Birch and Walker 2000).

Potassium and magnesium are the most significant essential inorganic elements, which affects the growth (Fig. 7.3 A). Inorganic-phosphate was expected to be the most important inorganic elements for yeast growth; however, magnesium and potassium are the most important for yeast growth. This occurred because of inorganic phosphate is transported as $MgPO_4$ that is involved in Mg-ATP complex (Lim *et al.* 2011, Maguire and Cowan 2002). Inorganic-phosphate intake is influenced by potassium presence and a lack of potassium inhibits phosphate intake and, as a result it affects yeast growth rate (Barreto *et al.* 2012, Sala 2015).

The most of aromas are formed during yeast growth (adaptation/exponential phase). Higher alcohols are formed and reacts to acetyl-CoA under growth conditions and therefore, acetate esters are generated. Acetate esters and higher alcohols are generated having inorganic-phosphate, potassium and magnesium as significant factors, which as expected were generated due to yeast growth via Ehrlich pathway; and therefore, are precursor of acetate esters. However, acetate esters and higher alcohols increased when supplemented with inorganic-phosphate, potassium and magnesium without affecting the yeast

growth (Fig. 7.3A), which suggests these inorganic elements can be involved in Ehrlich pathway. Higher alcohol equation is more complex than ethanol equation (Equation 7.6) because involves amino acid catabolism via Ehrlich pathway (Dack *et al.* 2017, Olaniran *et al.* 2017).

Acetate ester synthesis involves alcohol acetyl transferases including AATase I, AATase II and Lg-AATase I, which are encoded by the genes ATF1, ATF2 and Lg-ATF1, respectively (Xu *et al.* 2017, Mason and Dufour 2012, Saerens *et al.* 2010). Similarly to ethanol production, magnesium showed significant influence in higher alcohol production and as a consequence acetate ester formation. However, higher alcohol and acetate ester formation have also inorganic-phosphate and potassium as co-factors. Magnesium may be directly involved in enzyme activation. Potassium was also individually important for acetate ester formation due to the importance of potassium during the growth and acetate formation. Potassium is involved in pH control, which triggers to a formation and conversion of acetic acid to acetyl-CoA (Galdieri *et al.* 2014, Pronk *et al.* 1996) and involves Mg-ATP complex (Maguire and Cowan 2002). Finally, inorganic phosphate is involved in esterification reaction, which consumes inorganic phosphate generally from ATP. In conclusion, acetate ester formation (including isobutyl acetate, isoamyl acetate and 2-phenyl-acetate) are dependent of inorganic phosphate as a co-factor of ester reaction, magnesium as an alcohol co-factor and potassium as an acetate co-factor.

Fatty acid ester formation involves short- and medium-chain fatty acids, ethanol, acetyl-coA and their enzymes (EEB1 and EHT1). Fatty acid and fatty acids ester equations reflect the magnesium importance during yeast growth and ester formation. Magnesium is a significant factor for fatty acid reduction

due to fatty acid ester formation. Also, magnesium increases the formation of alcohols and, as a consequence, decreased the formation of fatty acids because the competition between higher alcohols and fatty acids via Ehrlich pathway (Olaniran *et al.* 2017, Yu *et al.* 2016, Hazelwood *et al.* 2008). Furthermore, inorganic-phosphate and magnesium were involved in esterification reaction, which consumes inorganic-phosphate and magnesium from Mg-ATP complex (Maguire and Cowan 2002).

Magnesium was very important for fatty acid ester formation because during its absence or limitation, yeast cell accumulates fatty acids and consequently decreased fatty acid ester formation. In contrast, in magnesium presence, yeasts can produce higher concentration of fatty acid esters and as a consequence decreases fatty acid formation. Therefore, the formation of fatty acid and fatty acid esters are inversely proportional. These may occur for two reasons: Firstly, the limitation of magnesium affects yeast growth and increases fatty acid accumulation. Second, the limitation of magnesium inhibits enzyme activation and as a consequence reduce the formation of cytosolic-ATP via glycolysis. The supplementation of inorganic-phosphate, potassium and magnesium increase active-flavour formation and decreases off-flavour formation (fatty acids). Brewers can use inorganic-phosphate, potassium and magnesium as fermentation supplement for increasing flavour formation without influence yeast growth and ethanol concentrations.

7.6. Conclusions

Magnesium is s most important inorganic element for fermentation performance and flavour formation (including the formation of ethanol, higher alcohols, acetate esters/fatty acid esters, and off-flavour reduction). Ethanol is magnesium dependent. Yeast growth is potassium/magnesium dependent. Supplementation level of inorganic phosphate, potassium and magnesium increase the flavour formation without affect the yeast growth. Potassium is vital for yeast growth, acetate esters and higher alcohols formation. Inorganic phosphate is important for fatty acid esters formation and fatty acid reduction and should be present in the wort in a minimal concentration of 250 mg/L for supporting fermentation performance. Optimal condition to guarantee yeast growth, increases active-flavour formation and decreases off-flavour formation is inorganic-phosphate (500 mg/L), potassium (680 mg/L) and magnesium (150 mg/L). All equations generated for all compound groups evaluated can be used for industrial application. PCA is helpful for explaining the formation of yeast metabolic aromas and their precursors via linear multivariate discriminations.

CHAPTER VIII
FINAL CONSIDERATIONS

8. CHAPTER VIII: CHAPTER 8: FINAL CONSIDERATIONS

This thesis has focussed on the impact of yeast nutrition on flavour formation during fermentation at laboratory-scale, as this is one of the key aspects, which can support brewers in their productions. As a result, a more complete understanding of the role of essential inorganic elements in flavour formation during yeast fermentation was provided, which allows brewers to more accurately predict and potentially control flavours. Industrially, understanding the impact of essential inorganic elements in flavour formation during fermentation is very important for improving/controlling and making decisions in industrial processes. Considering that essential inorganic elements are not easily measurable daily compounds for industries due to its time-consuming and expensive analysis. However, economically, it can affect fermentation costs by the characteristic of raw materials, the reduction of fermentation time and maintaining product quality.

From a sensory perspective, all aromas evaluated were identified above the typical threshold, except for fatty acids. All treatments generated acetic acid concentration under the typical threshold (1 g/L). However, treatments, which had more acetic acid accumulation reduced the production of ester and ethanol. Magnesium is the most important co-factor for the formation of ethanol, higher alcohols, acetate esters, fatty acid esters, and as a consequence involved in the reduction of acetic acid accumulation (vinegar taste) and fatty acids (off-flavours). Furthermore, Magnesium salts are a bitter taste stimulus and have bitter, salty and sour tastes (Lawless *et al.* 2003, Delwiche *et al.* 1999). Schiffman and Erickson (1971) classified magnesium chloride as bitter-salty.

Therefore, magnesium can be responsible for bitterness and also is important for human health.

8.1. CONCLUSIONS

The chapter 3 assesses the characterization of three-yeast strains for their differentiation, including (permissive growth temperature; capacity to grow on different carbon source; DNA fingerprint (physiologic differentiation); and mineral contents. Two ale-type (NCYC2592 and M2) and one lager-type (W34/70) brewing yeast strains were characterized based on differences in permissive growth temperature and the presence of melibiose (α-galactosidase). DNA fingerprint confirmed their genetically differences. NCYC2592 and M2 have similar mineral composition. The characterisation of brewing yeast strains provides a preliminary understanding to conduct the thesis investigation entitled: 'the impact of yeast nutrition on flavour formation during yeast fermentation'.

In chapter 4 provides a more complete understanding of flavour formation by considering the influence of initial carbohydrate composition and the mixture of carbohydrates/ethanol. Also, acetic acid accumulation and its importance on yeast-flavour formation. All strains applied consumed low quantity of maltotriose by 48 h. During the consumption of two third of fermented carbohydrates, acetic acid and ethanol are accumulated in parallel. At 16 h, ethanol concentration became higher than fermented carbohydrate concentration and acetic acid started being consumed. Acetic acid is accumulated by carbohydrates domain and consumed by ethanol domain. Also,

acetate esters and fatty acid esters are influenced by carbohydrates domain and ethanol domain. At 22°C, all strains grow similarly and the exponential phase finished at 20 h. NCYC2592 and W34/70 produced higher alcohols by 20 h (The end of exponential phase) and M2 produced 50% of higher alcohols at 20 h. All strains produced fatty acids (off-flavours) and were quantified with values under threshold levels.

Several studies have focused on flavour formation during yeast fermentation, which includes flavour production (Fairbairn 2012); metal ions influence on flavour stability (Zufall and Tyrell 2008); biosynthesis of higher alcohol (Vidal *et al.* 2014); volatile ester synthesis (Bilverstonem *et al.* 2015, Zhuang *et al.* 2015) and metabolic production of short chain fatty acids (Yu *et al.* 2016). Chapter 5 illustrated for the first time how flavour profiles is impacted when singular or a complex mix of eight different essential inorganic elements were added during fermentation. Ammonia-nitrogen, inorganic phosphate, potassium and magnesium significantly increased the production of target industrial compounds (ethanol and glycerol) and decreased acetic acid; furthermore increased the formation of higher alcohols and esters. Copper, iron, manganese or a composite mixture of all nutrient supplementations influenced negatively flavour formation. Zinc had less impact flavour formation.

To understand the role of several components present in wort a defined medium, which can 'mimic' wort composition was required for comparative yeast fermentation. Some researchers have developed a formulation of defined/semi-defined media (Taidi *et al.* 2003); however, any 'artificial wort' recipe published have not demonstrated enough similarity to natural wort composition, which includes key components: macronutrients (carbohydrates

and free amino acids), essential inorganic elements (including potassium, phosphorous, magnesium, calcium, iron, zinc, copper, manganese), vitamins and lipids. In the chapter 6 was created a synthetic sweet wort, which is defined medium and can imitate typical brewer's wort in terms of carbohydrates, amino acids, essential inorganic elements (micro inorganic nutrients and metal ions), vitamins and lipids concentrations. Amino acids and vitamins are the buffer compounds and essential inorganic elements increase the free hydrogen in wort. Yeast growth requires a minimal quantity of essential inorganic elements as a guarantee of growth. Phosphorus, potassium and magnesium are vital for yeast growth. During fermentations, inorganic elements influence yeast growth and the flavour formation. Synthetic wort can support fermentation studies of the yeast growth, carbohydrate consumption, essential inorganic elements assimilation, and flavour formation (including ethanol, glycerol, acetic acid, higher alcohols, short chain fatty acids, esters and fatty acids esters). SW is an important/useful tool for fermentation studies.

Chapter 7 brings the final findings and provides more evidence of inorganic element importance for yeast-flavour formation. Magnesium is s most important inorganic element for fermentation performance and flavour formation (including the formation of ethanol, higher alcohols, acetate esters/fatty acid esters, and off-flavour reduction). Ethanol is magnesium dependent. Yeast growth is potassium/magnesium dependent. Supplementation level of inorganic phosphate, potassium and magnesium increase the flavour formation without affect the yeast growth. Potassium is vital for yeast growth, acetate esters and higher alcohols formation. Inorganic phosphate is important for fatty acid esters formation and fatty acid reduction and should be present in

the wort in a minimal concentration of 250 mg/L for supporting fermentation performance. Optimal condition to guarantee yeast growth, increases active-flavour formation and decreases off-flavour formation is inorganic-phosphate (500 mg/L), potassium (680 mg/L) and magnesium (150 mg/L). All equations generated for all compound groups evaluated can be used for industrial application. PCA is helpful for explaining the formation of yeast metabolic aromas and their precursors via linear multivariate discriminations.

8.2. FUTURE WORK

This thesis has focussed on the impact of yeast nutrition on flavour formation during fermentation at laboratory-scale, as this is one of the key aspects, which can support brewers in their productions. Furthermore, data presented in chapters 4, 5, 6 and 7 showed evidence of the importance of essential inorganic elements such as inorganic-phosphate, potassium and magnesium on flavour formation during yeast fermentation. Thus, it is recommended further studies and opens a door for other research:

1. Magnesium role in the Ehrlich pathway and consequently higher alcohol/acetate ester formation. Higher alcohol formation and its synthesis via Ehrlich pathway and the importance of magnesium as an enzymatic co-factor is well known (Walker 2004; Briggs *et al.* 2004, Rosslyn *et al.* 2003). Magnesium has a vital importance for yeast division/growth, metabolic activities, respiro-fermentative metabolism, mitochondrial structure and function, response to environmental stress,

fermentation performance, ethanol production, etc. (Walker 2004, Walker et l. 1996). Rosslyn *et al.* (2003) demonstrated that low magnesium calcium concentration results in a reduction of the fermentation performance, which is similar response comparing to data that was presented in Chapter 6 and Chapter 7 of this thesis. Furthermore, Chapter 5 and 7 provide evidence for the importance of magnesium for higher alcohol and acetate ester formation. Therefore, further studies are required for a deep understanding of magnesium role in Ehrlich pathway and higher alcohol/acetate ester formation, which includes: a) the evaluation of magnesium and single amino acids during yeast growth using Tecan; and the evaluation of alcohol dehydrogenases (ADH) that are magnesium-dependent using polymerase chain reaction (PCR) technique analysis and b) higher alcohol analysis using gas chromatography with mass spectrum (GC-MS).

2. Magnesium-phosphate has a role in ATP. Magnesium activates over 300 enzymes including many glycolytic enzymes such as pyruvate kinase, hexokinase, phosphofructokinase, phosphoglycerate kinase and enolase (Walker *et al.* 2006, Walker 2004). Phosphorus is one of the key minerals required for yeast growth. In the brewery, wort contains phosphorus in the form of phosphate salts (Walker and Stewart 2016). Inorganic phosphate (Pi) is the major intracellular anion present in the yeast cell and is involved in cellular biosyntheses such as nucleic acids, nucleoproteins and phospholipids and ATP, as well as several metabolic pathways (Walker and Stewart 2016, Canadell *et al.* 2014).

Magnesium can be transported into cells via potassium and phosphorus transport systems, two plasma membrane transporters, ALR1 and ALR2, have been reported to be required for magnesium transport (Knoop *et al.*, 2005, Walker 2004). Therefore, magnesium-phosphate may have a role in as nucleic acids, nucleoproteins and phospholipids and ATP. During fermentation, ATP is generated via glycolysis because oxidative phosphorylation is inhibited and mostly of glycolytic reactions, which generates ATP have magnesium as an enzymatic co-factor that includes pyruvate kinase, hexokinase, phosphofructokinase, phosphoglycerate kinase and enolase (Walker *et al.* 2006, Walker 2004). Therefore, further studies are required for a deep understanding of magnesium-phosphate role ATP generation to evaluate magnesium-phosphate influx using polymerase chain reaction (PCR) technique by the activation of Pho84 and Pho89, which are responsible for Pi transport through plasma-membrane and have high-affinity for Pi uptake (Persson *et al.* 1999, Sala 2015). From a flavour perspective, it can support a deeper understanding of fatty acid ester formation, which utilizes ATP and magnesium as evidence provided in chapter 7 of this thesis.

3. Acetic acid and pantothenic acid as a precursor of acetyl-CoA and as a consequence ester formation. Acetyl-CoA is formed from glucose, fatty acid, and amino acid via catabolism (Shi and Tu 2015). Several metabolic activities involve acetyl-CoA, including the tricarboxylic acid cycle (TCA cycle), the glyoxylate cycle, fatty acid synthesis and β-oxidation, amino acid synthesis and sugar metabolism. Possibly, acetyl-

CoA, ATP and NADH share key position in cell metabolic activities, because they can regulate several enzymes activities and gene expression via acetylation/de-acetylation reaction (Pietrocola *et al.* 2015, Galdieri *et al.* 2014). Chapters 4, 5 and 7 of this thesis provided evidence, which clearly shows the role of acetic acid during aroma formation. Furthermore, inorganic-phosphate, potassium and magnesium have their influence on ester formation, which passes through acetic acid/acetate/acetyl-CoA formation and consequently esters. However, further studies are required for a deep understanding of the acetyl-CoA role, mainly, when considers pantothenic acid as a CoA precursor, acetic acid as a source of carbon units for Acetyl-CoA formation, its enzymatic co-factors and its effect on ester formation. Therefore, acetic acid/acetate/acetyl-CoA/CoA analysis can be carried out using high-performance liquid chromatography (HPLC) and the activation of Acs1p and Acs2p (acetyl-CoA synthetase) using polymerase chain reaction (PCR) technique.

4. Influence of individual amino acid their correlations/affinities with essential inorganic elements for generation of flavour formation during yeast fermentation. Ehrlich pathway is well known as a higher alcohol and short-chain fatty acid metabolic synthesis (Yu *et al.* 2016, Vidal *et al.* 2014, Ehrlich 1904). Chapters 5 and 7 of this thesis provided evidence that support the influence of essential inorganic elements such as inorganic-phosphate, potassium and magnesium in yeast growth and higher alcohol formation, which are both responses of amino acid consumption via Ehrlich pathway. According to Nies (2016) “organic

and inorganic particles present in natural environments also sequester metal cations". Amino acids as organic molecules have their individual metal-binding properties, which is related to chemical affinity (Nies 2016). The individual correlations/affinities between amino acids and inorganic elements can provide a deeper understanding of individual amino acid consumption and consequently yeast growth, cell wall structure, and flavour formation. Therefore, yeast characterization, which will involve the amino acid composition of yeast using gas chromatography with the mass spectrum (GC-MS); and the mineral composition of yeast using inductively coupled plasma mass spectrometry (ICP-MS). Afterwards, synthetic wort will be prepared with sole amino acid will be prepared with a different concentration of essential inorganic elements individually and yeast growth curves will be conducted using Tecan. Finally, considering the most important correlations/affinities between amino acids and essential inorganic elements, experiments focus on aromas formation will be conducted using gas chromatography with the mass spectrum (GC-MS).

5. Influence of individual vitamins utilization and their consumption by essential inorganic elements presence on flavour formation during yeast fermentation. Brewing yeasts require inorganic ions and vitamins for efficient growth and fermentation (Zhang *et al.* 2016, He *et al.* 2014). Thus, vitamins can affect the yeast flavour formation by altering their metabolic activities (He *et al.* 2014). Vitamin supplementation increases ethanol tolerance, the yield of yeast and yeast membrane phospholipids in terms of the amount of PUFA and polyunsaturated

fatty (Zhang *et al.* 2016). Experiments can be conducted using a synthetic wort developed in chapter 6 of this thesis. Synthetic wort will be prepared with a different concentration of vitamins/inorganic elements and yeast growth curves will be conducted using Tecan. Furthermore, unsaturated fatty acid formation and aroma formation analysis will be conducted using gas chromatography with mass spectrum (GC-MS). Finally, specific enzymes involved in vitamins and inorganic elements uptake and also specific enzymes involved in unsaturated fatty acid and aroma formation can be analysed using polymerase chain reaction (PCR) technique.

6. Influence of individual wort lipids and magnesium on flavour formation during yeast fermentation. The wort lipid can affect the ester formation because of a direct effect in ester enzymes (ATF1 and ATF2), which is directly inhibited by the supplementation of unsaturated fatty acid (He *et al.* 2014). Chapter 7 of this present thesis provided the evidence of magnesium involvement in the generation of fatty acid esters and as a consequence reduction of short-and medium-chain fatty acids. Therefore, magnesium can be involved in the lipids intake and break down because magnesium is one of the cations classified as a bond breaking (Keast and Breslin 2002), which is also related to the bitterness contribution of magnesium salts (Lawless *et al.* 2003). Experiments can be conducted using a synthetic wort developed in chapter 6 of this thesis. Synthetic wort will be prepared with a different concentration of individual lipids/magnesium and yeast growth curves will be conducted using Tecan. Furthermore, unsaturated fatty acid

formation and aroma formation analysis will be conducted using gas chromatography with mass spectrum (GC-MS). Finally, specific enzymes involved in vitamins and inorganic elements uptake and also specific enzymes (ATF1 and ATF2) involved ester formation can be analysed using polymerase chain reaction (PCR) technique.

7. Influence of individual carbohydrates in flavour formation during yeast fermentation. Glucose is the preferred source of carbon and energy for the yeast *Saccharomyces cerevisiae* (Galdieri *et al.* 2014). During brewing fermentation, glucose, fructose and sucrose are rapidly consumed during yeast fermentation and due to their easy consumption may be mostly responsible for active-flavour production; however, carbohydrates such as maltose and maltotriose need a little more energy for consumption and as a consequence they may be responsible for increasing fatty acids, sterols production and off-flavour (short-and medium-chain fatty acids). Experiments can be conducted using a synthetic wort developed in chapter 6 of this thesis. Synthetic wort will be prepared with single sugar (glucose, fructose, sucrose, maltose and maltotriose) for analysis yeast growth curves using Tecan and mini-fermentation vessels for CO2 weight loss, yeast viability and flavour analysis. Sugar/ethanol/glycerol and acetic acid formation can be analysed using high-performance liquid chromatography (HPLC) and aroma analysis using gas chromatography with mass spectrum (GC-MS).
8. Influence of low, medium, high and very high gravities in flavour formation during yeast fermentation. Saerens *et al.* (2006) demonstrated

that the initial wort gravity can determine the final concentration of important volatile aroma. Furthermore, authors emphasised that high and very high gravities induced ethanol, higher alcohols and as a consequence acetate esters. These may be related to acetic acid accumulation due to rapid sugars consumption and ethanol formation, which rapidly accumulates acetaldehyde and consequently generates acetic acid/acetate/acetyl-CoA. Therefore, acetyl-CoA reacts to alcohols forming acetate esters. Synthetic wort will be prepared with gravities at 10, 12, 14, 16, 18, 20 and 22 Plato. Furthermore, the influence of magnesium can be evaluated because as data presented in Chapter 7 of this thesis, magnesium is an important co-factor for alcohol and ester formation. Methodologically, yeast growth will be conducted using Tecan and mini-fermentation vessels for CO_2 weight loss, yeast viability and flavour analysis. Sugar/ethanol/glycerol and acetic acid formation can be analysed using high-performance liquid chromatography (HPLC) and aroma analysis using gas chromatography with mass spectrum (GC-MS). All influence in specific enzymes involved can be analysed using polymerase chain reaction (PCR) technique.

Finally, all future work described above can also consider using an experimental factorial design for evaluating interactions/optimizing fermentations and scale-up studies, which is always an issue for fermentation industries. Moreover, a deeper evaluation of all enzymes involved in flavour

formation is necessary, mainly, considering essential inorganic elements as enzymatic co-factors.

8.3. Final remarks

The importance of essential inorganic elements such as phosphate, potassium and magnesium in the brewing industry is clear. Furthermore, the influence of these essential inorganic elements for yeast-flavour production during alcoholic fermentation. The work completed in this thesis suggests that it is important to study the specific enzymes activation by magnesium; however, brewers can use all information from this work to improve, fix fermentation issues and classify their own products. Hence, this work raised the correlations between yeast physiology, mineral composition and carbohydrate consumption, which needs a deeper study in order to obtain a more complete understanding. In the future, brewers can use a real time-headspace analysis connect to fermenters to predict and reduce cost of mineral analysis during fermentation considering that this work generated several primary information, which correlates flavour to mineral presence.

9. REFERENCES

Abdi, H. and Williams, L. (2010). Principal component analysis. Wiley Interdisciplinary Reviews: Computational Statistics, 2 (4), pp.433–459.

Akram, M. (2014). Citric Acid Cycle and Role of its Intermediates in Metabolism. *Cell Biochemistry and Biophysics*, *68*(3), 475–478. http://doi.org/10.1007/s12013-013-9750-1

Anjum, M. F., Tasadduq, I., & Al-Sultan, K. (1997). Response surface methodology: A neural network approach. European Journal of Operational Research, 101, 65–73.

Anness, B. J. and Reed, R. J. R. Lipids in wort. *J. Inst. Brew.*, September-October, 1985, Vol. 91, pp. 313-317

Ansell R, Granath K, Hohmann S, Thevelein JM, Adler L (1997). The two isoenzymes for yeast NAD?-dependent glycerol 3-phosphate dehydrogenase encoded by GPD1 and GPD2 have distinct roles in osmoadaptation and redox regulation. EMBO J 16(9):2179–2187

Arino, J., Ramos, J. and Sychrova, H. (2010). Alkali Metal Cation Transport and Homeostasis in Yeasts. *Microbiology and Molecular Biology Reviews*, 74(1), pp.95-120.

ASBC Methods of Analysis, online. Beer Flavour Database. (2011). American Society of Brewing Chemists, St. Paul, MN, U.S.A.<http://methods.asbcnet.org/flavors_database.aspx >

Ashraf, N. (2011). Transcriptional and Metabolic Analysis of Flavour Development During Brewing Fermentation. Ph.D. Univerity of Nottingham.

Bailey, J. and Ollis, D. (1986). *Biochemical engineering fundamentals*. New York: McGraw-Hill.

Bamforth, C. (2001). PEER REVIEWED SUBMISSION pH in Brewing: An Overview Technical Q uarterly 1, *38*(1), 1–9. Retrieved from

http://www.lowoxygenbrewing.com/wp-content/uploads/2016/11/BAMFORTH-pH-in-brewing.pdf

Bamforth (2003). *Brewing yeast fermentation and performance - Chapter 7: Wort composition and beer quality*. 2nd ed. Oxford Brookes University Oxford, UK: Blackwell Science Ltd, pp.86-95.

Bamforth (2006). Brewing new technologies.Department of Food Science and Technology, University of California, Davis, CA 95616, USA.pp.484

Barker, R. L., Irwin, A. J. and Murray, C. R. (1992). The relationship between fermentation variables and flavor volatiles by direct gas chromatographic injection of beer. *Technical quarterly - Master Brewers Association of the Americas* 29(1): 11-17.

Barnett, J. A. (2003). A history of research on yeasts 6: The main respiratory pathway. *Yeast*, *20*(12), 1015–1044. http://doi.org/10.1002/yea.1021

Barreto, L., Canadell, D., Valverde-Saubí, D., Casamayor, A., & Ariño, J. (2012). The short-term response of yeast to potassium starvation. *Environmental Microbiology*, *14*(11), 3026–3042. http://doi.org/10.1111/j.1462-2920.2012.02887.x

Barros Neto, B.; Scarminio, I. S.; Bruns, R. E. (2003). Como Fazer Experimentos: Pesquisa e desenvolvimento na ciência e na indústria, Ed. da Unicamp: Campinas.

Baş, D. and Boyacı, İ. (2007). Modelling and optimization I: Usability of response surface methodology. Journal of Food Engineering, 78(3), pp.836-845.

Beltran, G., Esteve-Zarzoso, B., Rozès, N., Mas, A. and Guillamón, J. (2005). Influence of the Timing of Nitrogen Additions during Synthetic Grape Must Fermentations on Fermentation Kinetics and Nitrogen Consumption. *J. Agric. Food Chem.*, 53(4), pp.996-1002.Bezerra, M., Santelli, R., Oliveira, E., Villar, L. and Escaleira, L. (2008). Response

surface methodology (RSM) as a tool for optimization in analytical chemistry. Talanta, 76(5), pp.965-977.

Birch, R. M., & Walker, G. M. (2000). Influence of magnesium ions on heat shock and ethanol stress responses of Saccharomyces cerevisiae. *Enzyme and Microbial Technology*, *26*(9–10), 678–687. https://doi.org/10.1016/S0141-0229(00)00159-9

Birch, R. M., Ciani, M., & Walker, G. M. (2003). Magnesium, calcium and fermentative metabolism in wine yeasts. *Journal of Wine Research*, *14*(1), 3–15. https://doi.org/10.1080/0957126032000114973Birch et al. 2003

Blackwell, K., Tobin, J. and Avery, S. (1998). Manganese toxicity towards Saccharomyces cerevisiae: Dependence on intracellular and extracellular magnesium concentrations. Applied Microbiology and Biotechnology, 49(6), pp.751-757.

Blomberg (1992). Physiology of Osmotolerance in Fungi. *Advances in Microbial Physiology. Volume 33*, 1992, Pages 145-212

Bokulich, N. A., & Bamforth, C. W. (2013). The Microbiology of Malting and Brewing. *Microbiology and Molecular Biology Reviews*, *77*(2), 157–172. http://doi.org/10.1128/MMBR.00060-12

Boubekeur, S., Bunoust, O., Camougrand, N., Castroviejo, M., Rigoulet, M., & Guérin, B. (1999). A mitochondrial pyruvate dehydrogenase bypass in the yeast Saccharomyces cerevisiae. *Journal of Biological Chemistry*, *274*(30), 21044–21048. http://doi.org/10.1074/jbc.274.30.21044

Boubekeur, S., Camougrand, N., Bunoust, O., Rigoulet, M., & Guérin, B. (2001). Participation of acetaldehyde dehydrogenases in ethanol and pyruvate metabolism of the yeast Saccharomyces cerevisiaeBoubekeur, S., Camougrand, N., Bunoust, O., Rigoulet, M., & Guérin, B. (2001). Participation of acetaldehyde dehydrogenases in ethanol and. *European Journal of Biochemistry*, *268*, 5057–5065. http://doi.org/10.1046/j.1432-1327.2001.02418.x

Boulton C and Quain D (2001) Brewing Yeast and Fermentation. Blackwell Science, Oxford, UK.

Briggs DE, Boulton CA, Brookes PA & Stevens R (2004) Brewing: Science and Practice. Woodhead, Cambridge, UK.

Canadell, D. (2014). Potassium starvation responses in yeast highlight novel potassium-related functions, (April).

Canadell, D., González, A., Casado, C., & Ariño, J. (2015). Functional interactions between potassium and phosphate homeostasis in Saccharomyces cerevisiae. *Molecular Microbiology*, *95*(3), 555–572. http://doi.org/10.1111/mmi.12886

Chen, Y., Daviet, L., Schalk, M., Siewers, V., & Nielsen, J. (2013). Establishing a platform cell factory through engineering of yeast acetyl-CoA metabolism. *Metabolic Engineering*, *15*(1), 48–54. http://doi.org/10.1016/j.ymben.2012.11.002

Chen, Y., Siewers, V. and Nielsen, J. (2012). Profiling of Cytosolic and Peroxisomal Acetyl-CoA Metabolism in *Saccharomyces cerevisiae*. *PLoS ONE*, 7(8), p.e42475.

Conway, E. J., & Beary, M. E. (1962). A magnesium yeast and its properties. *The Biochemical Journal*, *84*(1957), 328–333. http://doi.org/10.1042/bj0840328Crapo et al. 1992

Cyert, M. and Philpott, C. (2013). Regulation of Cation Balance in Saccharomyces cerevisiae. Genetics, 193(3), pp.677-713.

Dack, R. E., Black, G. W., Koutsidis, G., & Usher, S. J. (2017). The effect of Maillard reaction products and yeast strain on the synthesis of key higher alcohols and esters in beer fermentations. *Food Chemistry*, *232*, 595–601. https://doi.org/10.1016/j.foodchem.2017.04.043

Dashko, S., Zhou, N., Compagno, C. and Piškur, J. (2014). Why, when, and how did yeast evolve alcoholic fermentation?. FEMS Yeast Research, 14(6), pp.826-832.

De Deken, R. H. (1966). The Crabtree Effect: A Regulatory System in Yeast. *Journal of General Microbiology*, *44*(2), 149–156. http://doi.org/10.1099/00221287-44-2-149

De Freitas, J., Wintz, H., Kim, J. H., Poynton, H., Fox, T., & Vulpe, C. (2003). Yeast, a model organism for iron and copper metabolism studies. *BioMetals*, *16*(1), 185–197. http://doi.org/10.1023/A:1020771000746

De Keukeleire, D. (2000). Fundamentals of beer and hop chemistry. *Química Nova*, 23(1), pp.108-112.

De Smidt, O., Du Preez, J. C., & Albertyn, J. (2008). The alcohol dehydrogenases of Saccharomyces cerevisiae: A comprehensive review. *FEMS Yeast Research*, *8*(7), 967–978. http://doi.org/10.1111/j.1567-1364.2008.00387.x

Deepak, V., Kalishwaralal, K., Ramkumarpandian, S., Babu, S., Senthilkumar, S. and Sangiliyandi, G. (2008). Optimization of media composition for Nattokinase production by Bacillus subtilis using response surface methodology. *Bioresource Technology*, 99(17), pp.8170-8174.Delwiche et al. 1999

De Virgilio, C., Bürckert, N., Barth, G., Neuhaus, J. -M, Boller, T., & Wiemken, A. (1992). Cloning and disruption of a gene required for growth on acetate but not on ethanol: The acetyl-coenzyme a synthetase gene of Saccharmoyces cerevisiae. *Yeast*, *8*(12), 1043–1051. http://doi.org/10.1002/yea.320081207

Dickinson, F. (1996). The purification and some properties of the Mg2+-activated cytosolic aldehyde dehydrogenase of *Saccharomyces cerevisiae*. *Biochemical Journal*, 315(2), pp.393-399.

Dickinson, J., Harrison, S. and Hewlins, M. (1998). An Investigation of the Metabolism of Valine to Isobutyl Alcohol in *Saccharomyces s cerevisiae*. Journal of Biological Chemistry, 273(40), pp.25751-25756.

Dickinson, J., Harrison, S., Dickinson, J. and Hewlins, M. (2000). An Investigation of the Metabolism of Isoleucine to Active Amyl Alcohol in *Saccharomyces cerevisiae*. Journal of Biological Chemistry, 275(15), pp.10937-10942.

Ehrlich F (1904) Uber das natürliche isomere des leucins. Ber Dtsch Chem Ges 37:1809–1840

Eide, D. J., S. Clark, T. M. Nair, M. Gehl, M. Gribskov (2005). Characterization of the yeast ionome: a genome-wide analysis of nutrient mineral and trace element homeostasis in *Saccharomyces cerevisiae*. Genome Biol. 6: R77.

Fairbairn, S. 2012. Stress, fermentation performance and aroma production by yeast. Thesis presented in partial fulfilment of the requirements for the degree of Master of Science at Stellenbosch University. Institute of Wine Biotechnology, Faculty of AgriSciences. *Supervisor:* Anita Smit and *Co-supervisor:* Prof Florian Bauer.

Field, L. S., Furukawa, Y., O'Halloran, T. V., & Culotta, V. C. (2003). Factors controlling the uptake of yeast copper/zinc superoxide dismutase into mitochondria. *Journal of Biological Chemistry*, *278*(30), 28052–28059. http://doi.org/10.1074/jbc.M304296200

Francis, I. & Newton, J. (2005). Determining wine aroma from compositional data. Aust. J. Grape Wine Res. 11, 114-126.

Galdieri, L., Zhang, T., Rogerson, D., Lleshi, R. and Vancura, A. (2014). Protein Acetylation and Acetyl Coenzyme A Metabolism in Budding Yeast. Eukaryotic Cell, 13(12), pp.1472-1483.

Gibson RB (2011). Improvement of higher gravity brewery fermentation via wort enrichment and supplementation. *Journal of the Institute of Brewing*. 117(3):268-284.

Gibson, B., Lawrence, S., Leclaire, J., Powell, C. and Smart, K. (2007). Yeast responses to stresses associated with industrial brewery handling: Figure 1. FEMS Microbiology Reviews, 31(5), pp.535-569.

Gombert, A., Moreira dos Santos, M., Christensen, B. and Nielsen, J. (2001). Network Identification and Flux Quantification in the Central Metabolism of Saccharomyces cerevisiae under Different Conditions of Glucose Repression. Journal of Bacteriology, 183(4), pp.1441-1451.

Hazelwood, L., Daran, J., van Maris, A., Pronk, J. and Dickinson, J. (2008). The Ehrlich Pathway for Fusel Alcohol Production: a Century of Research on Saccharomyces cerevisiae Metabolism. Applied and Environmental Microbiology, 74(12), pp.3920-3920.

Hazelwood, L., Tai, S., Boer, V., de Winde, J., Pronk, J. and Daran, J. (2006). A new physiological role for Pdr12p in *Saccharomyces cerevisiae*: export of aromatic and branched-chain organic acids produced in amino acid catabolism. FEMS Yeast Research, 6(6), pp.937-945.

He, Y., Dong, J., Yin, H., Zhao, Y., Chen, R., Wan, X., … Chen, L. (2014). Wort composition and its impact on the flavour-active higher alcohol and ester formation of beer - A review. *Journal of the Institute of Brewing*, *120*(3), 157–163. http://doi.org/10.1002/jib.145

Hellemond, J. J., Opperdoes, F. R., & Tielens, A. G. (2005). The extraordinary mitochondrion and unusual citric acid cycle in Trypanosoma brucei van. Biochemical Society Transactions, 33(5), 967–971.

Helen, A. and Watson, K. (1976). Thermal Adaptation in Yeast: Growth Temperatures, Membrane Lipid, and Cytochrome Composition of Psychrophilic, Mesophilic, and Thermophilic Yeasts. Journal of Bacteriology, 128(1), pp.56-68.

Heyland, J., Fu, J. and Blank, L. (2009). Correlation between TCA cycle flux and glucose uptake rate during respiro-fermentative growth of *Saccharomyces s cerevisiae*. Microbiology, 155(12), pp.3827-3837.

Huh, W., Falvo, J., Gerke, L., Carroll, A., Howson, R., Weissman, J. and O'Shea, E. (2003). Global analysis of protein localization in budding yeast. Nature, 425(6959), pp.686-691.

Ingledew, W. (2009). *The alcohol textbook*. Nottingham: Nottingham University Press.

Jaynes, J., Ding, X., Xu, H., Wong, W. and Ho, C. (2012). Application of fractional factorial designs to study drug combinations. *Statistics in Medicine*, 32(2), pp.307-318.

Jones, M. and Pierce, J. (1964). ABSORPTION OF AMINO ACIDS FROM WORT BY YEASTS. Journal of the Institute of Brewing, 70(4), pp.307-315.

Jones, C. B. (2005). What can we do (technically) to get “the right specification”? *Verified Software: Theories, Tools, and Experiments (VSTTE 2005). LNCS*, *4171*(1), 64–69. http://doi.org/10.1128/EC.3.1.1

Jones, M. and Pierce, J. (1964). ABSORPTION OF AMINO ACIDS FROM WORT BY YEASTS. Journal of the Institute of Brewing, 70(4), pp.307-315.

Jones, J. G., Sherry, A. D., Jeffrey, F. M., Storey, C. J., & Malloy, C. R. (1993). Sources of acetyl-CoA entering the tricarboxylic acid cycle as determined by analysis of succinate 13C isotopomers. Biochemistry, 32(45), 12240–12244.

Kennedy, A., Taidi, B., Dolan, J. and J.A., H. (1997). Optimisation of a fully defined medium for yeast fermentation studies. Food technology and biotechcnology, 35(4), pp.261-265.

Khuri, A. and Mukhopadhyay, S. (2010). Response surface methodology. Wiley Interdisciplinary Reviews: Computational Statistics, 2(2), pp.128-149.

Klipp, E., Nordlander, B., Krüger, R., Gennemark, P. and Hohmann, S. (2005). Integrative model of the response of yeast to osmotic shock. Nature Biotechnology, 23(8), pp.975-982.

Kneen, M., Stan, R., Yep, A., Tyler, R., Saehuan, C. and McLeish, M. (2011). Characterization of a thiamin diphosphate-dependent phenylpyruvate decarboxylase from Saccharomyces cerevisiae. FEBS Journal, 278(11), pp.1842-1853.

Knight, M., Bull, I. and Curnow, P. (2014). The yeast enzyme Eht1 is an octanoyl-CoA:ethanol acyltransferase that also functions as a thioesterase. Yeast, 31, pp.463–474.

Knoop, V., Groth-Malonek, M., Gebert, M., Eifler, K. and Weyand, K. (2005). Transport of magnesium and other divalent cations: evolution of the 2-TM-GxN proteins in the MIT superfamily. Molecular Genetics and Genomics, 274(3), pp.205-216.

Kocharin, K. and Nielsen, J. (2013). Specific growth rate and substrate dependent polyhydroxybutyrate production in Saccharomyces cerevisiae. AMB Express, 3(1), p.18.

Kopecká, J., Němec, M. and Matoulková, D. (2016). Comparison of DNA-based techniques for differentiation of production strains of ale and lager brewing yeast. Journal of Applied Microbiology, 120(6), pp.1561-1573.

Kratzer, S. and Schüller, H. (1995). Carbon source-dependent regulation of the acetyl-coenzyme A synthetase-encoding gene ACSI from *saccharomyces cerevisiae*. *Gene*, 161(1), pp.75-79.

Kresnowati, M. T. A. P., Van Winden, W. A., Almering, M. J. H., Ten Pierick, A., Ras, C., Knijnenburg, T. A., … Daran, J. M. (2006). When transcriptome meets metabolome: Fast cellular responses of yeast to

sudden relief of glucose limitation. *Molecular Systems Biology*, *2*. http://doi.org/10.1038/msb4100083

Krogerus, K. and Gibson, B. (2013). 125thAnniversary Review: Diacetyl and its control during brewery fermentation. Journal of the Institute of Brewing, (119), pp.86–97.

Kunau, W., Dommes, V. and Schulz, H. (1995). β-Oxidation of fatty acids in mitochondria, peroxisomes, and bacteria: A century of continued progress. *Progress in Lipid Research*, 34(4), pp.267-342.

Kunze, M., Pracharoenwattana, I., Smith, S. M., & Hartig, A. (2006). A central role for the peroxisomal membrane in glyoxylate cycle function. *Biochimica et Biophysica Acta - Molecular Cell Research*, *1763*(12), 1441–1452. http://doi.org/10.1016/j.bbamcr.2006.09.009

Kurtzman CP, Fell JW, Boekhout T, Robert V. 2011. Methods for isolation, phenotypic characterization and maintenance of yeasts. In The Yeasts: A Taxonomic Study, 5th edn, Kurtzman CP, Fell JW, Boekhout T (eds). Elsevier: Amsterdam; 87–110.

Lawless, H., Rapacki, F., Horne, J. and Hayes, A. (2003). The taste of calcium and magnesium salts and anionic modifications. Food Quality and Preference, 14(4), pp.319-325.

Layfield, J. B., & Sheppard, J. D. (2015). What Brewers Should Know About Viability, Vitality, and Overall Brewing Fitness: A Mini-Review. *Technical Quarterly*, *52*(3), 132–140. https://doi.org/10.1094/tq-52-3-0719-01

Lee Y, Jang J, Kim K, Maeng P (2011). TCA cycle-independent acetate metabolism via the glyoxylate cycle in *Saccharomyces cerevisiae*. *Yeast* 28(2):153 – 166.

Legras, J. and Karst, F. (2003). Optimisation of interdelta analysis for Saccharomyces cerevisiae strain characterisation. FEMS Microbiology, 221, pp.249-255.

Li, H., Liu, F., & Industries, F. (2015). Technological Factors Influencing Buffering Capacity of Wort. *Journal of the American Society of Brewing Chemists*, 236–239. https://doi.org/10.1094/ASBCJ-2015-0512-01

Li, H., Liu, F., Kang, L., & Zheng, M. (2016). Study on the buffering capacity of wort. *Journal of the Institute of Brewing*, *122*(1), 138–142. https://doi.org/10.1002/jib.286

Liesen, T., Hollenberg, C. and Heinisch, J. (1996). ERA, a novel cis-acting element required for autoregulation and ethanol repression of PDC1 transcription in Saccharomyces cerevisiae. Molecular Microbiology, 21(3), pp.621-632.

Lim, P. H., Pisat, N. P., Gadhia, N., Pandey, A., Donovan, F. X., Stein, L., … MacDiarmid, C. W. (2011). Regulation of alr1 mg transporter activity by intracellular magnesium. *PLoS ONE*, *6*(6). http://doi.org/10.1371/journal.pone.0020896

Lipinski, C., Lombardo, F., Dominy, B. and Feeney, P. (2001). Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Advanced Drug Delivery Reviews, 46(1-3), pp.3-26.

Ljungdahl, P. O., & Daignan-Fornier, B. (2012). Regulation of amino acid, nucleotide, and phosphate metabolism in Saccharomyces cerevisiae. *Genetics*, *190*(3), 885–929. http://doi.org/10.1534/genetics.111.133306

Luarasi, L., Troja, R., & Pinguli, L. (2016).T*he Relationship Between Yeast Viability and Concentration in the Fermentation Process of Wort*. *European Journal of Biotechnology and Genetic Engineering. 3*(1), 83–86.

Magalhães, F., Vidgren, V., Ruohonen, L. and Gibson, B. (2016). Maltose and maltotriose utilisation by group I strains of the hybrid lager yeast *Saccharomyces pastorianus*. FEMS Yeast Research, 16(5), p.fow053.

Maguire, M. and Cowan, J. (2002). Magnesium chemistry and biochemistry. BioMetals, 15, pp.203-210.

Marechal, P. A., & Gervais, P. (1994). Yeast viability related to water potential variation: influence of the transient phase. *Applied Microbiology and Biotechnology*, *42*(4), 617–622. https://doi.org/10.1007/BF00173929

Van Maris, A., Abbott, D., Bellissimi, E., van den Brink, J., Kuyper, M., Luttik, M., Wisselink, H., Scheffers, W., van Dijken, J. and Pronk, J. (2006). Alcoholic fermentation of carbon sources in biomass hydrolysates by Saccharomyces cerevisiae: current status. Antonie van Leeuwenhoek, 90(4), pp.391-418.

Mason, A. B., & Dufour, J. (2012). Alcohol acetyltransferases and the signi®cance of ester synthesis in yeast, (Figure 1), 1–12. Retrieved from papers2://publication/uuid/501859D4-A32E-4B47-AD90-2B584E5C6392

Meaden, P. G., Dickinson, F. M., Mifsud, A., Tessier, W., Westwater, J., Bussey, H., & Midgley, M. (1997). The ALD6 gene of saccharomyces cerevisiae encodes a cytosolic, Mg2+-activated acetaldehyde dehydrogenase. *Yeast*, *13*(14), 1319–1327. http://doi.org/10.1002/(SICI)1097-0061(199711)13:14<1319::AID-YEA183>3.0.CO;2-T

Morimoto RI, Kroeger PE & Cotto JJ (1996). The transcriptional regulation of heat shock genes: a plethora of heat shock factors and regulatory conditions. EXS 77: 139–163.

Myers, R., Montgomery, D. and Gunst, R. (2009). Response Surface Methodology: Process and Product Optimization Using Designed Experiments. Wiley, 38(3), p.705.

Myers, R.H., Montogomery, D.C. (2002). Response Surface Methodology: Process and Product Optimization Using Designed Experiments, second ed. John Wiley and Sons, NY.

Nevoigt, E., & Stahl, U. (1997). Osmoregulation and glycerol metabolism in the yeast Saccharomyces cerevisiae. *FEMS Microbiology Reviews*, *21*(3), 231–241. http://doi.org/10.1016/S0168-6445(97)00058-2

Olaniran, A. O., Hiralal, L., Mokoena, M. P., & Pillay, B. (2017). Flavour-active volatile compounds in beer: production, regulation and control. *Journal of the Institute of Brewing*, *123*(1), 13–23. https://doi.org/10.1002/jib.389

Page, M.J., and Di Cera, E. (2006). Role of Na^+ and K^+ in enzyme function. Physiol Rev 86: 1049–1092

Parker, N., James, S., Dicks, J., Bond, C., Nueno-Palop, C., White, C. and Roberts, I. (2015). Investigating flavour characteristics of British ale yeasts: techniques, resources and opportunities for innovation. *Yeast*, 32, pp.281–287.

Peyer, L. C., Bellut, K., Lynch, K. M., Zarnkow, M., Jacob, F., De Schutter, D. P., & Arendt, E. K. (2017). Impact of buffering capacity on the acidification of wort by brewing-relevant lactic acid bacteria. *Journal of the Institute of Brewing*, *123*(4), 497–505. https://doi.org/10.1002/jib.447

Pfeiffer, T. and Morley, A. (2014). An evolutionary perspective on the Crabtree effect. Frontiers in Molecular Biosciences, 1.

PIERCE, J. (1970). Institute of Brewing Analysis Committee: measurement of yeast viability. Journal of the Institute of Brewing, 76,442-443.

Pietrocola, F., Galluzzi, L., Bravo-San Pedro, J. M., Madeo, F., & Kroemer, G. (2015). Acetyl coenzyme A: A central metabolite and second messenger. *Cell Metabolism*, *21*(6), 805–821. http://doi.org/10.1016/j.cmet.2015.05.014

Pigeau, G. and Inglis, D. (2007). Response of wine yeast (Saccharomyces cerevisiae) aldehyde dehydrogenases to acetaldehyde stress during

Icewine fermentation. Journal of Applied Microbiology, 103(5), pp.1576-1586.

Pires, E. J., Teixeira, J. A., Brányik, T., & Vicente, A. A. (2014). Yeast: The soul of beer's aroma - A review of flavour-active esters and higher alcohols produced by the brewing yeast. *Applied Microbiology and Biotechnology*, *98*(5), 1937–1949. http://doi.org/10.1007/s00253-013-5470-0

Pisat, N. P., Pandey, A., & MacDiarmid, C. W. (2009). MNR2 regulates intracellular magnesium storage in Saccharomyces cerevisiae. *Genetics*, *183*(3), 873–884. http://doi.org/10.1534/genetics.109.106419

Porter, J. and Bamforth, C. (2016). NOTE: Manganese in Brewing Raw Materials, Disposition During the Brewing Process, and Impact on the Flavor Instability of Beer. Journal of the American Society of Brewing Chemists, 74(2), pp.87-90.

Pratt, P., Bryce, J. and Stewart, G. (2003). The Effects of Osmotic Pressure and Ethanol on Yeast Viability and Morphology. Journal of the Institute of Brewing, 109(3), pp.218-228.

Priest, F. and Stewart, G. (2006). *Handbook of brewing*. Boca Raton: CRC/Taylor & Francis.2ed. 853p.

Pronk, J., Yde Steensma, H. and Van Dijken, J. (1996). Pyruvate Metabolism in *Saccharomyces s cerevisiae*. Yeast, 12(16), pp.1607-1633.

Puligundla, P., Smogrovicova, D., Obulam, V. and Ko, S. (2011). Very high gravity (VHG) ethanolic brewing and fermentation: a research update. Journal of Industrial Microbiology & Biotechnology, 38(9), pp.1133-1144.

Remize, F., Andrieu, E. and Dequin, S. (2000). Engineering of the Pyruvate Dehydrogenase Bypass in *Saccharomyces s cerevisiae*: Role of the Cytosolic Mg2+ and Mitochondrial K+ Acetaldehyde

Dehydrogenases Ald6p and Ald4p in Acetate Formation during Alcoholic Fermentation. Applied and Environmental Microbiology, 66(8), pp.3151-3159.

Ribeiro, N., Pimenta Filho, E., Arandas, J., Ribeiro, M., Saraiva, E., Bozzi, R. and Costa, R. (2015). Multivariate characterization of the adaptive profile in Brazilian and Italian goat population. Small Ruminant Research, 123(2-3), pp.232-237.

Rodríguez-Navarro, A. (2000). Potassium transport in fungi and plants. Biochimica et Biophysica Acta (BBA) - Reviews on Biomembranes, 1469(1), pp.1-30.

Ruis, H.; Schüller, C. (1995). Stress signaling in yeast. *BioEssays*. 17, 959-965.

Saerens, S. M. G., Delvaux, F. R., Verstrepen, K. J., & Thevelein, J. M. (2010). Production and biological function of volatile esters in Saccharomyces cerevisiae. *Microbial Biotechnology*, *3*(2), 165–177. http://doi.org/10.1111/j.1751-7915.2009.00106.x

Saerens, S. M. G., Delvaux, F., Verstrepen, K. J., Van Dijck, P., Thevelein, J. M., & Delvaux, F. R. (2008). Parameters affecting ethyl ester production by Saccharomyces cerevisiae during fermentation. *Applied and Environmental Microbiology*, *74*(2), 454–461. http://doi.org/10.1128/AEM.01616-07

Sainson, D. (2006). Main flavour changes in aged speciality beers, 61–91. http://doi.org/10.1146/annurev.energy.31.020105.100253

Shi, L. and Tu, B. (2015). Acetyl-CoA and the regulation of metabolism: mechanisms and consequences. Current Opinion in Cell Biology, 33, pp.125-131.

Silva, J. A.; Damasceno, B.P.G.L.; Silva, F. L. H.; Madruga, M. S.; Santana, D. P. (2008). Application of the factorial design and response surface methodology for optimization of alcoholic fermentation. *Quim. Nova,* Vol. 31, No. 5, 1073-1077.

Silva, J. A.; Silva, F. L. H.; Alves, R. R. N.; Santana, D. P. (2006). Influence of nitrogen, phosphorus and ºbrix on the production of total secondary contaminant metabolites (MSCT) of alcoholic fermentation. *Quim. Nova,* Vol. 29, No. 4, 695-698.

Smart, K. A. (2003). Brewing yeast fermentation performance. Oxford, UK: Blackwell Science, chapter 8. 308p.

Spitaels, F., Wieme, A., Janssens, M., Aerts, M., Daniel, H., Van Landschoot, A., De Vuyst, L. and Vandamme, P. (2014). The Microbial Diversity of Traditional Spontaneously Fermented Lambic Beer. PLoS ONE, 9(4), p.e95384.

Stassi, P., Rice, J. F., Munroe, J. H., and Chicoye, E. (1987) Use of CO_2 evolution rate for the study and control of fermentation. Tech. Q. Master Brew. Assoc. Am. 24:44-50.

Stehlik-Tomas, V., Zetić, V. G., Stanzer, D., Grba, S., & Vahčić, N. (2004). Zinc, copper and manganese enrichment in yeast Saccharomyces cerevisae. *Food Technology and Biotechnology*, *42*(2), 115–120. http://doi.org/10.1080/17451000903042461

Stewart, G. (2017). The Production of Secondary Metabolites with Flavour Potential during Brewing and Distilling Wort Fermentations. *Fermentation*, *3*(4), 63. http://doi.org/10.3390/fermentation3040063

Swiegers, J., Bartowsky, E.J., Henschke, P., Pretorius, I.S. (2005). Yeast and bacterial modulation of wine aroma and flavour. Aust. J. Grape Wine Res. 11, 139-173.

Szczypka, M., Zhu, Z., Silar, P. and Thiele, D. (1997). Saccharomyces cerevisiae mutants altered in vacuole function are defective in copper detoxification and iron-responsive gene transcription. *Yeast*, 13(15), pp.1423-1435.

Taidi, B., Kennedy, A. and Hodgson, J. (2003). *Brewing yeast fermentation and performance - Chapter 8: Wort Substitutes and Yeast Nutrition*.

2nd ed. Oxford Brookes University Oxford, UK: Blackwell Science Ltd, pp.86-95.

Takahashi, H., McCaffery, J., Irizarry, R. and Boeke, J. (2006). Nucleocytosolic Acetyl-Coenzyme A Synthetase Is Required for Histone Acetylation and Global Transcription. *Molecular Cell*, 23(2), pp.207-217.

Thompson, C.C., Leedham, P.A. and Lawrence, D.R. (1973) Proc. Am. Soc. Brew. Chem., p. 137.

Titica M., Landaud S., Trelea I.C., Latrille E., Corrieu G., Chéruy A (2000). 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 American Society of Brewing Chemist, pp. 167-174

Turrens, J. F., and Boveris, A. (1980). Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. *Biochem. J.* 191, 421–427

Udeh, H. O., Kgatla, T. E., & Jideani, A. I. O. (2014). Effect of Mineral Ion Addition on Yeast Performance during Very High Gravity Wort Fermentation, *8*(11), 1208–1216.

Udeh, O. (2013). Role of magnesium ions on yeast performance during very high gravity fermentation. *Journal of Brewing and Distilling*, *4*(2), 19–45. http://doi.org/10.5897/JBD2013.0041

Van Maris, A., Abbott, D., Bellissimi, E., van den Brink, J., Kuyper, M., Luttik, M., Wisselink, H., Scheffers, W., van Dijken, J. and Pronk, J. (2006). Alcoholic fermentation of carbon sources in biomass hydrolysates by Saccharomyces cerevisiae: current status. Antonie van Leeuwenhoek, 90(4), pp.391-418.

Verbelen, P., Saerens, S., Van Mulders, S., Delvaux, F. and Delvaux, F. (2009). The role of oxygen in yeast metabolism during high cell density

brewery fermentations. Applied Microbiology and Biotechnology, 82(6), pp.1143-1156.

Verbelen, P., Saerens, S., Van Mulders, S., Delvaux, F. and Delvaux, F. (2008). The role of oxygen in yeast metabolism during high cell density brewery fermentations. Applied Microbiology and Biotechnology, 82(6), pp.1143-1156.

Verstrepen, K. J., Derdelinckx, G., Dufour, J. P., Winderickx, J., Thevelein, J. M., Pretorius, I. S., & Delvaux, F. R. (2003). Flavor-active esters: Adding fruitiness to beer. *Journal of Bioscience and Bioengineering*, *96*(2), 110–118. http://doi.org/10.1016/S1389-1723(03)90112-5

Vidal, E., de Morais Junior, M., François, J. and de Billerbeck, G. (2014). Biosynthesis of higher alcohol flavour compounds by the yeast *Saccharomyces s cerevisiae*: impact of oxygen availability and responses to glucose pulse in minimal growth medium with leucine as sole nitrogen source. Yeast 32: 47–56.

Vuralhan, Z., Morais, M. A., Tai, S. L., Piper, M. D. W., & Pronk, J. T. (2003). Identification and characterization of phenylpyruvate decarboxylase genes in Saccharomyces cerevisiae. *Applied and Environmental Microbiology*, *69*(8), 4534–4541. http://doi.org/10.1128/AEM.69.8.4534-4541.2003

Walker, G. M., Nicola, R. De, Anthony, S., & Learmonth, R. (2000). Yeast-metal interactions: impact on brewing and distilling fermentations. *Enzyme and Microbial Technology*, *26*, 678–687.

Walker GM (2004). Metals in yeast fermentation processes. Adv. Appl. Microbiol. 54:197-230.

Walker, G.M. Yeasts. *In Eukaryotic Microbes*; Schaechter, M., Ed.; Academic Press/Elsevier Science Publishers: Oxford, UK, **2011**; pp. 3–17.

Walker, G. and Stewart, G. (2016). Saccharomyces cerevisiae in the Production of Fermented Beverages. Beverages, 2(4), p.30.Walker et al. 2000).

Walker GM, De Nicola R, Anthony S, Learmonth R (2006). Yeast-metal interactions: impact on brewing and distilling fermentations. J. Inst. Brew Dist. APSC. pp.19-24.

Walker, G., Birch, R., Chandrasena, G. and Maynard, A. (1996). Magnesium, Calcium, and Fermentative Metabolism in Industrial Yeasts. Journal of the American Society of Brewing Chemists, 54(1), pp.13-18.

White, W. H., Skatrud, P. L., Xue, Z., & Toyn, J. H. (2003). Specialization of Function Among Aldehyde Dehydrogenases : *Genetics*, *163*(January), 69–77. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1462426&tool=pmcentrez&rendertype=abstract

Wietstock, P. C., Kunz, T.*;* Waterkamp, H., Methner F.J. (2015). Uptake and Release of Ca, Cu, Fe, Mg, and Zn During Beer Production. *Journal of the American Society of Brewing Chemists*, 73(2):179-184.

Wolf, F. I., and V. Trapani, 2008 Cell (patho)physiology of magnesium. Clin. Sci. (Lond.) 114: 27–35.

Wu, CFJ.; Hamada, M. (2009). Experiments: Planning, Analysis and Parameter Design Optimization. 2. Wiley; New York: 2009.

Xu, Y., Wang, D., Li, H., Hao, J. Q., Jiang, W., Liu, Z., & Qin, Q. (2017). Flavor contribution of esters in lager beers and an analysis of their flavor thresholds. *Journal of the American Society of Brewing Chemists*, *75*(3), 201–206. https://doi.org/10.1094/ASBCJ-2017-3007-01

Yoshioka, K., & Hashimoto, N. (1981). Ester Formation by Alcohol Acetyltransferase from Brewers' Yeast. *Agricultural and Biological Chemistry*, *45*(10), 2183–2190. https://doi.org/10.1271/bbb1961.45.2183

Yu, A., Pratomo Juwono, N., Foo, J., Leong, S. and Chang, M. (2016). Metabolic engineering of Saccharomyces cerevisiae for the overproduction of short branched-chain fatty acids. Metabolic Engineering, 34, pp.36-43

Zatta, P., Lain, E., & Cagnolini, C. (2000). Effects of aluminium on activity of Krebs cycle enzymes and glutamate dehydrogenase in rat brain homogenate. European Journal of Biochemistry, 267(10), 3049–3055

Zhang, L., Tang, Y., Guo, Z. and Shi, G. (2013). Engineering of the glycerol decomposition pathway and cofactor regulation in an industrial yeast improves ethanol production. Journal of Industrial Microbiology & Biotechnology, 40(10), pp.1153-1160.

Zhuang, S., Fu, J., Powell, C., Huang, J., Xia, Y., & Yan, R. (2015). Production of medium-chain volatile flavour esters in Pichia pastoris whole-cell biocatalysts with extracellular expression of Saccharomyces cerevisiae acyl-CoA:ethanol O-acyltransferase Eht1 or Eeb1. *SpringerPlus*, *4*(1). http://doi.org/10.1186/s40064-015-1195-0

Zufall, C. and Tyrell, T. (2008). The Influence of Heavy Metal Ions on Beer Flavour Stability. *Journal of the Institute of Brewing*, 114(2), pp.134-142.