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The impact of amino acid availability and gene transcription on aroma compound profiling in *Saccharomyces* yeast

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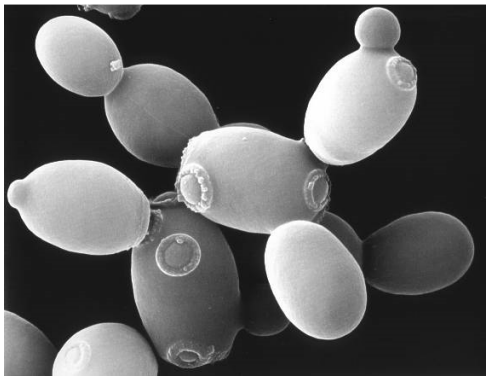

Crazy little thing called yeast!

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PUBLICATIONS

Peer reviewed publications

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3. Fischer, S., Procopio, S., Becker, T.: Self cloning brewing yeast: a new dimension in beverage production. European Food Research and Technology 237 (2013), 851–863.
4. **Procopio, S., Brunner, M., Becker, T.: Differential transcribed yeast genes involved in flavour formation and its associated amino acid metabolism during brewery fermentation. European Food Research and Technology 239 (2014), 421-439.**
5. **Procopio, S., Sprung, P., Becker, T.: Effect of amino acid supply on the transcription of flavour-related genes and aroma compound production during lager yeast fermentation. Food Science and Technology 63 (2015), 289-297.**
6. Steiner, J., Procopio, S., Becker, T.: Brewer's spent grain: source of value-added polysaccharides for the food industry in reference to the health claims. European Food Research and Technology (2015), DOI 10.1007/s00217-015-2461-7.
7. Kupetz, M; Procopio, S.; Sacher, B. and Becker, T.: Critical Review of the Methods of beta-Glucan Analysis and Its Significance in the Beer Filtration Process. European Food Research and Technology (2015). DOI 10.1007/s00217-015-2498-7.
8. Zhao X.; Procopio, S., Becker, T.: Flavor impacts of glycerol in the processing of yeast fermented beverages: a review. Journal of Food Science and Technology (2015). DOI 10.1007/s13197-015-1977-y.
9. Fischer, S., Engstle, C., Procopio, S., Becker, T.: EGFP-based evaluation of temperature inducible native promoters of industrial ale yeast by using a high throughput system. Food Science and Technology (2015). DOI 10.1016/j.lwt.2015.12.020.
10. Kerpes, R., Knorr, V., Procopio, S., Koehler, P., Becker, T.: Gluten-specific peptidase activity of barley as affected by germination and its impact on gluten degradation. Journal of Cereal Science (2016). DOI 10.1016/j.jcs.2016.01.004.

Non peer reviewed publications

1. Procopio, S., Brunner, M., Becker, T.: Sequentielle Aminosäure-Aufnahme von *Saccharomyces* Hefen. Der Weihenstephaner 82. Jahrgang 4 (2014), 146-151.
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CONFERENCE CONTRIBUTIONS

Oral presentations

1. Procopio, S., Qian, F., Becker, T.: Einfluss der Stickstoffassimilation und Hefegenexpression auf die Synthese sensorisch aktiver Aminosäuremetabolite. 44. Technologisches Seminar. Freising, Germany, 2011.
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4. Procopio, S., Becker, T.: Impact of nutrient availability on aroma-profiling during yeast fermentation. 13th International Congress on Yeasts. Madison, Wisconsin, USA, 2012.
5. Procopio, S., Becker, T.: Fermentative Glycerinerhöhung in Bier. 46. Technologisches Seminar. Freising, Germany, 2013.
6. Procopio, S., Becker, T.: Einfluss signifikanter Aminosäuren auf die Bildung von höheren Alkoholen und Estern im Bier. 47. Technologisches Seminar. Freising, Germany, 2014.
7. Procopio, S., Becker, T.: Einfluss der Aminosäureassimilation auf das Aromaprofil von *Saccharomyces* Hefen. GDL Kongress – Lebensmitteltechnologie. Rodgau, Germany, 2014.
8. Procopio, S., Becker, T.: Herausforderungen an den Fermentationsprozess malzbasierter Vinegar Drinks. 47. Technologisches Seminar. Freising, Germany, 2015.

Poster presentations

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CONTENTS

ABBREVIATIONS

AATase	alcohol acetyltransferase
AEATase	acyl-CoA:ethanol O-acyltransferase
B_S	regression coefficient
BCAA	branched chain amino acid
BCAATases	branched chain amino acid transferase
GATA	guanine-adenine-thymine-adenine
MCFA	medium chain fatty acids
NCR	nitrogen catabolite repression
mRNA	messenger ribonucleic acid
PLS	partial least square
PLSR	partial least square regression
qPCR	quantitative polymerase chain reaction
RSM	response surface methodology
UFAs	unsaturated fatty acids
VIP	variable importance in the projection

SUMMARY

Aroma is one of the most important quality characters of yeast fermented beverages. The production of beers with various aroma profiles is observed as a current trend in the brewing industry to offer a variety of beers to a new developing market. *Saccharomyces* yeast is responsible for fermentative activity and strongly influences the quality and aroma characters of the final product. Several studies have been performed to investigate the influence of different fermentation parameters, including osmotic pressure, oxygen content in wort and the nitrogen source and its availability on final sensory active metabolites. Amino acid assimilation by yeast during wort fermentation is linked to the aroma profile. However, many aspects of the genetic and metabolic processes that occur during alcoholic fermentation and the accompanying aroma compound production remain poorly understood.

Accordingly, in the first part of this thesis, the significance of wort amino acids in the production of important higher alcohols and esters during fermentation is discussed. The focus of the investigations was on amino acid variation in synthetic media, calculated by an experimental design that reduced primary insignificant parameter variation. Aroma-active higher alcohols and esters produced in each fermentation were quantified, and their correlation with the most significant amino acids for two different *Saccharomyces* yeast strains was determined. Multivariate data analyses were used to establish the relationship between amino acids and the resulting concentrations of aroma compounds by means of pattern recognition. Thus, a fingerprint of amino acid importance in the detected aroma compound spectrum was created.

In the second part of the study, DNA microarray analyses were performed. The results provide a comprehensive overview of the transcriptomic changes during model beer fermentation, with focus on the transcription of genes associated with amino acid assimilation and its derived aroma-active compounds and will serve as a reference for further studies. In subsequent experiments, the effect of amino acid supply on the transcription level of genes involved in the biosynthesis of aroma-relevant higher alcohols and esters was investigated. Amino acids were chosen according to the previous multivariate data analysis. These results show that the supply of single amino acids can determine the final concentrations of some important aroma-contributing metabolites. This provides evidence that the differential expression of genes participating in the biosynthesis of aroma-active compounds is affected by the concentrations of some amino acids. This information may help to improve understanding of the importance of specific aroma biosynthesis genes in the production of defined concentrations of aroma-active higher alcohols and esters during brewery fermentation.

ZUSAMMENFASSUNG

Aroma wird als eines der wichtigsten Merkmale für die Qualität hefefermentierter Getränke diskutiert. Ein derzeitiger Trend in der Brauindustrie besteht darin, möglichst viele Biere mit unterschiedlichen Aromaausprägungen zu produzieren, um den Verbrauchern eine variierende Auswahl an Bieren am sich stetig wandelnden Markt anbieten zu können. Hierbei haben die für die Fermentation verantwortlichen *Saccharomyces* Hefen entscheidenden Einfluss auf die Geruchs- und Geschmacksausprägung des finalen Produktes. Mehrere Studien haben sich bereits mit der Auswirkung der Fermentationsparameter, wie osmotischer Druck, Sauerstoffgehalt als auch der Stickstoffquelle und deren Verfügbarkeit in der Würze, auf die Synthese sensorisch aktiver Metaboliten beschäftigt. Gerade die Aminosäureassimilation der Hefe während der Fermentation scheint stark mit dem daraus resultierenden Aromaprofil verknüpft zu sein. Dennoch sind nicht alle genetischen und metabolischen Vorgänge während der Hefefermentation und der damit einhergehenden Synthese der Aromastoffe vollständig aufgeklärt.

In dem ersten Teil der vorliegenden Arbeit wurde zunächst die Signifikanz der in der Würze vorliegenden Aminosäuren für die Synthese der bedeutenden höheren Alkohole und Ester ermittelt. Der Schwerpunkt der Untersuchungen lag auf Aminosäurevariationen in einem synthetischen Medium. Die synthetischen Würzen wurden jeweils mit zwei verschiedenen *Saccharomyces* Hefestämmen fermentiert, die charakteristischen aroma-aktiven höheren Alkohole und Ester quantifiziert und deren Korrelation zu den signifikanten Aminosäuren ermittelt. Mittels multivariater Datenanalyse wurde die Beziehung zwischen den Aminosäuren und den daraus resultierenden Konzentrationen der einzelnen Aromastoffe evaluiert.

In einem zweiten Teil der Thesis wurden DNA-Microarrays angefertigt, deren Daten einen umfassenden Überblick über die Änderungen des Hefetranskriptoms während der Fermentation, mit Fokus auf die Expression von Genen des Aminosäuremetabolismus und der Aromastoffbiosynthese, bieten. In weiterführenden Experimenten wurde die Wirkung der zuvor als signifikant ermittelten Aminosäuren auf den Transkriptionslevel aroma-relevanter Gene untersucht. Hierbei konnte einigen Aminosäuren eine induzierende Wirkung hinsichtlich der Genexpression zugeschrieben werden. Auch die Endkonzentrationen der am Bieraroma maßgeblich beteiligten höheren Alkohole und Ester korrelieren mit den Aminosäuredotierungen. Diese Ergebnisse deuten darauf hin, dass eine gezielte Aminosäurezusammensetzung die Expression aroma-relevanter Gene beeinflusst und dazu beitragen kann, definierte Mengen an aroma-aktiven Metaboliten in fermentierten Getränken biosynthetisch zu generieren, um somit spezifische sensorische Eindrücke im Bier einzustellen.

1. INTRODUCTION

1.1 Consumer preferences for specific beer flavour characteristics

Odour and taste are two of the most important quality attributes of yeast fermented beverages. The current trend in the brewing industry consists of producing beers with different aroma profiles to offer a variety of beers to a developing market to meet specific consumer preferences. Beer aroma is the result of a plurality of interactions between chemical compounds that can exert synergistic or antagonistic effects. The chemical profile of beer is derived from the raw materials: barley and hops, the fermenting yeast and the ageing and storage conditions. The composition of beer has been intensively studied, and compounds have been identified that are responsible for certain individual aroma profiles of various beer types (Meilgaard, 1975a, 1975b; Saerens, Delvaux, Verstrepen, & Thevelein, 2010; Verstrepen, Derdelinckx, Dufour, Winderickx, Thevelein, et al., 2003a). The various flavours of the many styles of beer generally fall into two classes: lager and ale. Together, these two classes of beer collectively account for thousands of varieties of beer, such as India Pale Ale, Stout, Pilsner, Export and Wheat beers.

In general, the global beer market is around US$500 billion each year, and around 5% of this sum is captured by the fast-growing craft and specialty brewing sector. There is a global demand for new beers with novel flavours. It is predicted to grow further over the next few years as more breweries respond to this consumer demand. Several thousand unique varieties are created from different combinations of ingredients and added flavours. Fruit, vegetables and spices are added to both lagers and ales to create beverages with flavours such as cherry wheat and pumpkin spice. Spirit-flavoured brews such as rum, bourbon and tequila beers have become popular, particularly in the UK beer market.

Nowadays, hops are of great interest and used mainly to produce special citric, mandarin- and litchi-like aromas (Lutz, Kneidl, Kammhuber, & Seigner, 2013). Thus, brewers focus on identifying new varieties of hops that can deliver these desired flavour and aroma characteristics. Hop flavours and aromas come mainly from essential oils that are easily dissolved into hot wort and tend to fall into several categories: floral, fruity, citrus, herbal, earthy, piney and spicy. More specific descriptors include geranium, berry, grapefruit, orange, minty, grassy, woody, resinous, liquorice and pepper. The high volatility of these hop oils has led to the dry hopping technique. The basic dry hop technique is to add hops post-fermentation to capture the aroma oils in the beverage (De Keukeleire, 2000). However, when beers are divided into different categories, it turns out that lager is the most favoured beer in the world (see Table 1 - 1). Approximately 94% of the beer market is accounted for by lagers,

which are bottom-fermenting and have an alcohol content between 4.5% and 5.5% (v/v) with low hop bitterness (Eßlinger, 2009).

Table 1 – 1: Global beer styles 2004 (Eßlinger, 2009).

Style	Volume (THL)	Growth rate 1999–2004 (%)	Market share (%)
Specialities	22,497	2.90	1.46
Stout	14,181	2.10	0.92
Ale	34,389	-3.20	2.23
Premium lager	138,929	3.80	9.00
Mainstream lager	1,303,042	2.80	84.46
Non/low alcohol beer	29,791	3.20	1.93
Total market	1,542,829	2.70	
All premium	191,735	3.30	12.43

It is quite evident that many consumers do not prefer the over-fruity, grassy characteristics of strongly hopped beers but still want to enjoy different-tasting products. Besides these aroma hops, several aroma and flavour expressions in beer are based on the yeast fermentation process. Fermentation has the largest influence on flavour development of beer, wine, sake and cider (Boulton & Quain, 2001; Cordente, Curtin, Valera, & Pretorius, 2012). Along with ethanol and carbon dioxide as well as aldehydes, organic acids, organic sulphides and carbonyl compounds, brewing yeast cultures produce a broad range of aroma-active higher alcohols and esters (Trelea, Titica, & Corrieu, 2004; Vanderhaegen et al., 2003) (see Fig. 1 - 1).

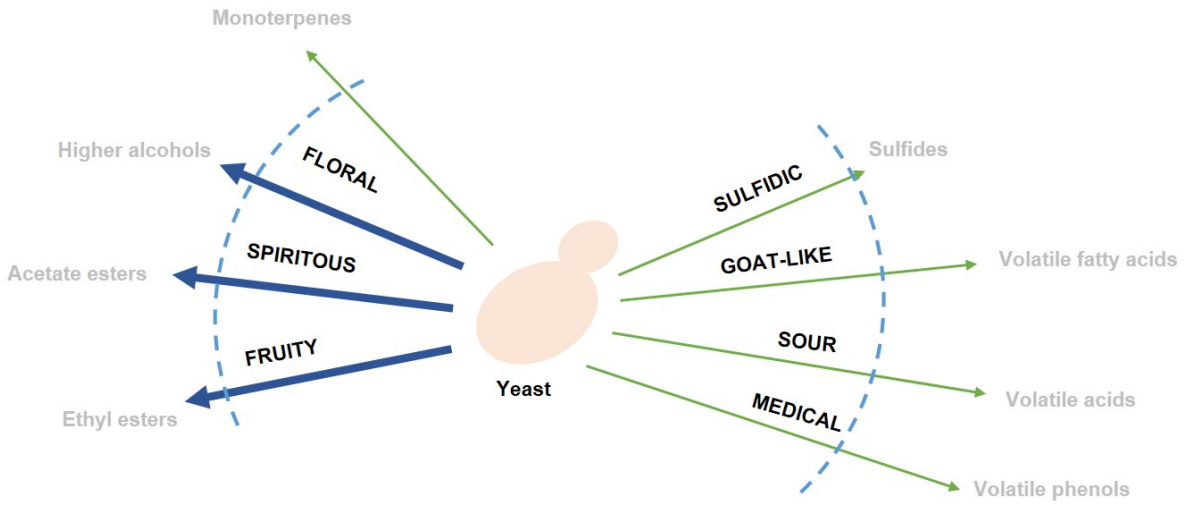

Figure 1 - 1: Different flavour compounds produced by brewing yeast (modified according to Cordente *et al*., 2012).

Aroma-active higher alcohols and esters are produced intracellularly by fermenting yeast cells and are of major industrial interest because of their contribution to the aroma in fermented beverages (Cristiani & Monnet, 2001; Dufour, Verstrepen, & Derdelinckx, 2002; Pisarnitskii, 2001; Quain & Duffield, 1985; Swiegers & Pretorius, 2005a). Of all secondary metabolites, higher alcohols are generally produced by yeast in the highest absolute concentrations. Volatile esters are also very important for aroma formation in fermented beverages. They are present in only trace quantities but can affect beer or wine flavour well below their threshold value (Verstrepen, Derdelinckx, Dufour, Winderickx, Thevelein, et al., 2003a; Verstrepen, Van Laere, et al., 2003b). The organoleptic characteristics of fermented beverages, particularly beer, depend mainly on the concentrations of aroma-active substances (Table 1 - 2).

Table 1 - 2: Concentration ranges of important higher alcohols and esters in different fermented beverages (Annemüller, 2009; Beech, 1993; Fukuda, Watanabe, Asano, Ouchi, & Takasawa, 1992; Furukawa, Yamada, Mizoguchi, & Hara, 2003; Hirooka, Yamamotoa, Tsutsuia, & Tanakab, 2004; Kobayashi, Ikeura, Odake, & Hayata, 2013; Krüger & Anger, 1990; Molina, Swiegers, Varela, Pretorius, & Agosin, 2007; Rankine, 1967; Rodríguez-Madrera, García-Hevia, Palacios-García, & Suárez-Valles, 2008; Sumby, Grbin, & Jiranek, 2010; Swiegers, Bartowsky, Henschke, & Pretotius, 2005b; Valles, Bedrinana, Tascón, Simon, & Madrera, 2007; Williams, Lewis, & Tucknott, 1980).

Aroma compound	Lager beer	Ale beer	Cider	Wine	Sake	Odour quality
Alcohols (mg/L)						
Propanol	5–10	15–30	13–15	16–23	5–114	Alcoholic, fusel-like
Isobutanol	30–50	20–60	7–15	52–110	15–31	Ethereal winey
Isoamyl alcohol	8–30	40–100	65–68	109–241	60–218	Fusel, alcoholic, pungent
2-Methyl butanol	15–25	15–30	53–62	34–45	unknown	Roasted onion, malty
Phenyl ethanol	8–35	8–35	1.3–2.1	18–66	9–100	Flowery, honey-like
Esters (mg/L)						
Ethyl acetate	15–25	10–50	15–35	5–64	3.7–15	Ethereal, Solvent-like
Isobutyl acetate	0.01–0.2	0.01–0.2	tr	tr–0.17	0–0.6	Sweet, fruity, ethereal, apple banana nuance
Isoamyl acetate	0.5–1.5	0.5–8	0.64–0.868	0.03–5.5	0.76–9.8	Sweet, banana, fruity
2-Methylbutyl acetate	0.04–0.053	0.1–0.4	0.02–0.2	0.8	unknown	Estery, ripe, tropical
Ethyl hexanoate	0.05–0.3	0.05–0.3	0.16	0.15–1.64	0.87	Pineapple, apple-like
Ethyl octanoate	0.1–1.5	0.1–1,5	0.19	0.14–2.61	0.77	Waxy, sweet, musty, pineapple
Ethyl decanoate	0.1–0.3	0.1–0.3	0.17	0.01–0.7	unknown	Sweet, waxy, fruity, apple

tr=trace

An important consideration in fermentation and flavour maturation is the fine tuning of the concentrations of all volatile compounds. In particular, the characterisation of different wine yeast species for byproduct formation has shown that the yeast species itself is a prominent factor determining flavour composition (Brandolini et al., 2002; Romano, 1997).

The characterisation of *Saccharomyces cerevisiae* has revealed that this yeast generates many secondary metabolites that are key determinants of quality (Fleet, 1990; Lambrechts & Pretorius, 2000). The balance of the resulting beer flavour metabolites is a consequence of the combination of the yeast strain and wort composition. In addition, a major aim of fermentation management is to control conditions in such a way as to ensure that these aroma-active compounds are produced in desired quantities (Boulton & Quain, 2001). As part of assuring the quality of the product, breweries maintain panels to ensure that the flavour profiles of their beers remain typical. In fulfilling specific consumer preferences, there may be a balance between the attempt to keep a specific aroma alive and the creation of a new aroma.

1.2 Transition from brewer's wort to the final product: fermentation

The biochemistry of brewery fermentation is complex, and many of its aspects remain to be fully clarified. In general, during fermentation, wort created from raw materials is converted to beer by *Saccharomyces* yeast. *S. cerevisiae* has been used for at least eight millennia in the production of alcoholic beverages (McGovern et al., 2004). All malt wort provides a medium with the potential to produce new yeast biomass, ethanol and flavour components in balanced and desired quantities. In a brewery fermentation, metabolism is fermentative, and respiration, in the sense of complete oxidation of sugars to carbon dioxide and water, does not occur. Thus, the major products of sugar catabolism are ethanol and carbon dioxide, which are produced in equimolar amounts (Boulton & Quain, 2001). The yield of carbon dioxide is reduced, with a small quantity used by the yeast cells for anabolic carboxylation reactions (Oura, Haarasilta, & Londesborough, 1980). Further, the quantity of oxygen that is supplied at the beginning of brewery fermentation acts to regulate the relation of carbohydrates used for yeast biomass and the transformation to ethanol. During batch fermentation there are changes in the rates of ethanol production. Thus, there is an initial lag phase, which corresponds with the yeast biomass from lag to exponential growth. During the latter period, the rate of ethanol formation reaches a maximum. Soon after yeast growth terminates, the rate of ethanol production declines (see Fig. 1 - 2) as a consequence of a combination of nutrient depletion and the toxic effects of ethanol.

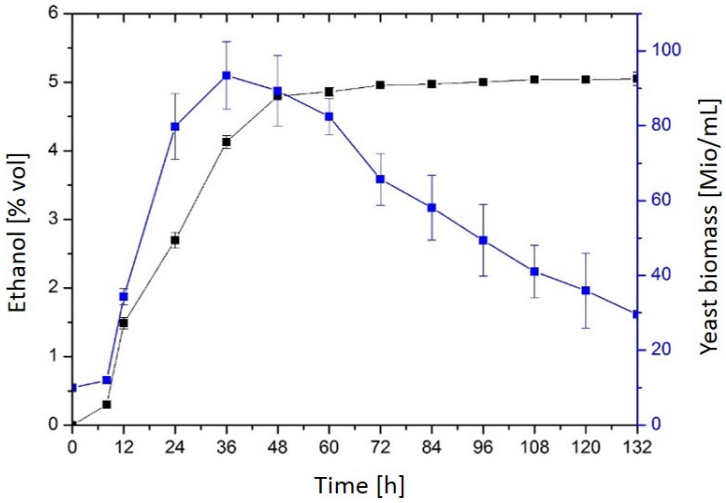

Figure 1 - 2: Alcohol formation and yeast biomass production under standard conditions in EBC tall-tube fermentations (Procopio, unpublished data).

Alcoholic fermentation is initiated by the dissimilation of glucose. The principal pathway predominantly used by all yeasts is the Embden–Meyerhoff glycolytic pathway (Fig. 1 - 3).The product of glycolysis, pyruvate, which is the simplest of the α-keto acids, occupies a major branch point in several metabolic pathways (Fig. 1 – 4). Pyruvic acid can be produced from glucose via glycolysis and converted back to carbohydrates via gluconeogenesis or to fatty acids via acetyl-CoA. It can also be used to construct the amino acid alanine and be converted into ethanol. With respect to sugar catabolism, carbon flow may be directed towards acetyl-CoA and subsequent oxidation via the tricarboxylic acid cycle and oxidative phosphorylation when oxygen is present during aerobic respiration or alternatively into ethanol formation via acetaldehyde when oxygen is lacking under anaerobic fermentation. *S. cerevisiae* is usually described as being facultatively fermentative. It may be assumed that the sole metabolic trigger that switches metabolism between these two modes is the availability of oxygen. Modulation of the concentration of dissolved oxygen in the fermentation medium is a central part of the strategy by which yeast growth is regulated (Boulton & Quain, 2001).

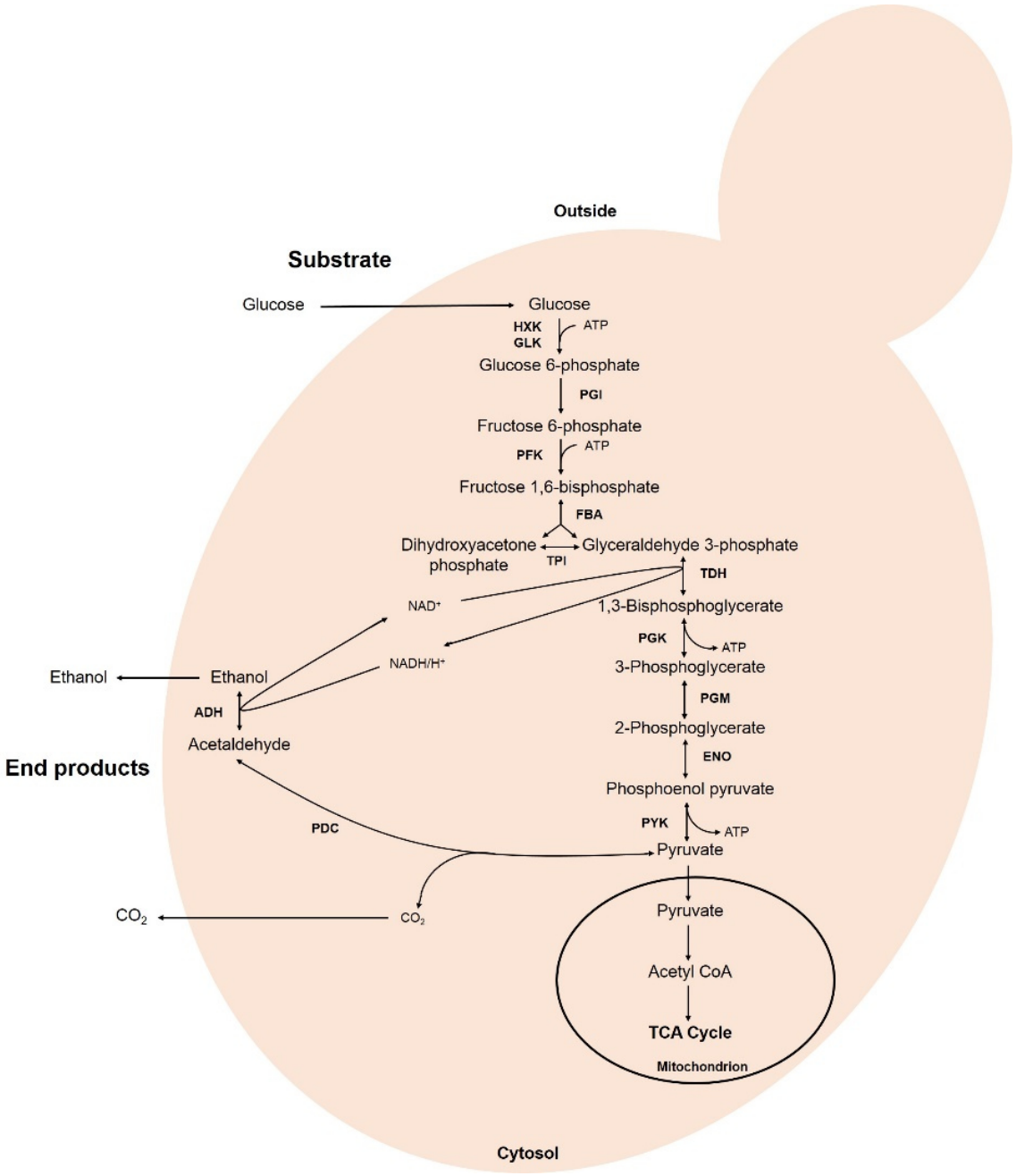

Figure 1 – 3: Emden-Meyerhoff pathway for dissimilation of glucose to ethanol in yeast via glycolysis.

Yeast growth normally requires added oxygen to synthesise sterols and unsaturated fatty acids (UFAs), which are essential components of membranes (Nes, Janssen, Crumley, Kalinoswki, & Akihisi, 1993). Lipid synthesis and optimal growth of *S. cerevisiae* cells during alcoholic fermentation require oxygen at approximately 5–7.5 mg/L (Rosenfeld, Beauvoit, Blondin, & Salmon, 2003). Under brewery and cider-making conditions, lower requirements of oxygen (between 1.5 and 2 mg/L) were found (David & Kirsop, 1973; O'Connor-Cox, Lodolo, & Axcell, 1993). However, if there is an exogenous source of UFAs and sterols, yeast is able to grow under strictly anaerobic conditions (Andreasen & Stier, 1953, 1954). The ability

to grow under strictly anaerobic conditions is sporadic in yeast. The quantity of oxygen required for fermentation is yeast strain dependent (Jacobsen & Thorne, 1980; Kirsop, 1974), possibly owing to differences in the necessity of mitochondrial functions. In particular, ale strains have been classified into several groups: half-air saturation, air saturation, oxygen saturation and more than oxygen saturation based, to achieve satisfactory fermentation performance (Kirsop, 1974). Similar strain-specific oxygen conditions have been observed for lager yeast strains (Jacobsen & Thorne, 1980).

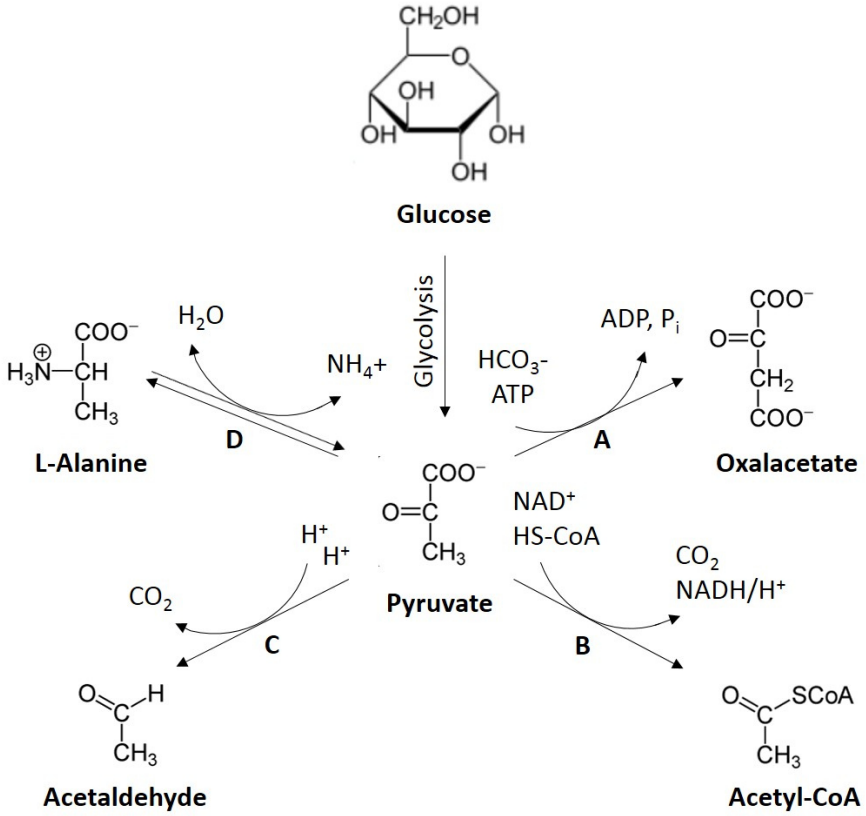

Figure 1 – 4: Pyruvate as branch-point for several metabolic pathways. Pyruvate (A) is carboxylated to oxalacetate - initial point for gluconeogenesis, (B) can enter into the fatty acid biosynthesis through acetyl-CoA, (C) can enter into the alcoholic fermentation, and (D) can be transaminated (with L-glutamic acid as reactant) to L-alanine.

Several parameters affect the process of brewery yeast fermentation. Some general areas of discussion that influence the biochemistry of fermentation are considered. These are wort nutrient composition and yeast regulatory mechanism as influenced by fermentation practice.

1.2.1 Catabolic route vs. anabolic route: the two routes of higher alcohol biosynthesis

In 1907, Ehrlich proposed that higher alcohols are derived from amino acid catabolism (Ehrlich, 1907). The structural molecular similarities of 2-methylbutanol and isoleucine as well as of isoamyl alcohol and leucine led Ehrlich to the research question of whether or not these amino acids were involved in higher alcohol synthesis. Neubauer and Fromherz suggested that α-keto acids were the intermediates in this process, completing the metabolic scheme (Neubauer & Fromherz, 1911). The elementary enzymatic chain reaction for the Ehrlich pathway and the related mechanism of the formation of higher alcohols from amino acids were demonstrated by Sentheshanmuganathan and Elsden (Sentheshanmuganathan & Elsden, 1958; Sentheshanuganathan, 1960). Thus, the key reaction sequence for the Ehrlich pathway: transaminase, decarboxylase and alcohol dehydrogenase, was established (Sentheshanuganathan, 1960) (Fig. 1 – 5).

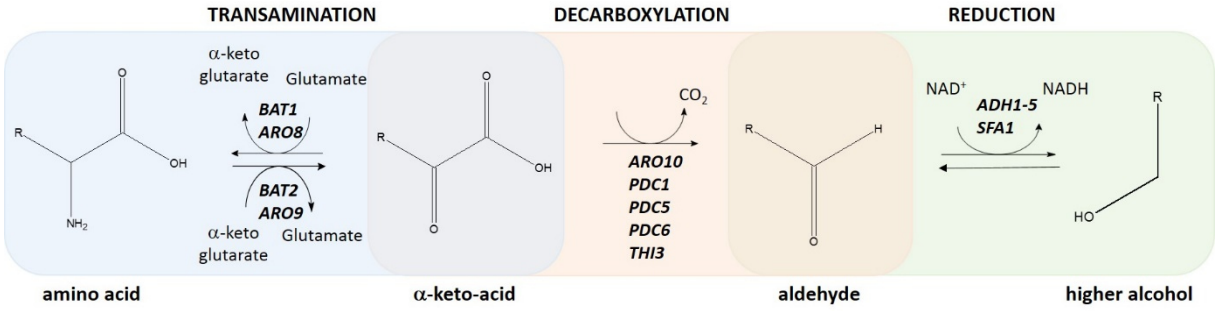

Figure 1 – 5: The Ehrlich pathway and the main genes involved in the synthesis of enzymes catalysing each reaction.

Amino acid uptake is performed by several amino acid transporters located in the cell membrane of yeast (Ahmad & Bussey, 1986; Amitrano, Saenz, & Ramos, 1997; Garrett, 2008; Jauniaux & Grenson, 1990; Poole et al., 2009). The amino acids that are assimilated by the Ehrlich pathway—valine, leucine, isoleucine, methionine, tyrosine, tryptophan and phenylalanine—are further transaminated to α-keto acids (Eden, Van Nedervelde, Drukker, Benvenisty, & Debourg, 2001; Hazelwood, Daran, Van Maris, Pronk, & Dickinson, 2008). This first reaction involves four enzymes encoded by the genes *BAT1*, *BAT2*, *ARO8* and *ARO9* (Kispal, Steiner, Court, Rolinski, & Lill, 1996; Pires, Teixeira, Brányik, & Vicente, 2014). Before α-keto acids are excreted into the growth medium, yeast cells decarboxylate them into fusel aldehydes followed by a reduction to form fusel/higher alcohols or by oxidation to form fusel acids (Hazelwood et al., 2008). In *S. cerevisiae*, there are five genes encoding decarboxylases *ARO10* and *THI3* and three pyruvate decarboxylases (*PDC1*, *PDC5* and *PDC6*) (Bolat,

Romagnoli, Zhu, Pronk, & Daran, 2013; Dickinson et al., 1997; Romagnoli, Luttik, Kötter, Pronk, & Daran, 2012). The last step of the Ehrlich pathway, in which the aldehydes are converted into their respective alcohols, involves the action of alcohol dehydrogenases. All six alcohol dehydrogenases of *S. cerevisiae* encoded by the genes *ADH1-5* and the formaldehyde dehydrogenase Sfa1 are able to catalyse the conversion of fusel aldehydes into higher alcohols (Dickinson, Salgado, & Hewlins, 2003).

This catabolic pathway of higher alcohol biosynthesis is the most studied and discussed one. However, higher alcohols are also formed during upstream biosynthesis (anabolic pathway) of amino acids (Chen, 1978; Dickinson & Norte, 1993; Oshita, Kubota, Uchida, & Ono, 1995). One important path is the de novo synthesis of branched-chain amino acids (BCAAs) via the isoleucine–leucine–valine pathway (Dickinson & Norte, 1993). Brewing wort normally has all proteinogenic amino acids feasible for yeast growth. However, during carbohydrate metabolism, α-keto acids are also generated via the biosynthesis of amino acids and may then be degraded to fusel alcohols, also followed by decarboxylation and reduction reactions (Chen, 1978). Already in 1957, Genevois and Lafon (Genevois & Lafon, 1957) pointed out the deficiencies of the Ehrlich–Neubauer–Fromherz scheme in not accounting for all higher alcohols formed, in particular with 2-butanol, 1-propanol and 1-butanol not corresponding to any naturally occurring amino acids. One year later, Thoukis showed, using radioactive carbon techniques, that isoamyl alcohol is formed in resting-cell fermentation from the carbon of glucose (Thoukis, 1958). Thus, many fermentation volatiles important to beer aroma may arise from yeast metabolism of carbohydrates, more precisely of hexoses, which provide the main carbon source for amino acid synthesis (Chen, 1978; Nisbet, Tobias, Brenna, Sacks, & Mansfield, 2014). Calculating the relative contribution of hexose sugars to individual aroma compounds is challenging, owing to high endogenous hexose substrate concentrations. In 1978, a [^{3}H]–[^{14}C] dual label tracer technique was tried for the first time to differentiate the metabolites from the anabolic and catabolic pathways of fusel alcohol biosynthesis (Chen, 1978). However, 97.7% of radioactive glucose was incorporated into ethanol and 0.8% into *n*-propanol, 0.4 into isobutanol, 0.3 into 2-methyl butanol and 0.7 into isoamyl alcohol, although the calculation of relative radiolabel distribution on a molar basis was not possible (Chen, 1978). Many years later, Nisbet et al. studied precursor–product relationships to measure high-precision ^{13}C/^{12}C isotope ratios of volatiles in wines produced from juices spiked with tracers of uniformly labelled [U^{13}C]-glucose. They showed that fusel alcohols and their acetate esters were also hexose derived. Further, a high proportion of higher alcohols associated with amino acid metabolism (isobutanol, isoamyl alcohol, 2-methyl alcohol) were up to 75% hexose derived, indicating a strong influence of the anabolic pathway in which

carbon skeletons are synthesised de novo from hexoses (Nisbet et al., 2014; Ugliano & Henschke, 2009a).

1.2.2 Esters: contributing to the fruity character of fermented beverages

Volatile esters are among the most important groups of aromatic compounds in fermented beverages. Some 100 distinct esters have been identified in beer (Boulton & Quain, 2001; Meilgaard, 1975b). They are crucial for the overall flavour profile in fermented beverages. Even if they are present in only trace quantities, they may define final aroma, being responsible for the highly desired fruity, candy and perfume-like aroma character (Aritomi et al., 2004; Debourg, 2000; Hiralal, Olaniran, & Pillay, 2013; Peddie, 1990; Saison, De Schutter, Uyttenhove, Delvaux, & Delvaux, 2009; Verbelen et al., 2009; Verstrepen, Derdelinckx, Dufour, Winderickx, Thevelein, et al., 2003a). Concentrations in beer are typically less than 1 mg/L particularly for ethyl esters and 10–50 mg/L for ethyl acetate (see Table 1 - 2). Because the concentration of most esters is under or around their threshold levels, even small changes in the concentrations of these secondary metabolites can have large effects on the sensory quality of the end product. Synergistic effects of different esters may explain why esters can affect the flavour of fermented beverages well below their threshold levels (Meilgaard, 1975a). However, it is important to balance the ester profile in fermented beverages; otherwise, overproduction may result in an overly fruity taste (Pires et al., 2014). It is of major industrial interest to understand the mechanism of their formation to be able to better control their levels in the end product.

Formation of esters by reactions between free organic acids and alcohols is unlikely; the rates of such esterification would be too slow (during storage up to 12 weeks) to account for the kinetics of their appearance during fermentation (Boulton & Quain, 2001). Instead, esters are formed during mainly primary fermentation intracellularly by yeast by an enzyme-catalysed condensation reaction of organic acids and alcohols (Nordström, 1963, 1964).

There are two main categories of flavour-active esters in fermented beverages: acetate esters and medium-chain fatty acid (MCFA) ethyl esters (Fig. 1 - 6). Both are synthesised in the cytoplasm and rapidly diffuse through the plasma membrane, as they are lipid soluble. Lipophilic acetate esters readily leave the cells, whereas the proportion of MCFA ethyl esters transferred to the medium decreases with increasing chain length (Pires et al., 2014; Verstrepen, Derdelinckx, Dufour, Winderickx, Thevelein, et al., 2003a). During brewery fermentation, UFA synthesis stops when oxygen is exhausted. Consequently, the membrane fluidity is reduced. Accordingly, it has been suggested that certain fatty acid ethyl esters with longer chains might act as UFA analogues (Mason & Dufour, 2000; Saerens et al., 2010).

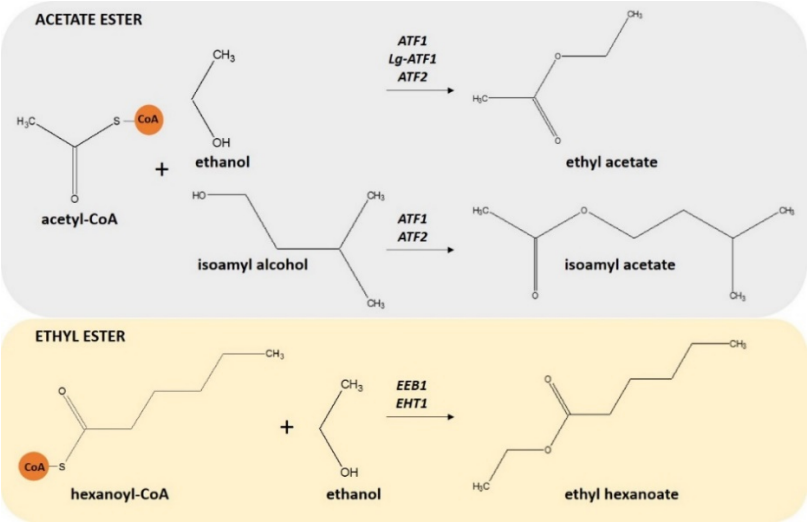

Figure 1 – 6: Simplified biosynthetic pathway and the main genes involved in ester production. Acetate esters are synthesised by higher alcohols or ethanol and acetyl-CoA, while ethyl esters are synthesised by ethanol and acyl-CoA.

For ester synthesis, organic acids must be linked to a coenzyme A to form a highly energetic acyl-CoA molecule. This molecule can be β-oxidised into a smaller acetyl-CoA entity. The majority of acetyl-CoA is still formed by oxidative decarboxylation of pyruvate and is esterified with ethanol or a higher alcohol to form acetate esters with the participation of alcohol acetyltransferases (AATases) I and II (Nordström, 1963). The second class of esters, MCFA ethyl esters, is formed from longer chains of acyl-CoA with ethanol with the involvement of acyl-CoA:ethanol *O*-acyltransferases (AEATases), Eeb1 and Eht1 (Saerens et al., 2010).
However, not only is the substrate concentration crucial for ester production but also the balance between synthesis and/or hydrolysis activity of the enzymes is an important factor, particularly in ethyl ester formation (Saerens et al., 2010; Sumby et al., 2010). Thus, all parameters that affect enzyme activity or substrate concentration will influence ester formation (Verstrepen, Derdelinckx, Dufour, Winderickx, Thevelein, et al., 2003a).

1.3 Regulatory mechanism of the production of sensory metabolites

Aroma-active metabolites derived by yeast can vary in their concentrations. One explanation for this variation is changes in expression levels of genes for enzymes involved in aroma synthesis during fermentation. Several stressors or fermentation parameters including oxygen influence the expression level of aroma-related genes, resulting in overexpression or downregulation. However, not only gene expression itself is important for controlling the aroma compound profile of fermented beverages; protein concentrations depend not only on mRNA level but also on its translation and degradation rates. Furthermore, considering the

complex nature of the expressed enzyme itself, it is reasonable to expect that many factors will affect the rate of its catalytic activity.

1.3.1 Regulation of gene expression

Of the 6,000 genes in the *S. cerevisiae* genome, only a fraction is expressed in a cell at any given time. Some gene products are present in very large amounts and others in much smaller amounts, depending on the changing requirements of gene products over the time. The need for enzymes in metabolic pathways switches as, for example, nutrient availability varies or cellular stresses appear. The cellular concentration of a protein is determined by seven transactions (Fig. 1 – 7), each of them containing several points to be regulated: transcription, posttranscriptional modification of mRNA, degradation of mRNA, translation, posttranslational modification of proteins, protein targeting and transport and protein degradation (Nelson & Cox, 2008).

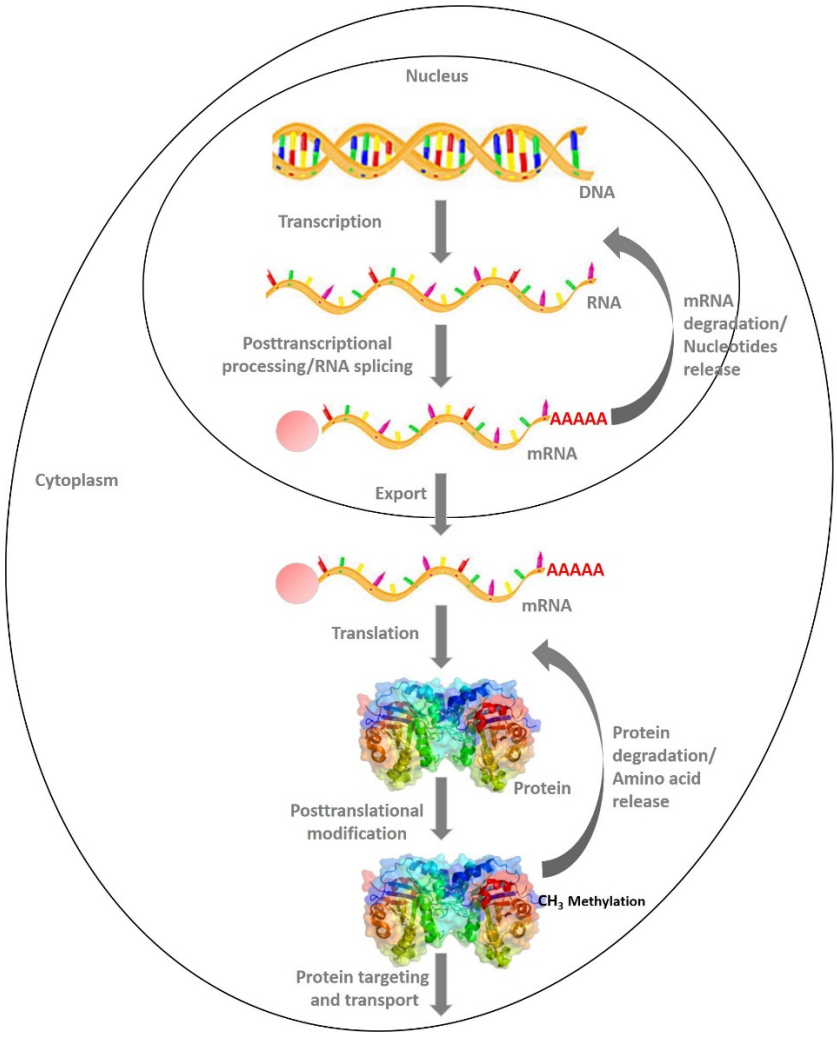

Figure 1 – 7: The seven transactions affecting the steady-state concentration of a protein.

Genes encoding proteins that are constantly required are expressed at a constant level—a process also known as constitutive gene expression. Such genes are referred to as housekeeping genes. All other genes vary in their products in response to gene expression regulation by molecular signals.

The regulation of many of the individual genes discussed for the production of aroma-active higher alcohols has already been exposed (some of them are summarised in Chapter 2.2). However, most studies have not taken into account the context of the Ehrlich pathway (Hazelwood et al., 2008). Iraqui et al. were the first to identify *ARO80* as the transcriptional activator involved in the induction of the *ARO9* and *ARO10* transaminase and decarboxylase genes in the presence of the aromatic amino acids tryptophan, phenylalanine and tyrosine (Iraqui, Vissers, Cartiaux, & Urrestarazu, 1998). Later studies have shown that the transcription of *ARO9* and *ARO10 also* requires the GATA activators Gat1 and Gln3, which play a role in nitrogen catabolite repression (NCR) in *S. cerevisiae* (Hofman-Bang, 1999; Lee & Hahn, 2013a). NCR acts as a global nitrogen quality control system, activating GATA genes in the presence of poor nitrogen sources and repressing them when nitrogen-rich materials are available (Cooper, 2002). Further, environmental conditions affect the transcription of *ARO9* and *ARO10* activated by *ARO80*, particularly under heat shock conditions (Lee, Sung, Kim, & Hahn, 2013b). Deletion of *ARO80* from the *S. eubayanus* subgenome in a lager yeast did not eliminate phenylalanine induction of LgSeubARO10, suggesting transregulation across the subgenomes (Bolat et al., 2013).

Transcriptional control may not be the only level of regulation of the Ehrlich pathway. Using a strong promoter in overexpression studies of the *ARO10* gene did not result in increased 3-phenylpyruvate decarboxylase activity during growth on a medium with glucose and ammonium sulphate, whereas the replacement of either glucose by ethanol or ammonium sulphate by phenylalanine led to a clear increase of 3-phenylpyruvate decarboxylase activity (Vuralhan et al., 2005). This result suggests that the expression of *ARO10* may be regulated at a posttranscriptional or posttranslational level in a carbon and nitrogen source-dependent manner (Hazelwood et al., 2008).

As already indicated, there are three regulatory factors important for the rate of ester formation: the individual concentrations of the two substrates and the total enzyme activity (Saerens et al., 2006; Verstrepen, Van Laere, et al., 2003b; Zhang et al., 2013). For the level of acetate esters during fermentation, the expressions of the AATases, Atf1 and Atf2, are most important (Lilly, Lambrechts, & Pretorius, 2000; Saerens, Verbelen, Vanbeneden, Thevelein, & Delvaux, 2008a; Verstrepen, Van Laere, et al., 2003b). The expression of these genes can be influenced by several parameters acting during the manufacturing of fermented beverages.

UFAs and oxygen in the fermentation medium directly repress the expression of *ATF1* (Fujii, Kobayashi, Yoshimoto, Furukawa, & Tamai, 1997). The repression of *ATF1* expression by UFAs is regulated through a so-called low-oxygen response element (Vasconcelles et al., 2001), which is activated under hypoxic conditions and repressed by the availability of UFAs (Chellappa et al., 2001; Jiang et al., 2001), whereas the repression due to oxygen is mediated mainly by the Rox1-Tup1–Ssn6 hypoxic repressor complex (Fujiwara, Kobayashi, Yoshimoto, Harashima, & Tamai, 1999). Besides the transferases, esterases are a group of hydrolysing enzymes that catalyse the cleavage and/or prevent the formation of ester bonds. The *IAH1* gene encodes a cytosolic esterase that hydrolyses the crucial acetate ester isoamyl acetate (Fukuda et al., 2000). Thus, accumulation of isoamyl acetate is controlled by a balance of these two enzyme activities (Fukuda et al., 1998). In addition to isoamyl acetate breakdown, Lilly et al. reported decreased production of ethyl acetate, phenyl ethyl acetate and hexyl acetate by the overexpressing *IAH1* mutant strain (Lilly et al., 2006). In a recent study, IAH1-encrypted enzyme was found to be more specific for shorter-chain esters (Ma et al., 2011).
For MCFA ethyl esters, it has been shown that the maximum expression level of *EEB1* is correlated with the final concentration of ethyl hexanoate but not with ethyl octanoate or decanoate (Saerens, Verbelen, et al., 2008a), a finding that may be associated with the decreasing transfer to the medium of ethyl esters with increasing chain length. However, the latter two esters seem to be negatively correlated with the expression of *EHT1* gene, as Eht1 has synthesis and hydrolysis activity, indicating that the esterase activity is the main factor determining these two ethyl esters (Saerens et al., 2006). Moreover, Saerens et al. showed that the addition of precursor levels increased ethyl ester formation. Octanoic acid induced the expression of *EEB1* and *EHT1*, whereas hexanoic, decanoic and dodecanoic acid did not affect gene expression (Saerens, Delvaux, et al., 2008b). They concluded that if the octanoic acid precursor does not specifically inhibit the esterase activity of the Eht1 enzyme, the cellular MCFA concentration must be rate limiting for ethyl ester synthesis (Saerens, Delvaux, et al., 2008b).

1.3.2 Effects on enzyme activity

Enzymes are central to every biochemical process. They act in organised sequences and catalyse the hundreds of stepwise reactions that degrade nutrient molecules, convert chemical energy and form biological macromolecules from simple precursors. Because organisms use enzymes ubiquitously and specifically, reliable and fast-acting mechanisms must be used to control the enzyme activity. There are essentially two possibilities available for the regulation of enzyme activity. First is the control of enzyme availability: here, the amount of enzyme (and thus, indirectly, the activity), depends on the balance between

synthesis and degradation. This process is regulated and controlled by various genetic measures including induction, repression and enzyme cascades. Second is the direct control of enzyme activity: here, conformation changes within the enzyme are responsible for the inactivation by regulating the substrate binding affinity. A common regulatory mechanism is the conformational change by allosteric effectors (normally small molecules) that bind to the allosteric centres of an enzyme. Activities can also be influenced by phosphorylation or dephosphorylation of specific amino acid side chains (Ser, Thr or Tyr residues). The covalent modifications elicit an activity change in the enzyme. Also, several factors affect the rate at which enzymatic reactions proceed: pH, enzyme concentration and substrate concentrations. With respect to the direct control of enzyme activity, little research has been performed, in comparison with that on the control of enzyme availability regulated by induction and repression in the production of aroma-active metabolites.

The best description of the activity of yeast enzymes involved in fermentation process has recently been demonstrated by Knight et al. (Knight, Bull, & Curnow, 2014), who developed methods to isolate and characterise recombinant AEATases after plasmid-based expression in a yeast host to investigate the biochemical synthesis of MCFA ethyl esters. They included a novel method of protein purification and a coupled enzyme assay to study protein function. They showed that recombinant Eht1 was functional as an acyltransferase with octanoyl-CoA as the preferred substrate. Eht1 was also active as a thioesterase, suggesting that the *S. cerevisiae* gene *EHT1* should be redefined as encoding an octanoyl-CoA:ethanol acyltransferase that can also function as a thioesterase (Knight et al., 2014). This shows that for Eht1 activity and thus for ethyl ester production, the substrate component is a very important factor, even if ethyl octanoate is not the most abundant ethyl ester in beer, because with increasing chain length, the transfer of MCFA ethyl esters to the medium decreases (Pires et al., 2014). Earlier studies dealing with the activity of enzymes involved in aroma metabolism focused on yeast alcohol dehydrogenase, containing a zinc atom in its catalytic site, which is essential for conversion of aldehydes to the corresponding alcohols. The metal ion, which may be limiting in malt worts, is a functional component of the enzymatic activity of the molecule (Boulton & Quain, 2001; Vallee & Hoch, 1995). The addition of zinc (0.1–0.15 mg/L) stimulated fermentation rate but not yeast growth (Bromberg et al., 1997). Also yeast alcohol dehydrogenase is inactivated by several biologically relevant oxidants (Abelidis, Moore, & Chakravarty, 1987; Fliss & Ménard, 1991), suggesting that zinc release from alcohol dehydrogenase as well as from other thiolate-ligated zinc proteins is particularly susceptible to oxidant attack (Fliss & Ménard, 1991, 1992).

Several fermentation parameters influence the expression of enzymes involved in aroma compound production, providing different opportunities for the brewing industry to offer several aroma profiles in beer. Wort oxygen and lipid content, fermentation temperature, fermenter design and top pressure, sugar and nitrogen content in wort and the yeast strain itself exert strong influence, particularly on the control of enzyme availability, which depends mainly on gene expression (Boulton & Quain, 2001; Verstrepen, Derdelinckx, Dufour, Winderickx, Thevelein, et al., 2003a). Still, it is important to remember that the enzyme availability depends not only on gene expression, because protein concentrations depend not only on mRNA level but also on the translation and degradation rate as well as on posttranslational modifications.

Fermentation temperature influences the expression of numerous genes and associated enzymes in the metabolic pathway of higher alcohol and ester formation. Rising fermentation temperatures increased the expression of the *BAP2* gene encoding BCAA permease, which promotes the transport of the BCAAs, valine, leucine and isoleucine in yeast (Didion, Grauslund, Kielland-Brandt, & Anderson, 1996; Yukiko, Fumihiko, Keiji, & Toshihiko, 2001). The higher availability of amino acids within the cell favours the Ehrlich pathway, increasing the production of fusel alcohols (Yukiko et al., 2001). High temperature also increases the expression of the BCAA aminotransferases encoded by the *BAT1* and *BAT2* genes (Saerens, Verbelen, et al., 2008a). It has been observed that high fermentation temperature increases the concentrations of propanol, isobutanol, isoamyl alcohol and phenyl ethanol, but that only the expression level of *BAT1* was strongly correlated with the final concentrations of these alcohols, particularly propanol (Saerens, Verbelen, et al., 2008a). As higher alcohols are a substrate for acetate ester formation, changes in higher alcohol concentrations promoted by temperature may also change ester levels, in particular those of acetate esters. Higher expression levels of *ATF1* and *ATF2* under high fermentation temperatures could be correlated with the end concentrations of ethyl acetate, isoamyl acetate and phenyl ethyl acetate (Mason & Dufour, 2000; Saerens, Verbelen, et al., 2008a). Even a slight increase of temperature raises ester production by up to 75% (Engan & Aubert, 1977). Further, the expression levels of *EEB1* and *EHT1*, encoding the AEATases, are temperature dependent. These changes were correlated only with the end concentration of ethyl hexanoate, indicating that the expression level is not the primary factor in ethyl ester production (Saerens, Delvaux, et al., 2008b; Saerens, Verbelen, et al., 2008a; Saerens et al., 2006).

Another influential fermentation parameter is the wort nutrient content. Besides the specific sugar profile, particularly the glucose maltose balance (Herrmann, 2005) and the glucose concentration itself, acting as positive inducer of several genes involved in aroma compound formation (Verstrepen, Moonjai, et al., 2003c), the wort nitrogen content is of major interest.

Several studies have revealed the effects of ammonium addition on the formation of aroma-active metabolites (Carrau et al., 2008; Hernández-Orte, Ibarz, Cacho, & Ferreira, 2005; Szlavko, 1974). Higher alcohols, acetate and ethyl esters are affected by the type as well as the concentration of nitrogen (Bell & Henschke, 2005). In particular, the amino acids have a large influence on aroma compound profiling in fermented beverages, given that several sensory-active higher alcohols depend directly on their metabolism (Chen, 1978; Hazelwood et al., 2008). Changes in availability of amino acids in the environment induce the *BAP2* gene (Wang, Jiang, & Jazwinski, 2010), and leucine even in micromolar concentrations acts as the main regulatory signal for the uptake of BCAAs in *S. cerevisiae* in various synthetic media (Didion et al., 1996). The BCAA permease Bap2 is also posttranslationally regulated by ubiquitination at its N terminus (Omura & Kodama, 2001, 2004). Thus, refined mechanisms regulate the cellular Bap2 level to control BCAA uptake, allowing response to varying external nitrogen sources (Usami et al., 2014). In a recent study, higher nitrogen levels in wort also induced the transcription of mitochondrial BCAATase encoded by the *BAT1* gene (Lei, Zhao, Yu, & Zhao, 2012). However, Vidal et al. reported a downregulation of the decarboxylase and alcohol dehydrogenase genes in *S. cerevisiae* under high ammonium sulphate supplementation of sugar cane juice (Vidal et al., 2013), causing a measurable decrease in higher alcohol content. This finding is similar to those in wine production (Bell & Henschke, 2005; Carrau et al., 2008) and in contrast to those in brewery fermentation, where either very high or low amino nitrogen content increases higher alcohol production (Boulton & Quain, 2001). The *ARO10* gene encoding a phenylpyruvate decarboxylase was recently reported as the key gene in higher alcohol formation in *S. cerevisiae* (Romagnoli et al., 2012). However, further investigations of preferred nitrogen source, higher alcohol formation and the relevant gene expression are needed. Moreover, the influence of nitrogen compounds on ester formation is very complex. Several theories have been advanced to explain gene and/or enzyme regulation under different nitrogen contents and its respective ester production (Malcorps, Cheval, Jamil, & Dufour, 1991; Peddie, 1990). High nitrogen levels in fermentation media caused higher expression levels of AATase-encoding genes, in particular *ATF1* (Lei et al., 2012; Verstrepen, Derdelinckx, Dufour, Winderickx, Pretorius, et al., 2003d). The transcription of the *ATF1* gene induced by nitrogen content corresponds to the protein kinase Sch9-mediated *ATF1* expression, which is involved in nitrogen sensing (Fujiwara et al., 1999; Verstrepen, Moonjai, et al., 2003c). Further, high nitrogen supply results in an upregulation of the *EEB1* gene in *S. cerevisiae* during cachaça fermentation, which correlates with ethyl octanoate (Vidal et al., 2013), possibly in contrast to that observed in brewery fermentation (Saerens, Delvaux, et al., 2008b). Owing to the contradictory literature on nitrogen content in

fermentation media and its influence on aroma compound production as well as the related gene expression, a systematic analysis of this metabolic network is suggested.

1.4 Motivation

The previous chapters provided an introduction to the major yeast-derived aroma-active higher alcohols and esters in fermented beverages, given that aroma is one of the most important quality attributes of beer. The survey showed that the biochemical pathways form a highly interlinked network and that changes in one component may have dramatic consequences. In particular, the nitrogen composition of the fermentation medium has a crucial influence on the metabolome and thus on the concentrations of sensory-active metabolic intermediates or their transformation rates in *Saccharomyces* yeast (Arias-Gil, Garde-Cerdán, & Ancín-Azpilicueta, 2007; Cruz, Cilli, & Ernandes, 2002; Saerens, Verbelen, et al., 2008a). The main sources are represented by amino acids (O´Connor-Cox & Ingledew, 1989). A decade-old categorisation introduced by Jones and Pierce in 1964 divides the amino acid uptake rates of a conventional fermentation medium into four groups (Jones & Pierce, 1964; Lekkas, Stewart, Hill, Taidi, & Hodgson, 2005; Ramos-Jeunhomme, De Keyser, & Masschelein, 1979). This original model was valid for only two defined yeast strains (*S. cerevisiae* Guinness No. 522 und No. 4220) and has not been transferred to other yeasts. This classification has begun increasingly to totter, given that this greatly simplified model of amino acid groupings no longer corresponds to the actual uptake mechanisms (Lekkas, Stewart, Hill, Taidi, & Hodgson, 2007; Wang & Brandriss, 1987). In this context, there is also the 70%–79% uptake of proline by a lager yeast strain (Gibson et al., 2009). These results provide evidence that strain-specific mechanisms or kinetics of amino acid absorption and metabolism exist, resulting in different aroma profiles caused by yeast-specific sequential amino acid uptake (Batistote, Cruz, & Ernandes, 2006; Cruz et al., 2002), which is performed by amino acid transporters located in the cell membrane (Garrett, 2008; Gibson et al., 2009; Poole et al., 2009). Further, the total concentration of all amino acids appears to influence fermentation kinetics (Arias-Gil et al., 2007). From amino acid metabolism, a large number of the aroma-active compounds, particularly higher alcohols, arise via the Ehrlich pathway (Etschmann, Bluemke, Sell, & Schrader, 2002; Hazelwood et al., 2008), and have a direct connection to the acetate esters (Mason & Dufour, 2000; Verstrepen, Derdelinckx, Dufour, Winderickx, Thevelein, et al., 2003a). Numerous studies have investigated the effect of nitrogen source and its availability on the final content of sensory-active metabolites (Torrea, Fraile, Grade, & Ancín, 2003; M. Ugliano et al., 2009b). However, little is known about how single amino acid concentrations affect the expression of the genes involved in the biosynthesis of aroma-active metabolites in industrial beer production. It is thus clear that this metabolic network is complex, and although it is reasonably well mapped, little information about the role of specific genes and their regulation within this network is available (Rossouw,

Naes, & Bauer, 2008). Also, the production of most aroma-active compounds is yeast strain dependent, and the genome of each strain is unique and finally defines the aroma profile of beer (Ramos-Jeunehomme, Laub, & Masschelein, 1991; Rossouw et al., 2008). Accordingly, one objective of this study was to identify key amino acids that influence the aroma compound profile of the most important yeast strains used in Bavarian beer production, *S. cerevisiae* strain S81 and *S. pastorianus* strain S23. Also, the study of the mechanism of amino acid absorption of these two yeast strains should provide more evidence explaining the different aroma profiles of lager beers and ales. Another objective was to elucidate the influence of the identified key amino acids on genes that affect the aroma profile of yeast and the formation of higher alcohols and their corresponding esters.

The thesis is accordingly organised into four parts:

1. Review of the fundamentals of the production and regulation of flavour-active esters and higher alcohols in *S. cerevisiae*.

2. New views of the importance of nitrogen content to aroma compound profile by variation of the amino acid pool.

3. Evaluation of key genes and the interrelation between amino acid uptake and the corresponding higher alcohol and ester products in brewing yeast strains.

4. Elucidation of the influence of key amino acids on the induction and/or repression of flavour-associated yeast genes.

A novel approach to extracting the specific amino acids in wort and their effects on the regulation of flavour-associated genes would enable innovative perspectives on aroma profiling, including a new possibility of producing beers with different aroma nuances with the aim of offering a variety of beers to a developing market.

2. RESULTS (THESIS PUBLICATIONS)

2.1 Summary of results

The thesis publications are summarised in this chapter, followed by full copies of the papers.

PART 1 Function and regulation of yeast genes involved in higher alcohol and ester metabolism during beverage fermentation.

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Aroma-active higher alcohols and esters are intracellularly produced by fermenting yeast cells. They are of major industrial interest because of their contribution to the aroma in fermented beverages. Higher alcohols are secondary metabolites produced by yeast in highest absolute concentrations and thus are of great interest. Esters, particularly acetate esters, whose synthesis is linked to the concentrations of their corresponding alcohol and of acetyl-CoA, are very important owing to their responsibility for the highly desired fruity, honey and perfume-like aroma in yeast fermented beverages. Even if volatile esters are present in only trace quantities, they can affect beer or wine flavour well below their threshold value. However, the absolute amount of aroma-active compounds is not substantial for the flavour of fermented alcoholic beverages. The relationships between the fine tuning of these different volatiles are more important. In the yeast fermentation process, numerous environmental factors affect the production of these volatile aroma compounds. Low or high temperature, carbon dioxide pressure and carbohydrate or nitrogen density are some of the most important (stress) factors in the beverage industry. These factors can act on the transcription level of flavour-associated yeast genes, resulting in concentration differences of these important flavour-active metabolites. Understanding of the function of genes involved in biosynthetic pathways of aroma-active substances as well as their regulatory mechanisms is needed to control the production of ester and higher alcohol synthesis to create specific aroma compound profiles in fermented beverages. This review discusses the regulation and function of several individual genes (*ATF1*, *ATF2*, *EEB1*, *EHT1*, *BAT1*, *BAT2* and *BAP2*) in fusel alcohol and ester synthesis in *Saccharomyces* yeast.

PART 2 Significant amino acids in aroma compound profiling during yeast fermentation analyzed by PLS regression

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Organoleptic characteristics of beer depend mainly on the flavour-active substances produced by yeast during fermentation. In particular, the volatile higher alcohols and esters are determined by the nitrogen and carbohydrate composition of wort. In particular, amino acid assimilation by yeast influences the synthesis of higher alcohols via the Ehrlich pathway. The concentration of higher alcohols and their corresponding esters can be influenced by the uptake efficiency of the corresponding amino acid and the sugar utilisation rate. For improved understanding of the relationship between amino acid composition in wort and its influence on synthesis of flavour-active metabolites, amino acid variations in a synthetic medium were established in an experimental design created with Design Expert to reduce primary, insignificant parameter variations. These variations were fermented by *S. cerevisiae* strain S81 and *S. pastorianus* strain S23, respectively. After fermentation, volatile compounds of each amino acid variation were quantified, and their correspondence with the most significant amino acids for *S. cerevisiae* and *S. pastorianus* was investigated. Some of the varied concentrations of aroma-active compounds could be related to the different amino acid compositions in synthetic media. The task of pattern recognition was analysed by partial least square (PLS) regression and variable importance in projection (VIP), a powerful tool of chemometrics for analysing multivariate data sets together with multiple targets. The most important explanatory variables affecting the synthesis of sensory compounds of S23 were leucine, isoleucine, valine, glutamine, cysteine and, surprisingly, proline, whereas those for S81 were leucine, isoleucine, valine, histidine, glutamine and again proline. Thus, a fingerprint of amino acid importance to the detected aroma compound profile was created. The BCAAs for both yeast strains seem to be the amino acids most important for the measured concentrations of aroma compounds. Proline identification as a key amino acid for S23 and S81, with respect to the aroma compound profile under anaerobic fermentation conditions, leads to a novel classification of sequential amino acid uptake during brewery fermentation, given that it has long been thought that proline is not taken up by yeast under brewing conditions.

PART 3 Differential transcribed yeast genes involved in flavour formation and its associated amino acid metabolism during brewery fermentation

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Amino acid concentration in wort and its use by yeast during fermentation can influence the synthesis of flavour-active higher alcohols and their corresponding esters in fermented beverages. For better understanding of the relationship between the biosynthesis of aroma-relevant metabolites and the importance of amino acids, an improved understanding of amino acid uptake and assimilation is essential for generating defined amounts of sensory metabolites to regulate specific sensory effects in fermented beverages. Besides, the knowledge of transcriptional responses of yeast genes involved in the biosynthesis of flavour compounds is limited. Accordingly, DNA microarray experiments were performed to analyse the global transcription profiles of the most important yeast strains, *S. cerevisiae* strain S81 and the *S. pastorianus* strain S23, used in Bavarian beer production. Changes in the transcription of genes associated with amino acid assimilation and its derived aroma-active compounds during fermentation were measured. The experiments revealed similarities as well as disparities in gene expression pattern when the two yeast strains were compared. In particular, the differences in sequential amino acid uptake suggest strain dependent amino acid assimilation. These differences could be due to the different transcription rates of genes encoding amino acid transporters, which could be associated with the various concentrations of higher alcohols. During lager yeast fermentation an increased proline uptake was found. This finding could be due to the lower fermentation temperature used for lager beer production, given that yeast with freezing tolerance tends to accumulate higher levels of proline. The expression levels of specific genes involved in the biosynthesis of aroma compounds revealed that *BAT1* and particularly *ARO8*, genes important for transamination reactions in the Ehrlich pathway, were highly expressed immediately after pitching. Several differences in the expression pattern of the two yeast strains were found to contribute to the different flavour compound profiles, which are influenced by, for instance, the genetic background of the yeast strain. These findings, in particular the differences in amino acid uptake, may help to generate defined amounts of sensory metabolites by manipulating substrate composition in wort to produce specific sensory effects in beer.

PART 4 Effect of amino acid supply on the transcription of flavour-related genes and aroma compound production during lager yeast fermentation

PAGE 71

To investigate the effect of amino acids on the formation of higher alcohols and esters, supplementation of synthetic wort with proline, leucine, cysteine, valine, glutamine and isoleucine was performed. The consequence of amino acid addition with respect to the expression levels of the main genes involved in the biosynthetic pathway of aroma-active compounds was investigated. The results showed that changes in selected amino acid levels had no strong effect on the general course of fermentation. The higher alcohols isobutanol, isoamyl alcohol and 2-methyl butanol were generated throughout the process of fermentation, whereas propanol was generated exclusively as a consequence of nitrogen metabolism. Leucine addition increased the production of isoamyl alcohol and isoamyl acetate and 2-methylbutyl acetate. Addition of valine and isoleucine increased the production of isobutanol and 2-methyl butanol, respectively. Within the range of supplementary amino acid concentrations used, *S. pastorianus* produced greater total amounts of higher alcohols. This difference could be associated with the upregulation of pyruvate decarboxylases (*PDC1*, *PDC5* and *PDC6*) and phenylpyruvate decarboxylase (*ARO10*). In contrast, most amino acid supplementations resulted in lower expression levels of *BAT1*, *BAT2* and also *ADH1*, suggesting a strong effect on the decarboxylation step in Ehrlich pathway. Amino acid addition resulted in a reduction in the final total concentration of esters. In particular, ethyl acetate was reduced in response to the supplementation with amino acids, indicating a strong possibility of reducing this acetate ester with a solvent-like odour. This reduction may have been caused by the downregulation of *ATF1* and *ATF2*, which participate in acetate ester biosynthesis. In general, these results show that during lager yeast fermentation, the production of aroma-active compounds can be markedly affected by a change in the supply of even one amino acid in the fermentation medium. Thus, amino acid supplementation and thereby amino acid composition in wort can affect the flavour of lager beer.

2.2 Function and regulation of yeast genes involved in higher alcohol and ester metabolism during beverage fermentation

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REVIEW PAPER

Function and regulation of yeast genes involved in higher alcohol and ester metabolism during beverage fermentation

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Abstract The biochemical formation of yeast-derived sensory-active metabolites like higher alcohols and esters determines the different characteristics of aroma and taste in fermented beverages. In yeast fermentation process, a large number of environmental factors affecting the production of volatile aroma compounds are abundant. Factors like substrate composition in fermentation media as well as process parameters influencing these flavor-active metabolites have already been described. These factors can act on the expression of yeast genes involved in aroma metabolism resulting in concentration differences in esters and higher alcohols important for flavor and taste. The understanding of the function of genes involved in biosynthetic pathways of aroma-active substances as well as their regulatory mechanisms is needed to control the production of ester and higher alcohol synthesis to create specific aroma profiles in fermented beverages. This review discusses the known regulation and function of several individual genes (*ATF1*, *ATF2*, *EEB1*, *EHT1*, *BAT1*, *BAT2* and *BAP2*) described in fusel alcohol and ester synthesis mainly in *S. cerevisiae* and *S. pastorianus* var. *carlsbergensis*. Also, different factors like oxygen and temperature that allow ester and higher alcohol synthesis to be controlled during yeast fermentation are described.

Keywords Fermentation · Yeast · Gene expression · Regulatory effects · Flavor profiling

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Introduction

Saccharomyces cerevisiae and other yeast have been widely used in industrial fermentations for the production of alcoholic beverages like beer, sake and wine. Since the genome DNA sequence of *S. cerevisiae* was reported by Mewes et al. [1], various studies including metabolomic, proteomic and transcriptomic analyses have been carried out for understanding the yeast fermentation process of alcoholic beverages. PCR is a common and often indispensable scientific technique used in molecular biology research for a variety of applications [2, 3]. These include DNA cloning for sequencing, DNA-based phylogeny or functional analysis of genes. Quantitative PCR (Q-PCR) is used to measure the quantity of a PCR product usually in real time. It quantitatively measures starting amounts of DNA, cDNA or RNA. Q-PCR is commonly used to determine whether a DNA sequence is present in a sample as well as the number of its copies in the sample. Quantitative real-time PCR has a very high degree of precision. QRT–PCR methods use fluorescent dyes, such as Sybr Green or fluorophore-containing DNA probes, such as TaqMan, to measure the amount of amplified product in real time. These days, DNA microarray technique is the powerful tool to comprehensively analyze the transcriptome and measure the expression levels of thousands of genes simultaneously using silicon chips marked with DNA fragments, which can hybridize with a fluorescently labeled complementary DNA [4]. These measured expression levels can give qualitative and quantitative information about the activity of individual genes.

In the field of biotechnology and molecular biotechnology, various experiments with yeast have been prepared using these techniques to define transcriptional levels of different genes to understand environmental stresses. Low

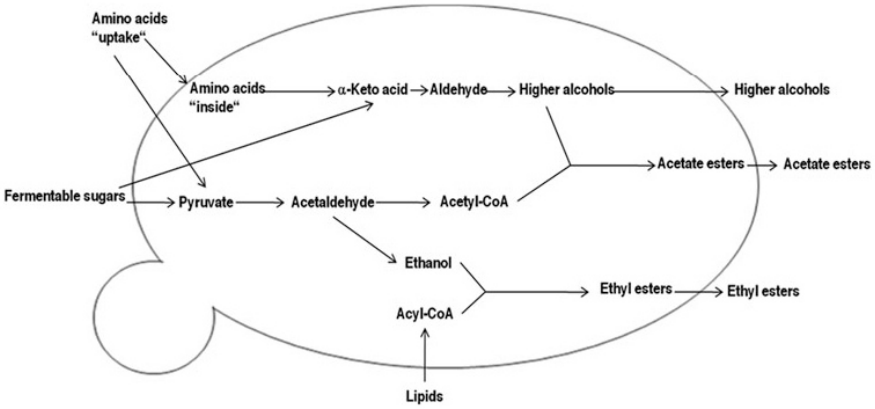

Fig. 1 Simplified biosynthetic pathway of ester and higher alcohol production in yeast fermentation. Synthesis of higher alcohols involves amino acid uptake and transamination as well as the decarboxylation of α-keto acid resulting in an aldehyde, which is followed by the reduction to higher alcohols. Acetate esters are synthesized by higher alcohols or ethanol and acetyl-CoA, while ethyl esters are synthesized by ethanol and acyl-CoA. Their export is discussed by diffusion through the membranes

or high temperature, carbon dioxide pressure and carbohydrate or nitrogen density are some of the most important stresses in the beverage industry [5, 15, 25, 38, 78]. Several important genes responding to low fermentation temperature in wine yeast which are related to cell cycle, cold stress and cytosolic fatty acid biosynthesis have been identified [5]. Also, dissolved oxygen concentrations of 7–8 mg/L regulate the transcription level of several genes during industrial propagation and fermentation of the industrial lager brewing yeast strain CB11 [6]. This transcriptional response of genes related to oxidative stress might be caused by the depletion of fermentable sugars in the media [6].

All these stresses caused by different fermentation conditions can alter the organoleptic characteristics of alcoholic fermented beverages, which mainly depend on the aroma-active metabolites produced by yeast. Aroma-active higher alcohols and esters are produced intracellularly by fermenting yeast cells (Fig. 1) and are of major industrial interest because of their contribution to the aroma in fermented beverages [7–13]. Higher alcohols are the secondary metabolites produced by yeast in the highest absolute concentrations (Table 1) and thus of great interest. Volatile esters are also very important for aroma formation in fermented beverages. They are only present in trace quantities but can affect beer or wine flavor well below their threshold value (Table 2) [14, 15]. However, the absolute amount of aroma-active compounds is not really relevant to the flavor of fermented alcoholic beverages. The relationship between the different volatiles is more important. This can be explained by synergy effects of the aroma-active substances [16]. In lager beer, a three- or four-to-one ratio of higher alcohols to esters is desired. An increase in this ratio triggered by rising higher alcohol concentrations results in a dry and aroma less beer character [17]. Another important aspect in fermentation process of alcoholic beverages and flavor maturation is the fine regulation of the concentration of these volatile compounds. Therefore, it is necessary to understand the biosynthetic formation of higher alcohols and esters as well as the regulation of their most important genes during stress situations caused by fermentation conditions. This will help to generate defined amounts of sensory metabolites to regulate specific sensory effects in fermented beverages.

Table 1 Standard and threshold levels for higher alcohols in lager beer [14]

Aroma-active higher alcohols	Standard value (ppm)	Threshold level (ppm)	Aroma impression
Propanol	2–10	21	Solvent-like
Isobutanol	5–10	10–100	Alcoholic
Isoamyl alcohol	30–50	60–65	Fruity, sweet
Phenyl ethanol	6–44	100	Rose, floral

Table 2 Standard and threshold levels for esters in lager beer [14]

Aroma-active esters	Standard value [ppm]	Threshold level [ppm]	Aroma impression
Ethyl acetate	15–25	21–30	Solvent-like
Isoamyl acetate	0.5–1.5	1–1.6	Banana
Phenyl ethyl acetate	1–5	3.0	Rose
Ethyl hexanoate	0.05–0.3	0.14	Sour apple
Ethyl octanoate	0.04–0.053	0.17	Sour apple

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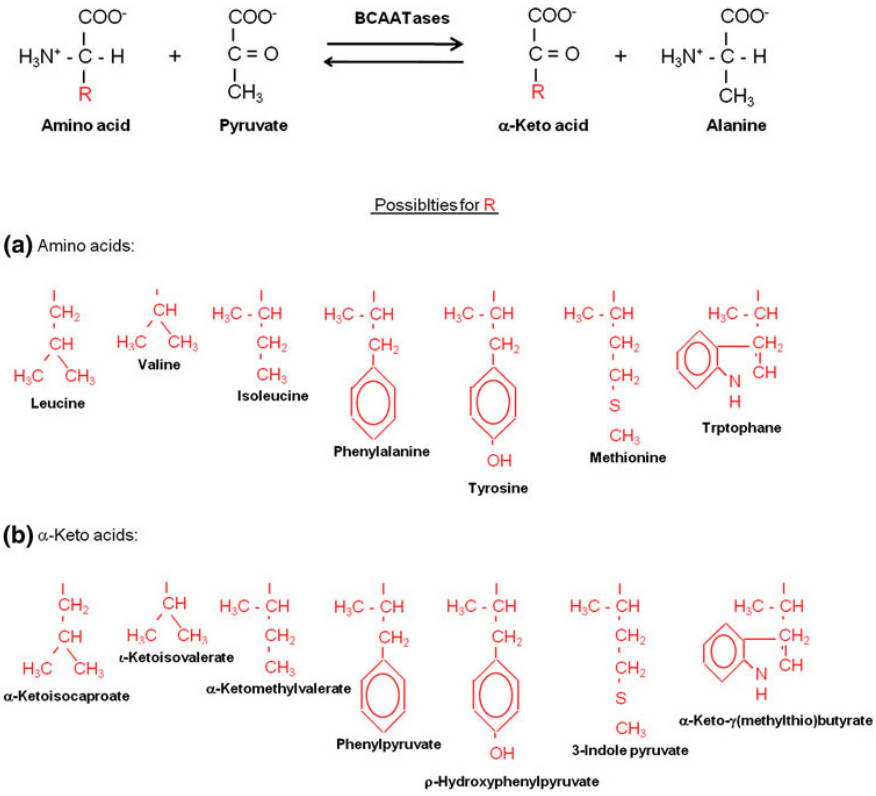

Fig. 2 Transamination reactions of different amino acids **a** resulting in certain α-keto acids **b** catalysed by amino acid aminotransferases (BCAATases)

Biosynthesis of aroma-active higher alcohols and esters

The most important sensory-active higher alcohols for beer aroma are propanol, isobutanol, isoamyl alcohol and phenyl ethanol [8, 16]. Their production is directly related to the metabolism of amino acids representing the main assimilable nitrogen source in cereal-based wort or unfermented grape juice. The amino acids are absorbed by yeast in a sequential order [18, 19]. Amino acid uptake is performed by a number of amino acid transporters located in the cell membrane of the yeast. The majority of these transporters have broad substrate specificity, while some are very high specific by transporting only one amino acid [20–25]. The branched chain amino acid permease of *S. cerevisiae* encoded by the *BAP2* gene has broad substrate specificity and mediates the assimilation of the branched chain amino acid valine, leucine and isoleucine [26]. These and other amino acids can be formed to higher alcohols by catabolic route [27]. The biosynthesis of higher alcohols starts with transamination reactions of an amino acid and an α-keto acid (Fig. 2) catalyzed by mitochondrial and cytosolic branched chain amino acid aminotransferases (BCAATases) encoded by *BAT1* and *BAT2*, respectively [28–32]. The resulting α-keto acid is converted into the corresponding fusel alcohol by decarboxylation to form an aldehyde and followed by a reduction to higher alcohols via the Ehrlich pathway [33, 34]. Simultaneously, the α-keto acids are derived from carbohydrate metabolism termed the anabolic pathway for higher alcohol formation [28]. Furthermore, several genes have been identified, which must be directly involved in the Ehrlich pathway and the production of higher alcohols [35]. The export mechanism of higher alcohols outside the cell into the culture medium is still unknown. Until now, no membrane transporter in *S. cerevisiae* is known. In fact, it is possible that export of these fusel alcohols occurs by simple passive diffusion across the lipid layer [36].

Beside these alcohols, esters represent the main class of flavor-active metabolites in alcoholic fermented beverages. Ethyl acetate, isoamyl acetate, isobutyl acetate, phenyl ethyl acetate and the C6 and C8 short-chain fatty acid ethyl esters, ethyl hexanoate and octanoate are of greatest importance because of their responsibility for the highly desired fruity aroma characters in beer, wine and sake [15, 37, 38]. Even though they are only present in trace quantities, they are extremely important for the flavor profile [14, 15]. Synergistic effects by the presence

Table 3 Genes involved in metabolic pathways of higher alcohol and ester formation in yeast whose expression levels are discussed under different fermentation conditions

Gene	Enzyme	Function
BAP2	Branched chain amino acid permease	Uptake of branched chain amino acids
BAT1	Mitochondrial branched chain amino acid aminotransferase	Branched chain amino acid transaminase activity
BAT2	Cytosolic branched chain amino acid aminotransferase	Amino acid catabolism and branched chain amino acid biosynthesis
ATF1	Alcohol acetyltransferase	Acetate ester production
ATF2	Alcohol acetyltransferase	Acetate ester production
EEB1	Acyl-coenzymeA/ethanol *O*-acyltransferase	Short-chain esterase activity
EHT1	Acyl-coenzymeA/ethanol *O*-acyltransferase	Short-chain esterase activity

of different volatile esters can explain the reason why esters can affect the flavor of fermented beverages well below their threshold value [16]. There are two main categories of flavor-active esters in fermented beverages, acetate esters and fatty acid ethyl esters (FAEEs). Their formation is mediated intracellularly by fermenting yeasts. Acetate esters are synthesized by an enzyme-catalyzed condensation reaction between a higher alcohol and an acetyl-CoA, which can be formed either by oxidative decarboxylation of pyruvate or by direct activation of acetate with ATP [39–41]. In *S. cerevisiae*, the involved enzymes are alcohol acetyltransferases I and II (AATase I and II; EC 2.3.1.84) encoded by the genes *ATF1* and *ATF2*, while in *S. pastorianus* strains, a closely related homologue Lg-*ATF1* gene next to *ATF1* was identified [41–44]. The formation of FAEEs, which are generated primarily during fermentation, is catalyzed by Eht1 and Eeb1 acyl-coenzymeA/ethanol O-acyltransferases (AEATases) encoded by the genes *EHT1* and *EEB1* [45–47]. The alcohol group is ethanol, and the required acyl-CoA compounds result from the fatty acid metabolism of organic acids and free coenzyme A under participation of acyl-CoA synthetase. Since esters are lipid soluble, their transport out of the cell is caused by diffusion through the plasma membrane into the fermenting medium. Furthermore, Nykanen and Nykanen determined that the transfer of ethyl esters decreases drastically with increasing chain length [48].

Aroma-active compounds derived by yeast can vary in their concentrations. This can be explained by changes in expression levels of genes involved in aroma synthesis during fermentation. To determine significant correlations between the expression levels of genes concerned in higher alcohol and ester synthesis and the changes in the end concentrations of these aroma compounds, statistical methods are necessary. Pearson correlation is popularly used for calculations. This will help to understand how the mentioned genes (Table 3) regulated under different substrates and fermenting conditions can alter the aroma profile in the surrounding medium.

Gene regulation and its physiological role in aroma profiling

BAP2—branched chain amino acid transport

Yeast amino acid permeases are responsible for transporting substrates into the cell in response to nutritional requirements of yeast. So far, 16 permeases are classified in the family of amino acid permeases in *S. cerevisiae* [49, 50]. For the industrial lager yeast strain CB 11, seven plasma membrane amino acid permeases coded by *AGP2*, *ALP1*, *MMP1*, *MUP1*, *SAM3*, *TAT1* and *TAT2* with specific substrate affinity and four with broad substrate specificity coded by *AGP1*, *GNP1*, *DIP5* and *BAP2* have been reported [51].

The *BAP2* gene encoding the branched chain amino acid permease is involved in the uptake of the three branched chain amino acids leucin, isoleucin and valine in *S. cerevisiae* [52, 53]. In 1995, Grauslund et al. [52] identified this permease by screening of a high-copy library on a low concentration of isoleucine. Furthermore, they found out that the deletion of *BAP2* gene resulted in a reduced uptake of leucine, isoleucine and valine by 20–50%. Due to the fact that the accumulation of amino acids is associated with entering the Ehrlich pathway, a correlation between the *BAP2* gene expression and the formation of higher alcohols has to be existent. The hypothesis was proved by constitutive expression of the *BAP2* gene using the industrial *S. cerevisiae* yeast strain BH-225 during wort fermentation [54]. This results in an increased isoamyl alcohol production but no increase in isobutanol or amyl alcohol production [54]. A constitutive gene expression of *BAP2* when using the brewing strain BH-225 can be achieved by rising temperatures what results in greater assimilation of valine, leucine and isoleucine promoting the production of isoamyl alcohol [54]. This temperature dependency of the *BAP2* gene can be confirmed by its decreased gene expression level using low fermentation temperature [55].

Otherwise, dissolved oxygen at 8 mg/L promoted an increasing expression level of *BAP2* gene together with an

increase in isobutanol and isoamyl alcohol using industrial lager brewing strain during brewery fermentation [56]. However, this metabolite increment did not correlate with *BAP2* gene expression [56]. Furthermore, changes in availability of amino acids in the environment result in the transcriptional induction of *BAP2* [57]. It was demonstrated that leucine in micromolar amounts acts as the main regulatory signal for the uptake of branched chain amino acids in *S. cerevisiae* using different synthetic media [58].

BAT1 and BAT2—formation of higher alcohols

Kispal et al. [59] identified the *BAT1* and *BAT2* genes in yeast encoding the two branched chain amino acid transaminases (BCAATases). Their corresponding enzymes Bat1p and Bat2p localize at mitochondria and cytosol, respectively, and catalyze the transfer of amino groups between branched chain amino acids and α-keto acids. These α-keto acids are precursors for the synthesis of higher alcohols, which significantly influence the taste and aroma of yeast-derived fermentation products.

The involvement of *BAT2* gene in the production of branched chain alcohols and isoamyl acetate has been shown by two genetic strategies [60]. The haploid *S. cerevisiae bat2* null mutant KY1058 showed reduction by 40 and 72% decrease in the production of isoamyl alcohol and isobutanol, respectively, compared to the wild type. On the other hand, the laboratory *S. cerevisiae* strain KY1060 harboring with multi-copy plasmids containing the *BAT2* gene showed a 1.3-fold and 2.2-fold increase in the production of isoamyl alcohol and isobutanol using synthetic fermentation medium [60]. Similarly, overexpression studies of *BAT2* in a laboratory wine yeast strain resulted in significant rising isobutanol concentrations [61]. Furthermore, it was demonstrated that a haploid *S. cerevisiae* bat1Δbat2Δ-double deletion strain was still able to produce isoamyl alcohol when growing on glucose [62]. This corresponds with the results that *BAT1* deletion is not essential for fusel alcohol production under glucose growth conditions, which indicates that other enzymes are able to compensate the loss of BCAATs. Otherwise, latest researches showed that Bat1p is preferentially involved in valine, isoleucine and leucine (VAL) biosynthesis, while Bat2p function is determinant for VAL catabolism, indicating functional diversification [63].

Increased higher alcohol formation due to overexpression of *BAT1* and *BAT2* genes can be achieved under different yeast fermentation conditions. The higher the expression of the *BAT1* gene, the higher is the production rate of isoamyl alcohol and isobutanol using high fermentation temperature [64]. It is known that overexpression of *BAT2* gene also results in significant increases in isobutanol using industrial *S. cerevisiae* strains [65]. However, in contrast to *BAT1*, the *BAT2* expression level is not dependent on temperature [64]. Besides the fermentation temperature, high fermentable sugar levels in the surrounding medium lead to an increased production of certain higher alcohols [66–68]. An increase in isoamyl alcohol and phenyl ethanol, which exceed their threshold when the specific sugar density increases from 14 to 18°P, has been observed during brewery fermentation using the industrial ale strain CMBS SS10 [64]. These results are in agreement with the fact that high carbohydrate concentrations positively affect the BCAATase activities [29, 30, 59, 69]. Also, isoamyl alcohol production correlates with the expression of *BAT1* [60]. Furthermore, concentrations of isoamyl alcohol and isobutanol increase in the presence of 0.5-, 1-fold amounts of nitrogen source in synthetic fermentation medium using haploid *S. cerevisiae* strain YHY554 [60]. These changes in isoamyl alcohol production by high nitrogen source are dependent on transcriptional co-regulation between BCAATase and L-leucine biosynthetic-encoding genes [60].

ATF1, Lg-ATF1 and ATF2—acetate ester synthesis

The *ATF1* and *ATF2* genes encode the alcohol acetyl transferases I and II (AATase I and II; EC 2.3.1.84), which catalyze the synthesis of acetate esters from acetyl-CoA and several kinds of higher alcohols in *S. cerevisiae* [41, 43, 70–72]. Beside these two genes, Yoshimoto et al. [42] identified a closely related homologue, the Lg-*ATF1* gene in *S. pastorianus* (formerly *Saccharomyces carlsbergensis*). The Lg-*ATF1* gene encodes Lg-AATase I, which is homologous to AATase I enzyme [42]. Many studies in *S. cerevisiae* and other microorganisms have been carried out to elucidate the function of genes involved in ester synthesis [42, 44, 72–75]. Several expression studies of *ATF1*, Lg-*ATF1* and *ATF2* genes have been realized in different yeast strains for understanding the role of the known alcohol acetyltransferases in acetate ester production [14]. Gas chromatography analysis of these acetate esters proved that the expression levels of *ATF1* and *ATF2* significantly affect their production [43, 44, 72, 76–78]. Deletion of *ATF1* and *ATF2* genes reduced the levels of ethyl acetate by 40 and 13%, respectively, during fermentation using the laboratory *S. cerevisiae* deletion yeast strains BY4742Δ*atf1* and BY4742Δ*atf2* and YPG medium compared with the wild-type strain BY4742 [14]. Verstrepen et al. [14] demonstrated the *ATF1*- and *ATF2*-encoding enzymes being also responsible for the synthesis of further aroma-active esters such as isoamyl acetate, isobutyl acetate, phenyl ethyl acetate, propyl acetate, pentyl acetate, hexyl acetate, heptyl acetate and octyl acetate, although *ATF2* gene seems to play a minor role in those syntheses.

These AATase activities involved in acetate ester production can be influenced drastically by different fermentation parameters. Several studies confirmed the

overproduction of acetate esters applied by fermentation of media with high carbohydrate concentrations [64, 79–84]. This often leads to unbalanced flavor profiles, which result in overfruity and solvent-like fermented alcoholic beverages [80–82]. It was observed that ethyl acetate production was increased by 25 and 45% when carbohydrate content was increased in wort fermentation cultures using diploid lager strain CMBS SS03 and diploid ale strain CMBS SS10, respectively [64]. Therefore, the inducibility of *ATF1* and *ATF2* caused by glucose is discussed as one possibility for enhanced ester synthase activities during brewing with high sugar concentrations [85, 86]. Other substrate concentrations like nitrogen compounds influence acetate ester production [87]. The most common explanation is the link between nitrogen metabolism and the production of higher alcohols [88]. As discussed, the addition of valine, leucine and isoleucine strongly increases the production of the corresponding fusel alcohols. Its higher levels may, in turn, lead to enhanced ester production [88, 89]. Another possibility for increased acetate ester concentration are nitrogen compounds affecting transcription of the *ATF1* gene, which fits with the protein kinase-mediated *ATF1* expression involved in nitrogen sensing [86, 90, 91]. Another positive parameter for AATase regulation is a high fermentation temperature [92]. Ethyl acetate and phenyl ethyl acetate for example are produced in their maximal concentrations at 20 °C [80, 93]. Higher expression levels of *ATF1* and *ATF2* are reported using high fermentation temperatures [45, 64, 94]. Calculating the Pearson product moment correlation coefficient, these results correlate with the end concentration of ethyl acetate, isoamyl acetate and phenyl ethyl acetate. This demonstrates that higher *ATF1* and *ATF2* expressions lead to a higher production of acetate esters [64]. Thus, the *ATF1* and *ATF2* expression levels can be used as a tool to predict total ester formation.

Beside the upregulation of the AATases, the expression level of *ATF* genes can be downregulated by different parameters in the fermentation medium. High concentrations of unsaturated fatty acids (UFA) and high amounts of dissolved oxygen in the medium are the best-known negative regulators for acetate ester production, which directly repress *ATF1* gene expression [70, 81, 95, 96].

EEB1 and EHT1—ethyl ester synthesis

The *EEB1* and *EHT1* genes encode two acyl-CoA:ethanol *O*-acyltransferases (AEATases), Eeb1 and Eht1. These enzymes catalyze the condensation reaction between an acyl-CoA component and ethanol to form medium chain fatty acid (MCFA) ethyl esters [47, 94]. It was shown in several deletion experiments that the loss of the *EEB1* gene results in reduced concentrations of ethyl butanoate, ethyl hexanoate, ethyl octanoate and ethyl decanoate using *S. cerevisiae* strains [47]. In contrast to these results, the laboratory *S. cerevisiae* deletion strain BY4741Δ*eht1* did not affect ethyl ester formation. Furthermore, the double mutant BY4741*Δeeb1Δeht1* resulted in similar ethyl ester concentrations of ethyl butanoate, ethyl hexanoate and ethyl decanoate as the single haploid deletion strain BY4741*Δeeb1* using YPD medium. This suggests that Eeb1 is the more important enzyme in ethyl ester synthesis compared with Eht1 [47].

Changes in the expression levels of the *EHT1* and *EEB1* genes will give further information how MCFA ethyl ester synthesis can be controlled during yeast fermentation. These variations of ethyl ester concentrations can be achieved by varying process parameters. It is known that concentrations of MCFA ethyl esters increase when high fermentation temperatures are used [97–99]. This fits with the temperature-dependent expression levels of *EEB1* and *EHT1* [64]. In contrast to *EHT1*, the maximum expression level of *EEB1* is greater under high temperature conditions. This confirms the previous results of *EEB1* being the most important gene for ethyl ester biosynthesis [47].

It is proved that fermentations realized by high maltose, maltotriose and glucose composition result in an increased ethyl octanoate concentration using an industrial ale strain [64]. Also, for an industrial lager strain, the highest ethyl hexanoate concentrations were found using the same sugar conditions. The maximum expression level of *EEB1* was correlated with the end concentration of ethyl hexanoate, but there was no correlation with the end concentration of ethyl octanoate and decanoate [64]. This could be explained by incomplete ethyl ester diffusion through the plasma membrane into the fermenting media. Nykanen and Nykanen noticed a decreased transfer of ethyl esters outside the cell by increasing chain length, from 100% for ethyl hexanoate to 54–68% for ethyl octanoate and 8–17% for ethyl decanoate [48]. Furthermore, Saerens et al. [64] proved a strong negative consent between *EHT1* expression level and the concentration of ethyl octanoate and decanoate during high initial carbohydrate fermentations. However, these results as well as overexpressing experiments of *EEB1* and *EHT1* genes only slightly affect ethyl ester production. The enzyme activity appears not to be the limiting factor for ethyl ester production. Moreover, the fatty acid precursor level for ethyl ester formation is discussed to be the rate limiting aspect [97].

Conclusions

Higher alcohols and especially esters represent the most important aroma-active substances produced by fermenting yeast due to their essential aroma impressions. Great advance has been made in elucidating their biochemical

pathways and their regulatory mechanisms. However, the knowledge about these genes has some gaps in this regard. Substrate concentrations and compositions are major limiting factors in the modulation of aroma-active compounds. Further investigations have to be done especially in analyzing the impact of nitrogen and amino acid composition on yeast gene regulation and aroma profiling. Gene regulatory effects of many amino acids especially proline have never been completely characterized. The traditional view that proline uptake during fermentation is limited due to the absence of oxygen has to be more and more rejected [51]. The role of proline uptake in aroma formation is still an unanswered question. Beside the nitrogen content, carbohydrates are widely discussed as aroma regulators in yeast fermentation. Since maltose is the main sugar in cereal-based wort, it is necessary to know which sugar ratio positively regulates gene expression toward specific aroma profiles. However, not only the substrate concentration is crucial for aroma production, but also the balance between synthesis and hydrolysis activity is an important factor especially in ethyl ester formation [96, 100]. Negative correlations between *EHT1* expression levels and the concentration of ethyl octanoate and decanoate can be explained by hydrolysis activity of Eht1 [96, 97]. For optimizing the ethyl ester concentration in the final fermentation product, it is necessary to know under which conditions synthesis or hydrolyses activity starts. Furthermore, the induction of *EEB1* gene suggests a detoxification pathway by the production of decanoate ethyl ester [96, 101]. Different options exist to handle the aroma profile during fermentation. Along with the use and the thoughtful combination of fermentation parameters like temperature and substrate composition, the yeast strain itself plays a major role on flavor formation. Especially, the profile of esters is highly strain dependent. Ramos-Jeunehomme et al. [102] suggested that these differences between distinct yeast strains are due to differences in AATase activity. It is generally admitted that *S. cerevisiae* strains mostly result in greater aroma concentration and composition than *S. pastorianus* strains. Beside the lower temperatures used in lager fermentation, the presence of further ester regulating genes could be responsible for the lighter taste. Furthermore, fusel alcohol synthesis can be well balanced in continuous systems by choosing suitable yeast strains [103]. Additional research is needed to improve the understanding of gene-regulating mechanisms and biosynthetic pathways of aroma-active substances during yeast fermentation.

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2.3 Significant amino acids in aroma compound profiling during yeast fermentation analyzed by PLS regression

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Significant amino acids in aroma compound profiling during yeast fermentation analyzed by PLS regression

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ABSTRACT

The biochemical formation of aroma-active metabolites determines different characteristics of aroma and taste in fermented beverages. Amino acid utilization by yeast during brewer's wort fermentation is seen as linked to flavor profile. For major understanding of the relationship between amino acid composition in wort and their impact on the synthesis of aroma-active metabolites amino acids were varied in synthetic medium and fermented by *Saccharomyces cerevisiae* strain S81 and *Saccharomyces pastorianus* var. *carlsbergensis* strain S23, respectively. After fermentation of different amino acid combinations higher alcohols and esters were detected by gas chromatography to determine specific aroma compound spectrum. Partial least square (PLS) regression and variable importance in the projection (VIP) were used to establish a relation between amino acids and the resulting concentrations of aroma compounds by means of pattern recognition, indicating most representative amino acids in aroma compound synthesis during fermentation. Thus, a fingerprint of amino acid importance on the detected aroma compound spectrum was created. The most important explanatory variables affecting the synthesis of aroma-active substances of *S. pastorianus* var. *carlsbergensis* are leucine, isoleucine, valine, glutamine, cysteine and surprisingly proline. In case of *S. cerevisiae* the significant amino acids are leucine, isoleucine, valine, histidine, glutamine and again proline.

1. Introduction

Fermentation media like cereal based wort contains several assimilable nitrogen sources, which are needed for cell growth. In first phase of fermentation the yeast hydrolates small peptides while in main phase mainly amino acids, representing the main assimilable nitrogen source in cereal based wort, are utilized. Brewing wort contains all proteinogenic amino acids, which vary depending on the wort type. This amino acid composition is known to influence the synthesis of higher alcohols and esters in fermented beverages (Äyräpää, 1971; Engan, 1970; Sablayrolles & Ball, 1995). Aroma-active higher alcohols and esters are produced intracellularly in the cytosol by fermenting yeast cells and are of major industrial interest because of their contribution to the aroma in fermented beverages (Cristiani & Monnet, 2001; Debourg, 2000; Dufour, Verstrepen, & Derdelinckx, 2002; Meilgaard, 1975; Pisarnitskii, 2001; Quain & Duffield, 1985; Swiegers & Pretorius, 2005). Biosynthesis of the higher alcohols involves the decarboxylation of α-keto acids to form aldehydes, followed by a reduction of the aldehydes to their corresponding higher alcohols (Dickinson et al., 1997). The α-keto acids can be formed by the degradation of amino acids. These amino acids are absorbed by yeast in a sequential order (Henschke & Jiranek, 1993; Jones & Pierce, 1964). Amino acid uptake is performed by a number of amino acid transporters located in the cell membrane of the yeast. The majority of these transporters have broad substrate specificity while some are very high specific by transporting only one amino acid (Ahmad & Bussey, 1986; Amitrano, Saenz, & Ramos, 1997; Garrett, 2008; Jauniaux & Grenson, 1990; Poole et al., 2009; Regenberg, Düring-Olsen, Kielland-Brandt, & Holmberg, 1999). The amino acids, valine, leucine, isoleucine, methionine, tyrosine, tryptophan and phenylalanine can be transaminated to α-keto acids (Chen, 1978; Dickinson, Harrison, & Hewlinsi, 1998; Eden, Simchen, & Benvenisty, 1996; Eden, Van Nedervelde, Drukker, Benvenisty, & Debourg, 2001; Hazelwood, Daran, Van Maris, Pronk, & Dickinson, 2008). The resulting α-keto acid is converted into the corresponding fusel alcohol by decarboxylation to form an

Abbreviations: DoE, Design of experiments; PLS, Partial least square; VIP, Variable importants in the projection; RSM, Response surface methodology; NIPALS, Nonlinear Iterative Partial Least Squares; HS-GC-FID, Headspace gas chromatography coupled with flame ionization detection.

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aldehyde and followed by a reduction to higher alcohols via the Ehrlich pathway (Dickinson et al., 1997; Ehrlich, 1907). In case of the degradation of the branched-chain amino acids valine, leucine and isoleucine the general order of biochemical reactions is similar, but the details for the formation of the individual higher alcohols are different (Dickinson, 2000). Thus, Dickinson et al. suggested four credible routes from α-ketoisovalerate to isobutyl alcohol by analyzing the metabolism of [2–13C] valine (Dickinson et al., 1998). Beside this catabolic route, the α-keto acids also derive from carbohydrate metabolism termed the anabolic pathway for higher alcohol formation (Chen, 1978). The anabolic route includes the biosynthesis of 1-butanol and 1-pentanol, which is especially active under anaerobic conditions (Mauricio, Moreno, Zea, Ortega, & Medina, 1997). The concentration of higher alcohols can therefore be affected by the uptake efficiency of the corresponding amino acid and sugar utilization rate. The contribution of each biosynthetic pathway can be influenced by wort amino acid composition, wort sugar composition, fermentation stage and yeast strain (Eden et al., 2001; Kruger, Pickerell, & Axcell, 1992; Sablayrolles & Ball, 1995).

Furthermore, esters, especially acetate esters, whose syntheses is linked to the concentration of their corresponding alcohol, are very important due to their responsibility for the highly desired fruity, honey and perfume-like aroma in yeast fermented beverages. They are the product of an enzyme-catalyzed condensation reaction between an acetyl-CoA and a higher alcohol, catalyzed by alcohol acetyltransferases (Verstrepen, Van Laere, et al., 2003). Even, volatile esters are only present in trace quantities they can affect beer or wine flavor well below their threshold value (Verstrepen, Van Laere, et al., 2003; Verstrepen, Derdelinckx, et al., 2003). However, the absolute amount of aroma-active compounds is not substantial for the flavor of fermented alcoholic beverages. The relationship and fine-tuning of these different volatiles are more important.

The aim of this study was to investigate the significance of the proteinogenic amino acids for the production of yeast aroma compounds during fermentation. Therefore, the important aroma-active higher alcohols propanol, isobutanol, isoamyl alcohol and 2-methylbutanol and esters ethyl acetate, isoamyl acetate, 2-methylbutyl acetate, isobutyl acetate and ethyl hexanoate were analyzed under different nitrogen conditions. The focus of investigations was on amino acid variations in synthetic media, calculated by a design of experiments (DoE) created with Design Expert® to reduce primary not significant parameter variations, since these are most important and most abundant nitrogen compounds in industrial wort. Volatile compounds of each fermentation were quantified and their correlation to the most significant amino acids for an ale and a lager yeast strain were investigated. This task of pattern recognition was analyzed by means of PLS, a powerful tool of chemometrics for analyzing multivariate datasets together with multiple targets. In this paper, we provide new evidence for the importance of different amino acids for the production of adequate quantities of the volatile aroma-active compounds in fermentation.

2. Materials and methods

2.1. Yeast strain and culture conditions

All experiments were carried out with an industrial lager brewing strain (W 34/70; Technische Universität München, Lehrstuhl für Brau- und Getränketechnologie, Freising, Germany) and an industrial ale brewing strain (W 68; Technische Universität München, Lehrstuhl für Brau- und Getränketechnologie, Freising, Germany). Yeast precultures were shaken overnight at 28 °C in test tubes with 10 ml of unhopped malt extract medium (Weyermann) (12° Plato). After 16 h of growth, this 10 ml overnight culture was used to inoculate 150 ml of unhopped malt extract medium in 180 ml glass bottle with an open swing stopper fixed with parafilm. The second preculture was shaken at 28 °C for 48 h followed by inoculation in 2 L medium in a Duran bottle and also shaken at 28 °C for further 48 h under semi-anaerobic conditions. Cells were cropped and washed with sterile, distilled water.

The standard synthetic medium used for aroma compound production was composed of (g/L): yeast nitrogen base w/o amino acids (Difco), 6.7; K_2HPO_4, 1.3; glucose, 12; maltose, 74; maltotriose, 17; fructose, 2.5; sucrose, 4; glycine (G), 0.04; alanine (A), 0.12; valine (V), 0.13; leucine (L), 0.17; isoleucine (I), 0.08; serine (S), 0.07; threonine (T), 0.08; asparagine (N), 0.16; glutamine (Q), 0.11; aspartic acid (D), 0.9; glutamic acid (E), 0.1; cysteine (C), 0.01; methionine (M), 0.02; lysine (K), 0.14; arginine (R), 0.15; histidine (H), 0.05; phenylalanine (F), 0.15; tyrosine (Y), 0.12; tryptophan (W), 0.02; proline (P), 0,36 and if necessary adjust the pH value with 20% lactic acid to pH 5.4 (Sacher, 2006). For the variation experiments amino acid concentrations were adjusted according to the experimental design in Table 1.

The cultured and washed yeast, with a pitching rate of 15×10^6 viable cells/ml for lager yeast and 10×10^6 viable cells/ml for ale yeast, was used to inoculate 400 ml of fresh, prewarmed (12 °C or 20 °C) synthetic medium (pH level 5,4) in 500 ml glass bottle with an open swing stopper fixed with parafilm to create anaerobic conditions. Static fermentation was carried out at 12 °C for the lager strain and 20 °C for the ale strain. Samples for chromatographic analysis were taken after 96 h of fermentation and immediately cooled on ice.

2.2. Design of experiments of amino acid variations in synthetic medium

For varying the concentrations of the twenty proteinogenic amino acids in synthetic medium over three levels (standard concentration; maximum concentration, +50% of standard concentration; and minimum concentration, −50% of standard concentration, see Table 2) Response surface methodology (RSM; Design-Expert package, version 8.0.4, 2010; Stat-Ease, Minneapolis, MN, USA) was used. RSM is an optimization approach permitting to determine the input combination of factors that maximize or minimize a given objective function. Here, RSM modeling was used for reduction of primary not significant parameter variations. In this paper D-optimal design was employed. This computer-generated design is an alternative to consider an irregular experimental region for a nonstandard model or an unusual sample size requirement (Montgomery, 2001). A design is said to be D-optimal if $|X'X^{-1}|$ is minimized. This implies that 34 fermentations were done by varying the concentrations of all twenty amino acids (independent variables) (see Table 1). Here, only single fermentations of each of the 34 treatments were performed for the ale and lager yeast, so a source of errors can be occurred from the single fermentations as well as from the analysis.

2.3. Analytical method

Headspace gas chromatography coupled with flame ionization detection (HS-GC-FID) was used for measurement of higher alcohols and esters in the fermentation products. Samples (5 ml) were collected in 15-ml precooled glass tubes, which were immediately closed and cooled on ice. The samples were analyzed with a calibrated Hewlett–Packard 6890 gas chromatograph equipped with a headspace sampler (HP 7694; Hewlett–Packard, Waldbronn, Germany) and with an HP-5 column (Crosslinked 5% Ph – 95% Me-Si; length, 50 m; inside diameter, 0.32 mm; layer thickness, 0.52 μm; Waldbronn, Germany). Samples were heated for 20 min at 65 °C in the headspace autosampler. The injection

Table 1
DoE of amino acids in synthetic medium calculated by design Expert. Concentration of amino acids in mg/l.

Sample	G	A	V	L	I	S	T	N	Q	D	E	C	M	K	R	H	F	Y	W	P
1	40	120	130	170	80	70	80	160	110	90	100	10	20	140	150	50	150	120	20	360
2	20	60	195	255	40	35	120	80	165	45	150	15	30	210	75	75	225	180	10	540
3	20	60	195	255	120	35	40	240	55	135	50	5	10	210	75	25	225	60	10	540
4	40	120	130	170	80	70	80	160	110	90	100	10	20	140	150	50	150	120	20	360
5	60	60	65	85	40	105	40	80	165	135	150	15	10	210	75	25	75	60	10	540
6	20	180	195	255	120	105	40	240	55	135	50	15	10	70	225	75	225	180	10	540
7	60	60	65	255	120	35	120	80	55	45	150	5	30	70	225	25	225	60	30	180
8	20	180	65	85	120	35	120	80	165	135	50	15	10	210	75	75	75	180	10	180
9	60	180	195	255	40	105	120	240	165	45	50	5	30	70	225	75	75	60	30	540
10	20	180	65	85	40	105	120	80	55	45	50	5	10	70	75	25	225	180	30	180
11	60	180	65	255	40	35	40	240	165	135	50	15	30	210	75	25	225	180	30	180
12	20	180	195	255	120	35	40	240	55	45	150	5	10	210	75	75	225	60	30	180
13	20	60	65	255	120	105	120	240	165	135	150	5	10	210	225	25	75	180	30	540
14	60	180	65	255	40	35	40	240	165	135	50	15	30	210	75	25	225	180	30	180
15	60	60	195	85	120	105	40	80	165	135	150	5	30	70	75	75	225	180	30	180
16	20	60	195	85	40	35	40	80	55	135	50	5	30	210	225	25	225	60	10	540
17	20	60	65	85	40	35	40	240	165	45	50	5	10	210	225	75	75	180	10	180
18	60	180	65	85	40	35	120	80	165	135	150	15	10	70	225	75	225	60	10	180
19	20	60	195	255	40	105	120	240	55	45	150	15	30	70	75	25	75	60	10	180
20	60	180	195	255	120	105	120	80	165	135	50	5	10	210	75	25	75	60	10	180
21	60	180	195	85	40	105	40	80	55	45	150	15	10	210	225	25	75	180	30	540
22	20	60	65	255	40	35	40	80	55	135	50	15	10	70	75	75	75	60	30	540
23	60	180	195	85	40	35	120	240	55	135	150	5	10	70	75	25	75	180	10	540
24	20	60	65	255	40	35	40	80	55	135	50	15	10	70	75	75	75	60	30	540
25	20	180	65	85	120	105	40	80	165	45	150	15	30	70	75	25	75	60	10	540
26	60	60	195	255	120	35	40	80	165	45	50	15	10	70	225	25	75	180	10	180
27	20	180	195	85	120	35	120	80	55	135	50	15	30	210	225	75	75	180	30	180
28	60	60	65	85	120	105	120	240	55	45	50	15	10	210	75	75	225	60	10	540
29	20	180	65	255	40	35	40	80	165	45	50	5	10	210	75	25	225	60	10	540
30	60	180	65	255	40	105	40	80	55	135	150	5	30	210	225	75	75	180	10	180
31	20	60	195	85	40	105	40	240	165	135	150	15	10	210	225	25	225	60	30	180
32	60	60	65	85	120	105	120	240	55	45	50	15	10	210	75	75	225	60	10	540
33	60	180	65	255	40	105	120	240	165	135	150	5	10	70	225	25	225	60	30	180
34	60	180	195	85	40	35	120	240	55	135	150	5	10	70	75	25	75	180	10	540

block and flame ionization detector temperatures were kept constant at 150 and 250 °C, respectively; helium was used as the carrier gas. The oven temperature was held at 50 °C for 11 min, then increased to 120 °C at a rate of 10 °C per min hold for 5 min and then increased to 220 °C at a rate of 20 °C per min, and finally held at 220 °C. The results were analyzed with Agilent Technologies Chemstation Rev. A.10.01 software.

2.4. PLS data analysis

PLS regression techniques were used to develop a mathematical relationship between the chemical data, represented by a matrix **X**, and the mean sensory scores, represented by a matrix **Y**. The basis for the PLS-2 algorithm is to express the relationship between **Y** and **X** (i.e., $\mathbf{Y} = f(\mathbf{X})$) through latent variables $\boldsymbol{t}$ and $\boldsymbol{u}$, such that covariance between $\boldsymbol{t}$ and $\boldsymbol{u}$ is maximized (inner relationship). Data from the D-optimal design shown in Table 1 were used as **X**-matrix for determination of significant model terms in aroma compound profiling of *Saccharomyces pastorianus* var. *carlsbergensis* strain S23 and *Saccharomyces cerevisiae* strain S81 by PLS regression. **Y**-matrix, data shown in Tables 3 and 4 comprises the concentrations of the measured higher alcohols and esters establishing the aroma compound profile for yeast strain S23 and S81. The calculations on the 34 objects in each type of yeast strain were programmed and carried out applying the most commonly used Nonlinear Iterative Partial Least Squares (NIPALS) Algorithm developed by Wold (Abdi, 2010; Henrion & Henrion, 1995; Kessler, 2007; Krause, Schöck, Hussein, & Becker, 2011; Mitzscherling, 2004; Wold, 1974).

2.5. Variable importance

The aim of this study was to extract a fingerprint of amino acid importance with respect to aroma compound profile established by yeast fermentation. Extracting informative variables in multivariate regression using PLS can be done analyzing regression coefficients combined with VIP (Chong & Jun, 2005; Eriksson, Johansson, Kettaneh-Wold, & Wold, 2006; webpage, 2011). Therefore, the "autoscaled" regression coefficients (Eq. (1)) and the VIP (Eq. (2)) were calculated for each model and variable, respectively.

$$B_s = W(P \cdot W)^{-1} \cdot Q^T \quad (1)$$

Table 2
The three levels of amino acids concentrations in mg/l used for DoE.

Amino acid	Standard concentration [mg/l]	Minimum concentration [mg/l]	Maximum concentration [mg/l]
G	40	20	60
A	120	60	180
V	130	65	195
L	170	85	255
I	80	40	120
S	70	35	105
T	80	40	120
N	160	80	240
Q	110	55	165
D	90	45	135
E	100	50	150
C	10	5	15
M	20	10	30
K	140	70	210
R	150	75	225
H	50	25	75
F	150	75	225
Y	120	60	180
W	20	10	30
P	360	180	540

Table 3
Final aroma concentrations in the single 34 fermentations performed with the lager yeast, determined by HS-GC-FID.

Sample	Propanol [mg/l]	Isobutanol [mg/l]	Isoamyl alcohol [mg/l]	2-Methyl butanol [mg/l]	Ethyl acetate [mg/l]	Isobutyl acetate [mg/l]	Isoamyl acetate [mg/l]	2-Methyl butyl acetate [mg/l]	Ethyl hexanoate [mg/l]
1	4.7	3.8	20.7	6.5	8.4	0.02	0.3	0.04	0.16
2	9.5	4	26.3	3.5	8.4	0.02	0.3	0.01	0.2
3	3.8	4.1	23.9	6.9	8.8	0	0.3	0.01	0.2
4	5.2	4.3	22.7	7.3	9.5	0	0.4	0.01	0.2
5	5.9	3.8	19.4	5.6	9.3	0.02	0.3	0.03	0.16
6	5.3	4.4	25.7	7.2	10.4	0.03	0.4	0.04	0.17
7	4.3	3	31.5	9.5	7.8	0	0.4	0.05	0.16
8	3.3	3.3	15.3	15.8	6.9	0	0.2	0.08	0.16
9	9.9	4.5	31	3.1	11.2	0.03	0.5	0.01	0.2
10	6.2	3.8	16.8	7.1	6.7	0.03	0.2	0.01	0.1
11	8.2	3.87	40.7	3.6	8.2	0.02	0.6	0	0.1
12	4.2	4.6	28.8	7	7.3	0.03	0.5	0.05	0.15
13	3.3	2.7	34.1	7.8	8.4	0	0.6	0.05	0.14
14	6.9	3.3	34.9	3.3	7.4	0	0.5	0.01	0.13
15	4.1	5	12.2	9.2	7.7	0.03	0.2	0.1	0.1
16	10.5	9.9	18.9	6.3	10.5	0.03	0.3	0.04	0.14
17	8	5.6	19.8	5.9	6.2	0	0.2	0	0.1
18	6.1	4.2	14.6	6.2	6	0	0.2	0	0.1
19	14.5	6.3	38.3	3.2	4.7	0.03	0.5	0.02	0.11
20	4	4.8	28.7	7	5.1	0.02	0.4	0.04	0.13
21	16.6	19	36.4	11	18.7	0.1	0.8	0.1	0.2
22	7.1	3.2	65.6	3.6	17.9	0.02	1.4	0.03	0.23
23	11.9	7.2	15.6	4.3	9.2	0.02	0.2	0.01	0.2
24	6.5	3	60.2	3.3	14.3	0	0.01	0.2	0.2
25	3.2	2.6	12.4	11.8	9.3	0	0.2	0.06	0.18
26	3.8	3.4	30.8	6.7	8.2	0.04	0.4	0.03	0.22
27	4.3	5.5	13.9	12.4	10.8	0.03	0.2	0.08	0.23
28	5.2	6	33.6	18.1	19.9	0	0.9	0.2	0.31
29	5.8	2.5	38	3.3	11.2	0	0.7	0.01	0.2
30	5.4	2.4	35.8	3.2	7.7	0.03	0.4	0	0.22
31	8	5.2	16.1	4.9	11.1	0.03	0.3	0.01	0.3
32	3.9	4	20.2	13.6	14.4	0	0.4	0.1	0.2
33	5.4	2.4	31.8	3.3	7.1	0	0.4	0	0.2
34	11.6	7.7	17.3	5	12.7	0.05	0.3	0.04	0.22

$$VIP_j = \sqrt{n \sum_{a=1}^{A} \left(q_a^2 t_a^T t_a (W_{ja}/\|W_a\|)\right)^2 / \sum_{a=1}^{A} q_a^2 t_a^T t_a} \quad (2)$$

The matrix $\boldsymbol{B}_s$ contains the scaled regression coefficients, $\boldsymbol{W}$ the weighted scores, $\boldsymbol{P}$ the loadings of matrix $\boldsymbol{X}$ and Q the loadings of matrix Y. Further, q and w represent column vectors (single components) out of the matrices $\boldsymbol{Q}$ and $\boldsymbol{W}$, respectively, vector t out of matrix $\boldsymbol{T}$ containing scores of matrix $\boldsymbol{X}$. The index a indicates the number of the component, index j the number of the respective variable. All the presented methods reaching to the final model output are collected in a final scheme for better understanding (see Fig. 1).

To compare between variables in a data matrix appropriate preprocessing of the data is recommended. The method of autoscaling is widely used to scale each (independent) variable into comparable ranges. Therefore, the dataset is transferred to the center of the multidimensional coordinate system and scaled, such that the absolute value of each variable does not influence the result. After PLS decomposition and model development it is of interest, which variable of the regressor matrix $\boldsymbol{X}$ is of importance to the target of interest. Next to others, the method of VIP can be used to estimate the importance of each variable in the established PLS model. Therefore, the scores and loadings of components used in the model are scaled and summed up for each variable independently (see Eq. (2)). Nevertheless, the question of importance of single variables should not be addressed only by using one method (webpage, 2011). The second approach used considers the influence of each variable by scaled coefficients or regression parameters in terms of prediction models (Eq. (1)). Both presented methods are applied in combination in order to estimate the importance of the used variables (amino acids) influencing the detected concentrations of aroma compounds.

3. Results and discussion

The industrial lager yeast strain S23 and ale yeast strain S81 were used to evaluate the influence of nitrogen composition on flavor formation, in relation to determine the significant amino acids in aroma compound profiling. Before the amino acid variations were realized the standard synthetic medium was compared to an industrial unhopped wort. The characteristics budding index, alcohol formation, extract decrease as well as the aroma compound production were comparable between both media (data not shown) (Procopio, Qian, & Becker, 2011a, 2011b). The different fermentations were carried out in glass bottles under anaerobic conditions at 12 °C for the lager yeast and 20 °C for the ale yeast. These 34 fermentation vessels contained 400 ml sterile synthetic medium according to their amino acid compositions calculated by DoE with Design Expert (see Table 1).

3.1. Flavor formation

After main fermentation of 96 h, the production of nine most important aroma-active compounds was analyzed by HS-GC. The results of the HS-GC analysis are shown in Tables 3 and 4. The *S. cerevisiae* strain S81 and *S. pastorianus* var. *carlsbergensis* strain S23 strains synthesized a variety of flavor compounds, at which most

Table 4
Final aroma concentrations in the single 34 fermentations performed with the ale yeast, determined by HSspace GC-FID.

Sample	Propanol [mg/l]	Isobutanol [mg/l]	Isoamyl alcohol [mg/l]	2-Methyl butanol [mg/l]	Ethyl acetate [mg/l]	Isobutyl acetate [mg/l]	Isoamyl acetate [mg/l]	2-Methyl butyl acetate [mg/l]	Ethyl hexanoate [mg/l]
1	6.8	29.7	33.7	9.6	24.5	0.31	1.5	0.17	0.08
2	7.4	23.7	45.8	6.2	20	0.3	2	0.1	0.1
3	7.4	34.1	51.3	15.8	22.3	0.3	2	0.2	0.1
4	9.6	34.3	33.8	10.9	14	0.2	1.2	0.2	0.1
5	7.7	29.2	35	8.7	17.8	0.2	0.8	0.09	0.09
6	8.1	36.4	45	12.7	19.5	0.3	1.5	0.18	0.1
7	6.3	24.9	30.2	12.1	9.4	0.14	0.9	0.16	0.07
8	5.7	24.2	21	19.7	8.7	0.12	0.5	0.26	0.06
9	8.6	33.5	48.7	8.6	27.1	0.42	2.7	0.19	0.13
10	7.5	32.5	26.6	10.3	11.4	0.2	0.8	0.1	0.1
11	6.3	19.7	32.9	5.5	11.7	0.1	1.2	0.1	0.1
12	5.3	22.2	31.4	9.3	13.4	0.18	1.2	0.15	0.08
13	4.8	17.1	34.8	9	15.3	0.14	1.2	0.14	0.09
14	6.5	19	31.5	5.4	10.1	0.12	1	0.07	0.07
15	5.3	16.4	15	4.8	9.4	0.1	1	0.1	0.1
16	6.6	31.1	22.9	7.3	14.9	0.26	0.6	0.12	0.08
17	6.4	23.6	22.6	7	10.9	0.2	0.6	0.1	0.1
18	5.6	20.4	19.5	6.7	9.7	0.1	0.5	0.1	0.1
19	6.4	23.6	32.3	8.8	9.1	0.13	0.9	0.11	0.06
20	5.5	23.8	34	6.9	9.8	0.14	1	0.09	0.07
21	6.4	30.8	22.8	7	18.4	0.3	0.8	0.1	0.1
22	5.9	20	58.3	5.5	13.6	0.1	1.7	0.1	0.1
23	6.3	32.3	21.8	7.7	11.2	0.19	0.3	0.09	0.09
24	5.9	20.3	60.9	5.7	13.2	0.12	1.7	0.07	0.06
25	6.4	17.1	18	8.9	7.7	0.1	0.6	0.1	0
26	6	27	39	13	30	0.2	1.2	0.2	0.1
27	5.5	30.8	18.7	12.5	10.5	0.2	0.5	0.2	0.1
28	5	29.4	20.2	14.2	10.7	0.2	0.5	0.2	0.1
29	6.4	19.5	41.8	5.2	10.2	0.1	1.1	0.06	0.06
30	6.7	24	44	22	33	5.2	1.7	0.1	0.1
31	6.5	29.7	19.7	5.7	8.7	0.15	0.5	0.06	0.06
32	4.9	27.1	19.9	13.5	11.3	0.16	0.5	0.18	0.06
33	7.1	25.9	37.9	6.8	8.4	1	0.9	0.06	0.05
34	6.2	30.1	20.8	7.2	12.9	0.2	0.5	0.1	0.1

were present in normal range of concentrations (see Tables 3 and 4).

All the higher alcohols measured were accumulated throughout the fermentation for lager and ale yeast. According to amino acid composition in synthetic medium different concentrations of propanol, isobutanol, isoamyl alcohol, and 2-methylbutanol were detected. Also, the esters were detected by HS-GC. All esters measured were accumulated throughout the fermentation by using the ale yeast strain S81. Different concentrations of ethyl acetate, isoamyl acetate, 2-methylbutyl acetate, isobutyl acetate and ethyl hexanoate were accumulated throughout the fermentation according to amino acid composition in synthetic medium. 2-Methylbutyl acetate and isobutyl acetate by using the lager strain were not detected under all amino acid variations. Isobutyl acetate concentrations in lager yeast fermentations are always very low. This thesis can be confirmed in all 34 variation experiments. In many amino acid compositions no isobutyl acetate was detected. Isobutyl acetate is the corresponding ester to the higher alcohol isobutanol, which can be synthesized by catabolic route from valine via the Ehrlich pathway. The results may suggest, that in case of low valine concentrations (65 mg/l) only little amount of isobutyl acetate is detectable for lager yeast fermentations. Also, 2-methylbutyl acetate was not detected in all of the fermentations. 2-methylbutyl acetate is the corresponding ester to the higher alcohol 2-methyl butanol, which can be synthesized by degradation of the amino acid isoleucine via the Ehrlich pathway. No concentrations of 2-methylbutyl acetate were analyzed, when low concentrations of isoleucine 40 mg/l were used in synthetic media. However, this could not be confirmed in all experiments. Increased concentrations of isoamyl acetate were detected especially for the ale yeast strain, in samples where more leucine was present in the medium. Further, samples with high isoleucine concentrations added to the medium showed the highest 2-methylbutanol concentrations in ale yeast fermentation. It has been shown that addition of valine, leucine and isoleucine strongly increase the production of the corresponding fusel alcohols isobutanol, isoamyl

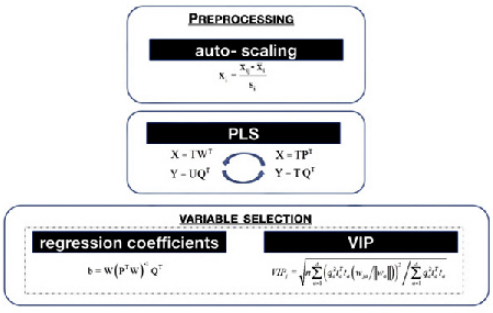

Fig. 1. Schematic summary of methods used for processing of the data set for model investigation; = variable (column) in matrix X, = mean of variable (column) in matrix X, = standard deviation of variable (column) in matrix X, X = regressor matrix, Y = target matrix, T = score matrix for X, W = weighted loading matrix, U = score matrix for Y, P = loading matrix for X, Q = loading matrix for Y, b = vector of regression coefficients in prediction of Y, VIP = variable importance in the projection, n = number of samples.

alcohol and 2-methyl butanol and this may in turn lead to enhanced ester production (Äyräpää, 1971; Calderbank & Hammond, 1994; Engan, 1970; Sablayrolles & Ball, 1995). Also, for both yeast a trend is seen for increasing ethyl acetate production, when using high proline concentrations in synthetic medium. Thus, some of the varied concentrations of aroma-active compounds could be related to the different amino acid compositions in synthetic media. Nevertheless, these higher alcohols are also formed by sugars via anabolic route and most higher alcohols like propanol can just be synthesized by carbohydrates. Therefore, no clear stoichiometry of amino acid concentration and the concentration of the corresponding higher alcohol can be computed. The relative contribution of Ehrlich pathway and the anabolic pathway to the formation of higher alcohols was shown by Chen (Chen, 1978). Here, he showed when [l4C(U)]-labeled valine, isoleucine, or leucine was used as tracer, the contribution of Ehrlich pathway to the formation of isobutanol, 2-methyl butanol and isoamyl alcohol was found to be 34%, 75%, and 80%, respectively. The results clearly indicate that the relative contributions of the Ehrlich and anabolic pathways vary with each higher alcohol.

3.2. Chemometric analysis

In this contribution the chemometric method is used to investigate the single importance of amino acids ("variables") on the resulting aroma compound profile. Therefore, PLS was used to calculate the latent variables using all 9 aroma compounds as target matrix (each in a single column). In this work, those latent variables are used to calculate variable importance in the projection (VIP) as well as the regression coefficient vector of prediction model for single aroma compounds. The biggest challenge in chemometric analysis for regression or correlation purposes is to find the

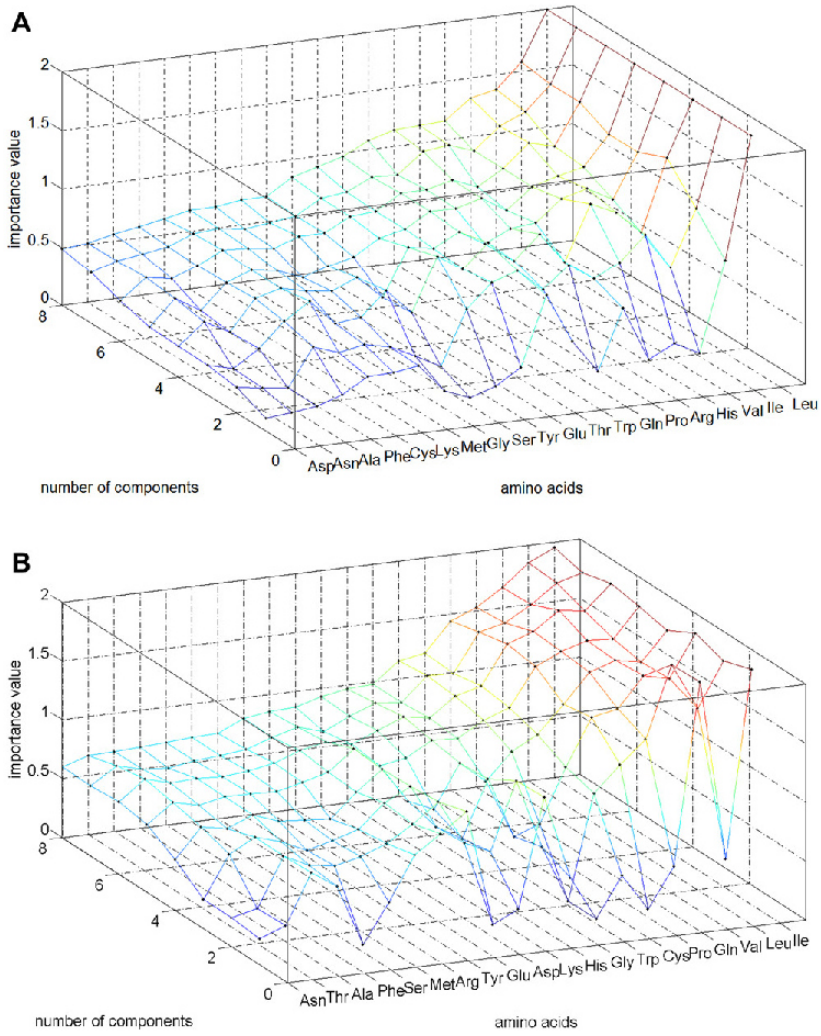

Fig. 2. Statistical importance with rising number of components of each amino acid on the concentrations of aroma compounds of (A) ale yeast strain S81 and (B) lager yeast strain S23; to achieve comparability, each importance measure was summed up over each individual aroma-active compound, normalized to the maximum value and finally summed between both importance measures used.

representative number of components (latent variables). The first step in this study was to iterate over the number of components. The result presented in Fig. 2 indicates, that isoleucine, leucine, valine, glutamine, proline and cysteine are the six most important amino acids for the lager yeast strain S23, leucine, isoleucine, valine, histidine, proline and glutamine the six most important amino acids for the ale yeast strain S81 fermentation in an aroma compound profile – fingerprint sense.

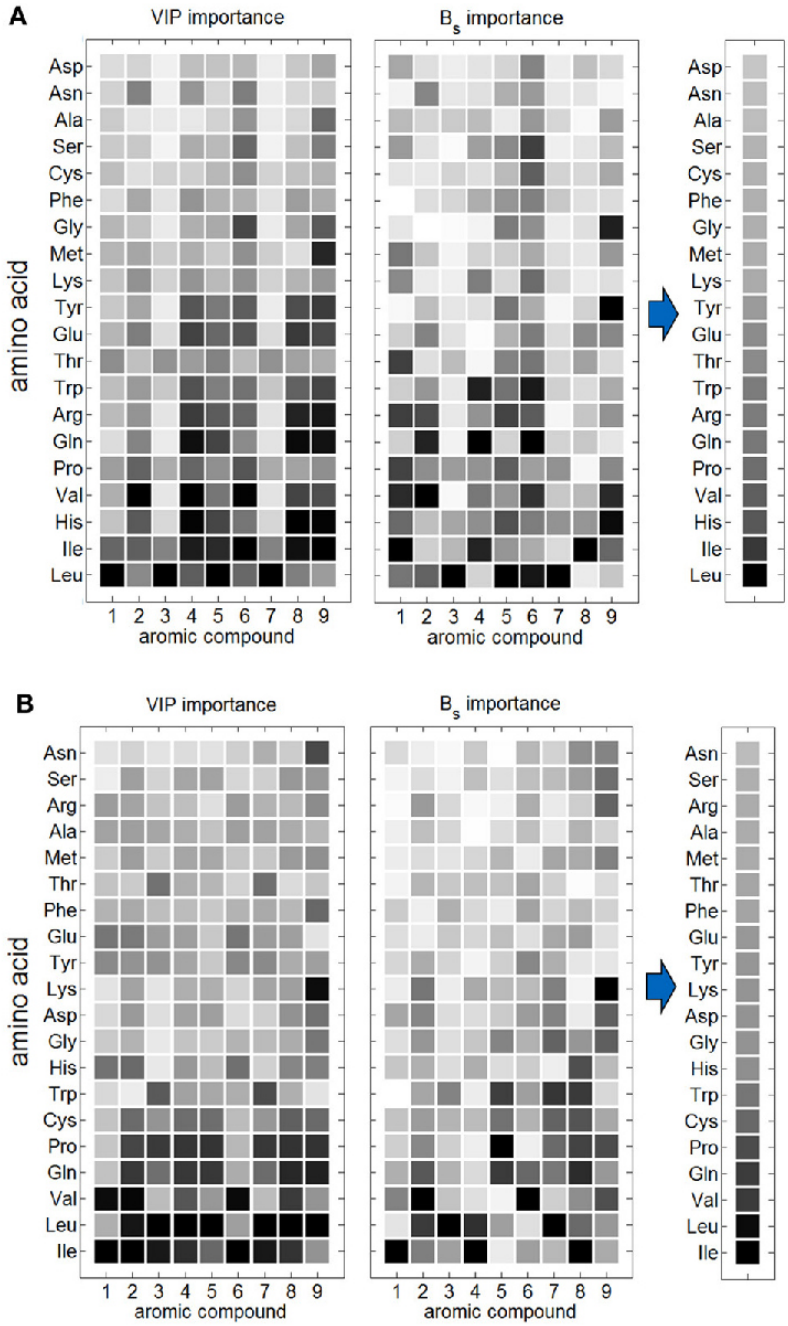

Fig. 3. Statistical importance of each amino acid on the concentrations of aroma compounds of (A) ale yeast strain S81 and (B) lager yeast strain S23; *x* axis labels are 1 - propanol, 2 – isobutanol, 3 – isoamyl alcohol, 4 - 2-methyl butanol, 5 – ethyl acetate, 6 – isobutyl acetate, 7 – isoamyl acetate, 8 – 2-methyl butylacetate and 9 – ethyl hexanoate; (A,B – left) – variable importance in the projection (VIP) map; (A,B – right) – standardized regression coefficient map – amino acids were sorted by their absolute VIP and B_s values; (A,B – single column) – summed and normalized importance measure of both methods; black indicates high statistical importance on the resulting concentrations of aroma compounds.

However, decision making for the amount of components is typically carried out monitoring the root mean square error (RMSE) of the resulting predictive model. Since the aim of this study was not to establish predictive models but using PLS as tool to develop a fingerprint, the number of components investigated were chosen on their explanation in variance of targets (Y) (Jørgensen & Goegebeur, 2007). The explained variance calculated on 8 components resulted in more than 80% (data not shown). Furthermore, the importance values did not change significantly increasing the number from 6 to 8 components (Fig. 2). Thus, 8 components were taken for investigating the influence of each amino acid on the aroma compound profile analyzed. The final results are presented in Fig. 3A for *S. cerevisiae* strain S81 and Fig. 3B for *S. pastorianus* var. *carlsbergensis* strain S23, whereas each regression parameter/VIP value is represented in a gray scale according to its magnitude. Therefore, each calculated numerical value was normalized to the maximum importance measure of its corresponding aroma compound.

Each amino acid causing a value representing a black dot in both shown color maps might have high impact on aroma compound concentrations established in the corresponding fermentation product. Those amino acids are leucine, isoleucine, valine, glutamine, cysteine and surprisingly proline for *S. pastorianus* var. *carlsbergensis* strain S23 while for *S. cerevisiae* strain S81 leucine, isoleucine, valine, histidine, glutamine and proline. However, a clear differentiation of importance in the whole 20 amino acids is not possible. This counts especially for the results calculated for top fermenting yeast. The importance values for glutamine, arginine, tryptophan, threonine and glutamic acid did not differ significantly compared to leucine. This could be caused due to the enhanced synthesis of individual aroma-active components in ale yeast fermentation due to the higher fermentation temperature. Therefore, it can be assumed that the spectrum of amino acids is utilized to a greater extend which results in almost equal importance values.

The amino acids leucine, isoleucine and valine for both yeast strains seem to be the most significant amino acids for the measured concentrations of aroma compounds. These findings match with existing results reported in the literature. Leucine, isoleucine and valine are the branched chain amino acids taken up by yeast, being transaminated, decarboxylated and then reduced to the higher alcohols isobutanol, isoamyl acetate and 2-methyl butanol via Ehrlich pathway (Chen, 1978; Dickinson et al., 1997; Ehrlich, 1907; Hazelwood et al., 2008). The esters isoamyl acetate, isobutyl acetate and 2-methylbutyl acetate are synthesized via corresponding higher alcohol and acetyl coA (Malcorps & Dufour, 1992; Nordström, 1963, 1964). So decreased concentrations of isobutyl acetate, isoamyl acetate and 2-methylbutyl acetate were detected in response to low levels of valine, leucine and isoleucine in the medium as well as vice versa.

Furthermore, the influence of addition of different amino acid concentrations in fermentation medium on several flavor compounds has been shown (Arias-Gil, Garde-Cerdán, & Ancín-Azpilicueta, 2007; Hernández-Orte, Ibarz, Cacho, & Ferreira, 2005). Also there is a great effect of total available nitrogen content on aroma formation. Hernández-Orte et al. showed that the addition of any kind of nitrogen to must causes reduction of different higher alcohols like isoamyl alcohol, methionol and phenylethanol while a combined supplementation of amino acids and ammonium results in high fruity and fusel notes (Hernández-Orte et al., 2005). There is also a positive correlation between the content of nitrogen and concentration of esters, due to higher alcohol acyl transferase activity (Bell, Ough, & Kliewer, 1979; Guitart, Hernández Orte, Ferreira, Peña, & Cacho, 1999; Torrea, Fraile, Grade, & Ancín, 2003; Ugliano, Travis, Francis, & Henschke, 2010; Yoshimoto, Fukushige, Yonezawa, & Sone, 2002). Thus, Torrea et al. showed that addition of amino acids and ammonium nitrogen increases acetate and ethyl esters to a greater extent and decreased higher alcohols to a lesser extent than ammonium nitrogen alone (Torrea, Valera, Ugliano, Ancín-Azpilicueta, & Francis, 2011). Nevertheless the nitrogen base content in the presented experiments was kept constant. Just the amino acids where varied to investigate their significance on aroma compound profiling, what indeed could affect total nitrogen content in the medium.

However a surprising finding of the presented study was the significance of proline for the measured aroma compound profile of *S. pastorianus* var. *carlsbergensis* strain S23 and *S. cerevisiae* strain S81. Given that proline is not converted to an higher alcohol by Ehrlich-pathway its significance in aroma formation could be explained by the synthesis of glutamate from proline. Therefore proline is taken up by yeast during fermentation which has been shown by different working groups (Gibson et al., 2009; Ingledew, Magnus, & Sosulski, 1987; Ough & Stashak, 1974; Poole et al., 2009). Proline is taken up during growth phase and is excreted at the end of fermentation (Gibson et al., 2009; Ough, Huang, & An, 1991).

4. Conclusion

Under the presented conditions it could be shown, that it is possible to establish a fingerprint between the available amino acid concentrations and the detected concentrations of different aroma compounds. In conclusion, leucine, isoleucine, valine, glutamine, cysteine and proline for *S. pastorianus* var. *carlsbergensis* strain S23 while for *S. cerevisiae* strain S81 leucine, isoleucine, valine, histidine, glutamine and proline were calculated as the most important amino acids. These amino acids seem to have impact on the concentrations of the measured aroma compound spectrum. Proline being identified as significant amino acid, with respect to the aroma compound profile under anaerobic fermentation conditions, leads again to a novel classification of sequential amino acid uptake during brewing yeast fermentation. New classification of amino acid uptake for brewing yeast has already been shown (Schmitt & Boivin, 2011). Furthermore, the proline uptake of brewing yeast strains seems to be strain dependent, especially by comparison of lager and ale yeast strains (Procopio & Becker, 2012). Genetic analysis of yeast during the fermentation process would help to identify and understand the possible strain dependent proline uptake under fermentation with limited oxygen content. In addition, marking experiments with stable isotopes will help to study the catabolism of proline and its anabolism of daughter products during yeast fermentation. It is known that under anaerobic yeast fermentation little to no proline is degraded, such as to glutamate, since this oxidative step requires an oxidase which needs molecular oxygen (Wang & Brandriss, 1987). Thus, oxygen, even if present in very low concentrations, can support proline catabolism during fermentation (Ingledew et al., 1987). Also, proline utilization could be supported under anaerobic conditions when no other source of amino acids is present in the fermentation medium (Wang & Brandriss, 1987).

Nevertheless, higher alcohols like tyrosol and tryptophol also formed via Ehrlich mechanisms were not taken into chemometric analysis, cause of not being most relevant for aroma compound profiling in beer. Therefore, further amino acids could be classified as significant for the aroma compound profile of lager or ale yeast fermented beverages taking these other higher alcohols (tyrosol, methionol and tryptophol) into account. Though, the presented approach successfully describes the use of multivariate analysis, namely PLS establishing a fingerprint on provided amino acids and resulting aroma compound profile in fermentation product.

Nevertheless, literature presents several different methods with respect to other aspects estimating variable importance or selecting informative variables from datasets (Sorol, Arancibia, Bortolato, & Olivieri, 2010; Teófilo, Martins, & Ferreira, 2009). Those possibilities should be investigated in further studies. However, the results indicate the possibility using chemometrics to uncover synergies between input variables and output variables in a statistical manner.

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2.4 Differential transcribed yeast genes involved in flavor formation and its associated amino acid metabolism during brewery fermentation

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ORIGINAL PAPER

Differential transcribed yeast genes involved in flavour formation and its associated amino acid metabolism during brewery fermentation

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Abstract During fermentation, *Saccharomyces* yeast produces various aroma-active metabolites which determine the different characteristics of aroma and taste in fermented beverages. Amino acid utilisation by yeast during brewer's wort fermentation is considered to be linked to flavour profile. For a better understanding of the relationship between the biosynthesis of aroma-relevant metabolites and the importance of amino acids, DNA microarrays were carried out on the *Saccharomyces cerevisiae* strain S81 and the *Saccharomyces pastorianus* strain S23. Changes in the transcription of those genes were measured which are associated with amino acid assimilation and its derived aroma-active compounds during fermentation. Genes were selected whose average expression level increased or decreased more than 1.5-fold at 8, 12, 24, 48, 72, 96 or 120 h and showed a significant ($P \leq 0.05$) differential expression pattern during the period of fermentation. For the ale strain, 57 of the detected genes involved in flavour and amino acid metabolism were selected, whereas 46 significant genes were evaluated for the lager strain. Among these, genes were those involved in transcriptional regulation as well as others associated with amino acid transport, such as *PUT4* which is accountable for the uptake of proline. Other genes whose expression decreased or increased during fermentation were evaluated—including those participating in amino acid metabolism, for example glutamate and proline metabolism—as well as enzymes involved in the biosynthesis of aroma-active higher alcohols and esters, which are most important for typical beer flavour. This study provides information that might help to improve the understanding and production of defined concentrations of specific aroma compounds during brewery fermentation.

Keywords Gene expression · Microarray analysis · Amino acids · Aroma-active metabolites · Yeast fermentation

Abbreviations

BCAA	Branched-chain amino acid
CEL	(RNA signal intensity) files
HS-GC-FID	Headspace gas chromatography coupled with flame ionisation detection
RMA	Robust multichip average algorithm

Introduction

Saccharomyces yeast has been used in the production of alcoholic beverages for a long time. Along with ethanol and carbon dioxide as well as aldehydes, organic acids, organic sulphides and carbonyl compounds, fermenting yeast cultures produce a broad range of aroma-active higher alcohols and esters [1, 2]. These metabolites represent the characteristics of flavour and taste in fermented beverages such as beer, wine, cider and sake [3–6]. Numerous environmental factors such as substrate composition in fermentation media can affect the production of these higher alcohols and esters during yeast fermentation processes [7–9]. The most important sensory-active higher alcohols for beer aroma are propanol, isobutanol, isoamyl alcohol, 2-methyl butanol and phenyl ethanol [10, 11]. The amino acid composition in wort is particularly known to influence the

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synthesis of higher alcohols in fermented beverages [12–14]. Amino acids represent the major source of assimilable nitrogen in wort and are absorbed by yeast in a sequential order [15, 16]. Amino acid uptake is performed by a number of amino acid transporters located in the cell membrane of the yeast. The majority of these transporters have broad substrate specificity, whereas some are very highly specific in that they transport only one amino acid [17–22]. Amino acids assimilated by the Ehrlich pathway (valine, leucine, isoleucine, methionine, tyrosine, tryptophane and phenylalanine) are taken up slowly throughout the fermentation time [16]. The initial transamination reaction is catalysed by mitochondrial and cytosolic branched-chain amino acid aminotransferases (BCAATases) [23–27]. The resulting α-keto acid is converted into an aldehyde by decarboxylation and followed by reduction to a higher alcohol [28, 29]. The Ehrlich pathway for methionine catabolism represents a special case [26, 30]. Beside this catabolic pathway proposed by Ehrlich, higher alcohols can also result from sugars as by-products of amino acid synthesis, which is termed the anabolic pathway [23, 31, 32].

In addition, esters, especially acetate esters, represent the main class of flavour-active metabolites in alcoholic fermented beverages. The synthesis is linked to the concentration of its corresponding higher alcohol, except for ethyl acetate, which is linked to ethanol. Acetate esters are the product of an enzyme-catalysed condensation reaction between an acetyl-CoA and a higher alcohol. This reaction is catalysed by alcohol acetyltransferases [33]. The fatty acid ethyl esters represent the second class of esters produced by fermenting yeast. With the participation of acyl-CoA synthetase, ethyl esters are formed by ethanol and acyl-CoA compounds resulting from the fatty acid metabolism of organic acids and free coenzyme A [34, 35]. Ethyl acetate, isoamyl acetate, isobutyl acetate, phenyl ethyl acetate and the C6 and C8 short-chain fatty acid ethyl esters ethyl hexanoate and octanoate are responsible for the highly desired fruity aroma characteristics in beer, wine and sake [36–38]. Even though esters are only present in trace quantities, they are extremely important for the flavour profile [36, 39]. The synergistic effects of the presence of different volatile esters can explain why esters can affect the flavour of fermented beverages well below their threshold value [11].

It has been shown that amino acid concentrations can influence the synthesis of aroma-active higher alcohols and their corresponding esters during yeast fermentation [9, 40, 41]. Thus, an improved understanding of amino acid uptake and assimilation is therefore essential to generate defined amounts of sensory metabolites in order to regulate specific sensory effects in fermented beverages. Beside the influence of amino acids on the aroma compound profiling, the understanding of transcriptional responses of yeast genes involved in the biosynthesis of flavour compounds is limited. Furthermore, different *Saccharomyces* yeast strains contribute to the chemical composition with an impact on the aroma properties of the resulting beverage [42, 43]. Beer is usually manufactured from wort by brewer's yeast, which can usually be divided into two types based on flocculation behaviour: lager yeast (*Saccharomyces pastorianus*) and ale yeast (*Saccharomyces cerevisiae*) [44]. Lager beers are relatively new to the brewing industry. Lager yeast, *S. pastorianus*, was first isolated and described in 1904 by Emil Christian Hansen. The lager yeast genome exhibits many similarities to that of ale yeast, due to being a hybrid of *S. cerevisiae* and *Saccharomyces eubayanus* [45], which is assumed to be an early ancestor of lager yeast. The aim of this study was to investigate differentially expressed genes participating in aroma compound profiling and its associated amino acid metabolism in the course of brewery fermentation. Therefore, we have analysed the global transcription profile of the most important yeast strains used in Bavarian beer production, the *S. cerevisiae* strain S81 and the *S. pastorianus* strain S23, by means of microarray technology. Furthermore, amino acid uptake was monitored, and volatile compounds were quantified at various different stages of fermentation. Fermentations were carried out in a synthetic medium under specific industrial brewing conditions used for traditional lager and ale beer fabrication. This will provide new evidence for the importance of amino acid assimilation and the expression of specific genes involved in flavour biosynthesis during yeast fermentation.

Materials and methods

Yeast strains and fermentation conditions

The industrial yeast strains used in this study were the ale yeast *S. cerevisiae* (strain S81; Hofbräuhaus, Freising, Germany) and the lager yeast *S. pastorianus* (strain S23; Hofbräuhaus, Freising, Germany). Yeast cells were cropped and washed with sterile, distilled water and pitched into synthetic wort. The synthetic medium used for aroma compound production was composed of: yeast nitrogen base w/o amino acids (Difco), 6.7 (g/L); K_2HPO_4, 1.3 (g/L); glucose, 12 (g/L); maltose, 74 (g/L); maltotriose, 17 (g/L); fructose, 2.5 (g/L); sucrose, 4 (g/L); glycine (G), 0.53 (mmol/L); alanine (A), 1.35 (mmol/L); valine (V), 1.1 (mmol/L); leucine (L), 1.3 (mmol/L); isoleucine (I), 0.61 (mmol/L); serine (S), 0.67 (mmol/L); threonine (T), 0.67 (mmol/L); asparagine (N), 1.21 (mmol/L); glutamine (Q), 0.75 (mmol/L); aspartate (D), 0.68 (mmol/L); glutamate (E), 0.68 (mmol/L); cysteine (C), 0.08 (mmol/L); methionine (M), 0.13 (mmol/L); lysine (K), 0.96 (mmol/L);

arginine (R), 0.86 (mmol/L); histidine (H), 0.32 (mmol/L); phenylalanine (F), 0.91 (mmol/L); tyrosine (Y), 0.66 (mmol/L); tryptophane (W), 0.1 (mmol/L); proline (P), 3.13 (mmol/L)—and, if necessary, the pH value was adjusted with 20 % lactic acid to pH 5,4 [46]. The specific gravity of the synthetic wort was 12°P. This medium was compared to an industrial un-hopped wort [47]. The cropped yeast cells were used to inoculate a fresh synthetic medium (pH level 5,4) with a pitching rate of 15×10^6 viable cells/mL for lager yeast and 10×10^6 viable cells/mL for ale yeast. Fermentations were carried out in tall 2-L EBC tubes. These experiments were carried out in triplicate for both yeast strains. Static fermentation was carried out at typical industrial temperature conditions of 12 °C for the lager strain and 20 °C for the ale strain. The fermentation tubes sample point was located at a height of approximately 50 cm. Samples were taken at intervals for up to 120 h after pitching and immediately cooled on ice. Synthetic wort and cells were separated by centrifugation at 0 °C. Cell pellets for RNA isolation were flash-frozen in liquid nitrogen and stored at −80 °C.

Fermentation analysis

The course of fermentation and yeast growth was followed by withdrawing samples, cooling them on ice and separating the yeast and fermenting wort by centrifugation. Before that, cell suspensions were diluted to an appropriate volume, and density was calculated using a counting chamber (Neubauer improved, BRAND GMHB + CO KG) and a standard light microscope (Zeiss) at 200× magnification. The extract and alcohol content of the fermenting media were measured with the DMA 4500 density analyser and Alcoholyser Plus (Anton Paar, Graz, Austria).

Headspace GC analysis

Headspace gas chromatography coupled with flame ionisation detection (GC-FID) was used for the measurement of higher alcohols and esters in the fermentation products. Samples (5 mL) were collected in 15 mL pre-cooled glass tubes, which were immediately closed and cooled on ice. The samples were analysed with a calibrated Hewlett-Packard 6890 gas chromatograph, using an internal standard. The gas chromatograph was equipped with a headspace sampler (HP 7694; Hewlett-Packard, Waldbronn, Germany) and an HP-5 column (cross-linked 5 % Ph −95 % Me–Si; length, 50 m; inside diameter, 0.32 mm; layer thickness, 0.52 µm; Waldbronn, Germany). Samples were heated for 20 min at 65 °C in the headspace auto sampler. The injection block and flame ionisation detector temperatures were kept constant at 150 and 250 °C, respectively; helium was applied as the carrier gas. The oven temperature was held at 50 °C for 11 min, then increased to 120 °C at a rate of 10 °C per min and held for 5 min, then increased to 220 °C at a rate of 20 °C per min, and finally held at 220 °C. The results were analysed by means of Agilent Technologies Chemstation Rev. A.10.01 software.

Amino acid analysis

The samples were centrifuged, and the upper layer was used for amino acid analysis. Amino acids were analysed according to a modified analysis by Krömer [48] using an HPLC (U3000 Dionex, Dionex, Sunnyvale, USA), utilising a Gemini C18 column (5 µm, 110A, 150 × 4.6 mm; Phenomenex, Aschaffenburg, Germany) with automated online derivatisation (*o*-phthaldialdehyde plus 3-mercaptopropionic acid) at a flow rate of 1 mL/min and with fluorescence detection. Modifications: Quantification was calculated with an external calibrating curve. Proline was analysed photometrically according to a modified analysis of Ough [49]. Quantification was carried out by a calibrating curve, and modifications were performed according to Schönberger [50].

RNA isolation, hybridisation and microarray analysis

Seven time points (8, 12, 24, 48, 72, 96, 120 h after pitching) and the reference sample (time point 0 h) were chosen for microarray analysis using Yeast 2.0 whole-genome GeneChips from Affymetrix. All analyses were carried out in triplicate, with each replicate processed separately. Twenty-four samples were used for each yeast strain (*S. cerevisiae* and *S. pastorianus*), making a total of 48 GeneChips. RNA was isolated applying the Trizol method (Invitrogen, Carlsbad, CA, USA) and further purified using an RNeasy mini kit (Qiagen, Hilden, Germany) for microarray analysis. RNA yield and quality were determined employing a bioanalyzer (2100 Bioanalyzer, Agilent Technologies, Inc.). Sample preparation, hybridisation and scanning were performed according to the manufacturer's instructions described in the technical manual (GeneChip Expression Analysis, Affymetrix [www.affymetrix.com]). Following scanning, RNA signal intensity (CEL) files were generated using the GeneChip operating system (GCOS, Affymetrix, Santa Clara, CA, USA). CEL files contain the raw signal intensity values for each probe on the array, generated from the scanned image of the GeneChip array. The CEL file therefore contains signal intensity values for the 11 perfect match probes and 11 mismatch probes within each probe set, which is more than 100,000 signal intensity values for the Yeast Genome 2.0 GeneChip array. Quality control analysis was performed to exclude any anomalies in the probe-level data (the data are available upon request).

Data analysis

For data analysis, the CEL files, representing replicates from each time point, were normalised using the robust multi-array average (RMA) algorithm. RMA is an algorithm used to create an expression matrix from Affymetrix data. Here, the raw intensity values are background corrected, log2 transformed and then quantile normalised. Next, a linear model is fitted to the normalised data to obtain an expression measure for each probe set on each array [51]. In order to establish the optimal hybridisation threshold for interpreting transcription data, the CEL files were normalised as a single experimental group for the two strains. For identifying differentially expressed genes during the time period, the analysis of variance (ANOVA) was applied. In these data sets, one-way ANOVA was performed to identify genes that were differentially expressed between time points ($P < 0.05$), and genes that were at least 1.5-fold up- or down-regulated were selected. Array data are available from the GEO database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE50728). In this study, only genes which are involved in flavour formation and its associated amino acid metabolism are mentioned. All calculations were performed by programming using the freely available R packages (http://www.r-project.org) and Bioconductor (https://www.bioconductor.org). Principal component analysis was performed with Unscrambler from CAMO ASA (Oslo, Norway).

Results and discussion

Fermentation performance

Typical industrial production conditions were used for the EBC tube fermentations. The progress of the fermentations is shown in Fig. 1. The fermentation kinetics of extract decrease (Fig. 1a) and alcohol increase (Fig. 1b) for the lager and ale yeast proceeded normally. The fermentation profiles can be divided into three phases (Fig. 1c). During the lag phase, cells grew slowly, after 8 h, cells entered the exponential growth phase where cells grew fast, extract decreased and ethanol was biosynthesised rapidly. No change in cell density was observed for both yeast strains until 96 h. After 96 h, cell numbers were reduced to 14×10^6 cells/mL with respect to the ale yeast and to 26×10^6 cells/mL with respect to the lager yeast fermentation, a result of yeast flocculation and sedimentation.

Amino acid uptake and flavour formation

Amino acid assimilation was monitored during the course of fermentation of the lager strain S23 and the ale strain S81. A detailed analysis of the uptake of the important amino acids is shown in Table 1. A reduction in the concentration of all amino acids was observed during fermentation. The highest consumption occurs during the first 48 h for most of the amino acids, after which the variation is very small. During the first 24 h of fermentation, methionine, asparagine, threonine, glutamate, glutamine, histidine and leucine were largely consumed concerning both strains. Although sequential uptake could not be accurately determined, a number of amino acids were reduced to negligible levels relatively early during fermentation. These included methionine (undetectable at 48 h), asparagine, glutamine and histidine (0.08, 0.007 and 0.10 mmol/L at 48 h, respectively) concerning the lager yeast (Table 1). With regard to the ale yeast, these included methionine (undetectable at 24 h), lysine and arginine (both undetectable at 48 h), and threonine, glutamine, isoleucine and histidine (0.003, 0.01, 0.12 and 0.11 mmol/L at 48 h, respectively) (Table 1). The concentration of a number of amino acids during lager fermentation increased between 72 and 120 h, including threonine, leucine, isoleucine, arginine, valine, proline and glycine (Table 1). A similar finding was reported for the lager yeast CB11 [52]. With regard to ale yeast fermentation, only the concentrations of tyrosine and valine increased (Table 1). During S23 fermentation in terms of per cent utilisation, the most assimilated amino acids were methionine, glutamine, asparagine and lysine (90–100 % uptake), followed by threonine, histidine, serine and aspartate (70–89 % uptake), isoleucine, leucine, glutamate, phenylalanine, tyrosine, arginine, valine and proline (50–69 % uptake). The lowest net uptake was observed with alanine (28.33 %) and glycine (4.46 %). In addition, methionine, lysine, asparagine, threonine, glutamine and isoleucine (90–100 % uptake) were found to be the most assimilated amino acids by S81, followed by arginine, glutamate, phenylalanine, leucine (70–89 % uptake), serine, histidine, aspartate, tyrosine and alanine (50–69 % uptake). The lowest net uptake was observed with valine (36.39 %), proline (24.70 %) and glycine (12.14 %). These results show that the amino acid uptake by S81 and S23 did not follow a defined course, which might be due to the different genetic backgrounds between lager and ale yeast. Some amino acids which should be completely absorbed according to the A to D grouping of Jones and Pierce [16] were not taken up in total (Table 1). Also, the critical time value as discussed by Perpète et al. [53], in which amino acids of the previously described group A (glutamine, glutamate, serine, asparagine, aspartate, threonine, lysine and arginine) should be taken up, could not be confirmed in this study. Obviously, certain amino acids are favoured by the two different yeast strains. The exact course of uptake as well as the sequence differs with regard to S81 and S23, respectively, which might be due to the differences in the

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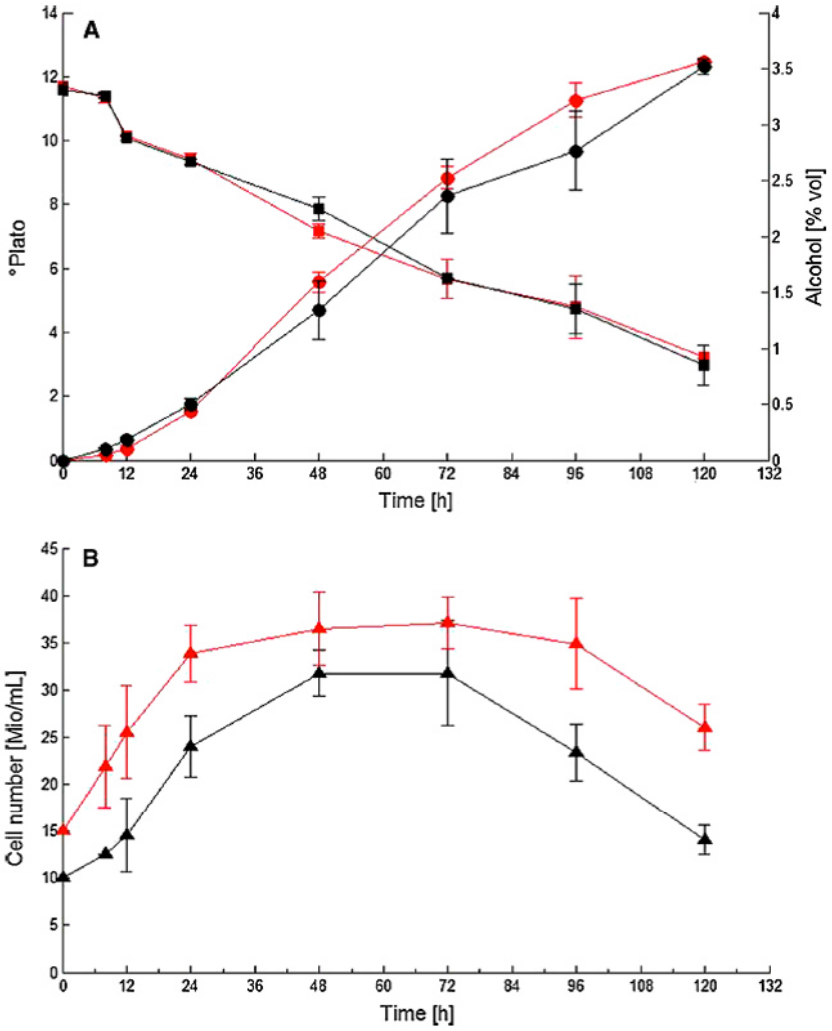

Fig. 1 Fermentation profile of EBC tall-tube fermentations with the lager (*red colour*) and the ale (*black colour*) brewing yeast strain. **a** Course of sugar content expressed as °Plato (*squares*) and alcohol formation (*circles*). **b** Cell number development (*triangles*) throughout the fermentations. Results are the averages of three independent fermentations. *Error bars* indicate ± SD. Lager yeast: 12°P 12°C, ale yeast: 12°P 20°C

permease genes characteristics between lager and ale yeast. In particular, methionine was rapidly and completely assimilated by S81 and S23 at 24 and 48 h, respectively. Group B amino acids (valine, methionine, leucine, isoleucine and histidine) and group C amino acids (glycine, phenylalanine, tyrosine, alanine and tryptophane) [16] could not be differentiated due to being utilised without lagging. Furthermore, proline was assimilated by the two strains. Certainly, the amount of proline uptake and the chronological sequence differ when comparing the strains. S23 showed 52.86 % of uptake, while for S81, an uptake of only 24.70 % was evaluated (Table 1).

In order to exhibit a relation between the amino acid uptake and the higher alcohol and ester formation, the production of nine volatile compounds was analysed by HS-GC during fermentation. The results of the HS-GC analysis are shown in Figs. 2 and 3. S23 and S81 synthesised a variety of flavour compounds, of which some were present in concentrations above their threshold levels (Table 2). The higher alcohols measured were accumulated throughout the fermentation and reached their maximum concentrations between 96 h and 120 h of fermentation (Fig. 2). After fermentation, the most present higher alcohols detected for S81 and S23 were isobutanol and isoamyl alcohol, respectively (Table 2). Furthermore, the esters were detected by HS-GC (Fig. 3). Ethyl acetate (Fig. 3a), isobutyl acetate (Fig. 3b), isoamyl acetate (Fig. 3c), 2-methylbutyl acetate (Fig. 3d) and ethyl hexanoate (Fig. 3e) were accumulated throughout the fermentation. Ethyl acetate was detected as the most present ester for S81 and S23 (Table 2). As higher alcohols and esters are of particular importance for the taste of beers, principal component analysis was performed on the final concentrations produced by the two yeast strains (Fig. 4). The scores and loadings plot for the first and

Table 1 Amino acid concentrations in synthetic wort and their per cent depletion at 120 h during fermentation with *S. pastorianus* strain S23 and *Saccharomyces cerevisiae* strain S81

Amino acid	Concentration (mmol/L)[a]								Per cent (%)
	0 h	8 h	12 h	24 h	48 h	72 h	96 h	120 h	
S. pastorianus (S23)									
Methionine	0.14	0.12	0.05	0.02	0.00	0.00	0.00	0.00	100.00
Asparagine	1.24	1.18	1.10	0.65	0.08	0.00	0.00	0.00	100.00
Glutamine	0.75	0.48	0.24	0.13	0.007	0.00	0.00	0.00	100.00
Lysine	0.96	0.84	0.76	0.63	0.39	0.11	0.01	0.01	99.19
Threonine	0.67	0.68	0.52	0.32	0.06	0.00	0.08	0.09	86.45
Histidine	0.33	0.32	0.30	0.22	0.10	0.07	0.04	0.04	86.36
Serine	0.66	0.60	0.46	0.41	0.36	0.24	0.19	0.15	77.29
Aspartate	0.68	0.61	0.44	0.41	0.35	0.27	0.21	0.18	73.11
Isoleucine	0.61	0.54	0.50	0.46	0.31	0.19	0.17	0.18	69.86
Leucine	1.31	1.20	0.90	0.71	0.61	0.44	0.41	0.43	66.96
Glutamate	0.68	0.60	0.43	0.39	0.34	0.28	0.25	0.24	64.47
Phenylalanine	0.91	0.82	0.74	0.66	0.50	0.39	0.30	0.30	61.03
Tyrosine	0.66	0.51	0.47	0.40	0.34	0.27	0.28	0.28	57.53
Arginine	0.86	0.79	0.72	0.59	0.50	0.35	0.27	0.38	55.92
Valine	1.10	0.97	0.94	0.81	0.72	0.60	0.42	0.50	54.48
Proline	3.16	2.92	2.90	2.31	1.70	1.16	1.38	1.49	52.86
Alanine	1.35	1.27	1.21	1.09	1.02	0.95	0.94	0.97	28.50
Glycine	0.53	0.57	0.50	0.60	0.52	0.46	0.42	0.51	4.46
S. cerevisiae (S81)									
Methionine	0.13	0.05	0.02	0.00	0.00	0.00	0.00	0.00	100.00
Lysine	0.96	0.84	0.82	0.35	0.00	0.00	0.00	0.00	100.00
Asparagine	1.21	1.04	1.00	0.56	0.00	0.00	0.00	0.00	100.00
Threonine	0.64	0.48	0.45	0.23	0.003	0.00	0.00	0.00	100.00
Glutamine	0.75	0.45	0.20	0.09	0.01	0.004	0.003	0.00	100.00
Isoleucine	0.61	0.45	0.43	0.29	0.12	0.09	0.05	0.05	91.82
Arginine	0.86	0.57	0.57	0.39	0.21	0.18	0.12	0.11	86.95
Glutamate	0.69	0.44	0.40	0.31	0.19	0.19	0.18	0.18	73.54
Phenylalanine	0.91	0.82	0.76	0.63	0.43	0.40	0.28	0.25	71.90
Leucine	1.29	1.10	0.73	0.54	0.50	0.49	0.41	0.39	70.04
Serine	0.66	0.46	0.43	0.32	0.31	0.27	0.22	0.22	67.33
Histidine	0.33	0.28	0.27	0.19	0.11	0.12	0.10	0.11	66.97
Aspartate	0.68	0.59	0.58	0.43	0.31	0.28	0.25	0.24	64.44
Tyrosine	0.66	0.50	0.46	0.40	0.37	0.27	0.27	0.28	58.38
Alanine	1.35	1.20	1.23	1.01	0.84	0.77	0.66	0.58	56.92
Valine	1.11	0.88	0.95	0.84	0.77	0.68	0.61	0.71	36.39
Proline	3.13	3.07	2.90	2.86	2.78	2.78	2.62	2.35	24.70
Glycine	0.54	0.51	0.47	0.40	0.52	0.53	0.49	0.47	12.14

[a] Each value is the mean of three replicate measurements. Standard errors were always lower than 20 % of mean value

second components (PC1, PC2) are especially useful, since these two components summarised more variation in the data than any other pair of components (see Fig. 4). The score plot (Fig. 4a) reveals the existence of differences between samples fermented with different yeast strains. For lager yeast, the replicates are well clustered, while those for ale yeast are slightly scattered. As the loadings plot shows (Fig. 4b), the higher alcohols and their corresponding esters cluster together, and the largest effect is clearly along PC1 which explains 89 % of the variation. Ethyl acetate and ethyl hexanoate did not clearly cluster with the other aroma-active compounds. It is obvious that the end product produced with the ale yeast was the richest in all higher alcohols and esters. A reverse composition was found for the end product produced with the lager yeast. The concentration of all measured flavour volatiles in case of S81 was higher than that in case of S23 (Table 2), which was caused by the differences in the fermentation temperature and

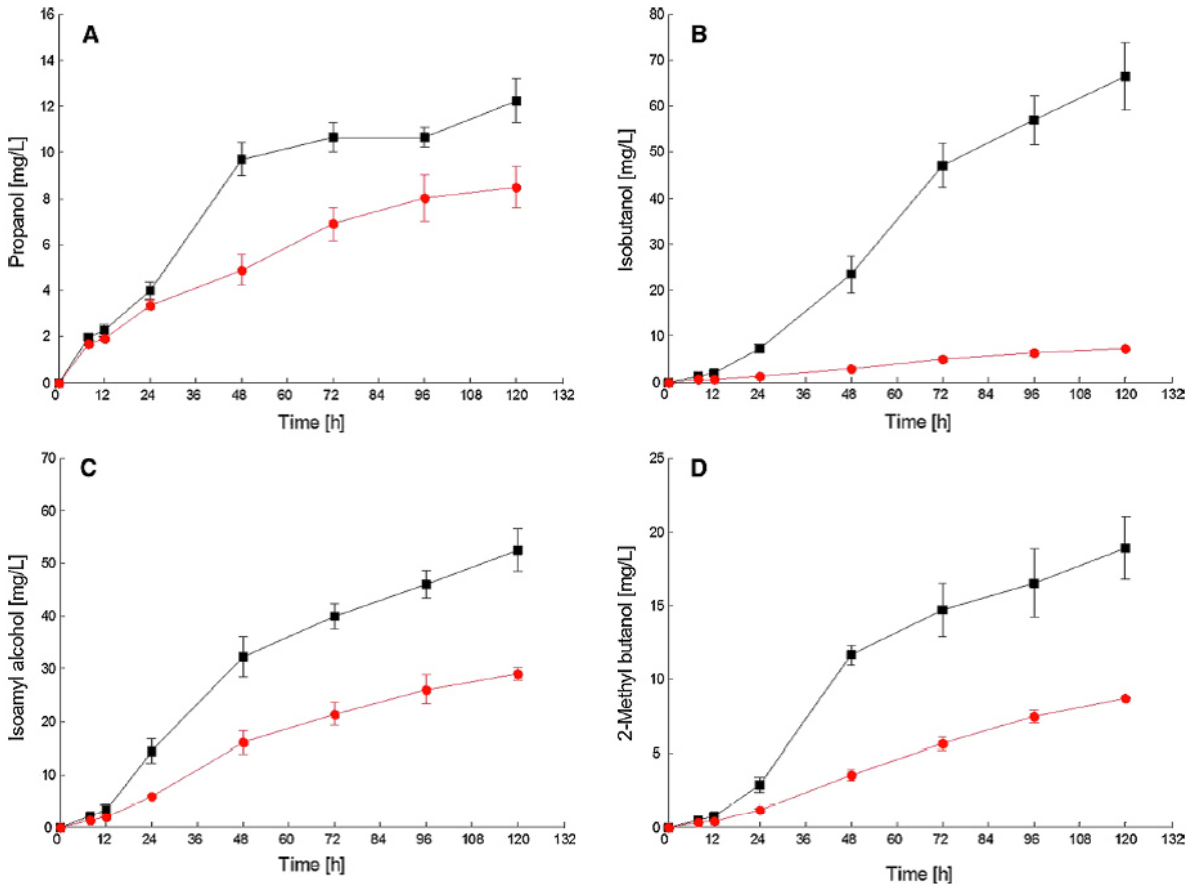

Fig. 2 Higher alcohols synthesised in EBC tall-tube fermentations with the lager (*circle*) and an ale (*square*) brewing yeast strain. Production of propanol (**a**), isobutanol (**b**), isoamyl alcohol (**c**), and 2-methyl butanol (**d**). Results are the averages of three independent fermentations. *Error bars* indicate ± SD. Lager yeast: 12°P 12°C, ale yeast: 12°P 20°C

physiological properties between the two yeast strains [43]. Fermentation of lager beers requires a lower temperature compared to ale beers. These temperatures cause the clean taste and the lack of fruity esters. Fermentation of brewery wort at such a low temperature is only possible due to the cryotolerant nature of *S. pastorianus* [54]. The Patagonian species characterised as *S. eubayanus* in 2011 gives lager yeast its interesting cold tolerance and is responsible for the characteristic flavour of lager beer [45].

According to the results in Tables 1 and 2, a relationship between BCAA assimilation and the formation of isobutanol, isoamyl alcohol and 2-methyl butanol and its corresponding esters, isobutyl acetate, isoamyl acetate and 2-methylbutyl acetate was particularly distinguishable for the lager yeast. Of these higher alcohols, isobutanol was detected in lowest concentrations, which could be explained by the small uptake of valine. Isobutanol can be synthesised by catabolic route from valine via the Ehrlich pathway [26]. The results may suggest that in the case of low valine uptake, only a small amount of isobutanol would be synthesised. In contrast, a high concentration of isobutanol was determined in top-fermenting fermentations, along with low valine uptake. This observation could be due to the synthesis of higher alcohols formed by sugars via anabolic pathway [23]. Therefore, no clear stoichiometry of amino acid uptake and the concentration of the corresponding higher alcohol can be computed. Besides, the metabolism of other amino acids cannot be ignored [55]. The transamination reaction which is the first step in the synthesis of higher alcohols needs an amino acid and an α-keto acid [26]. This includes the transamination of an amino group ($-NH_2$) to an α-keto acid. The α-keto acid thereby becomes an amino acid, and the amino acid becomes an α-keto acid which can take part in further transamination reactions. In this manner, amino acids which are not involved in the Ehrlich pathway can indirectly participate in fusel alcohol

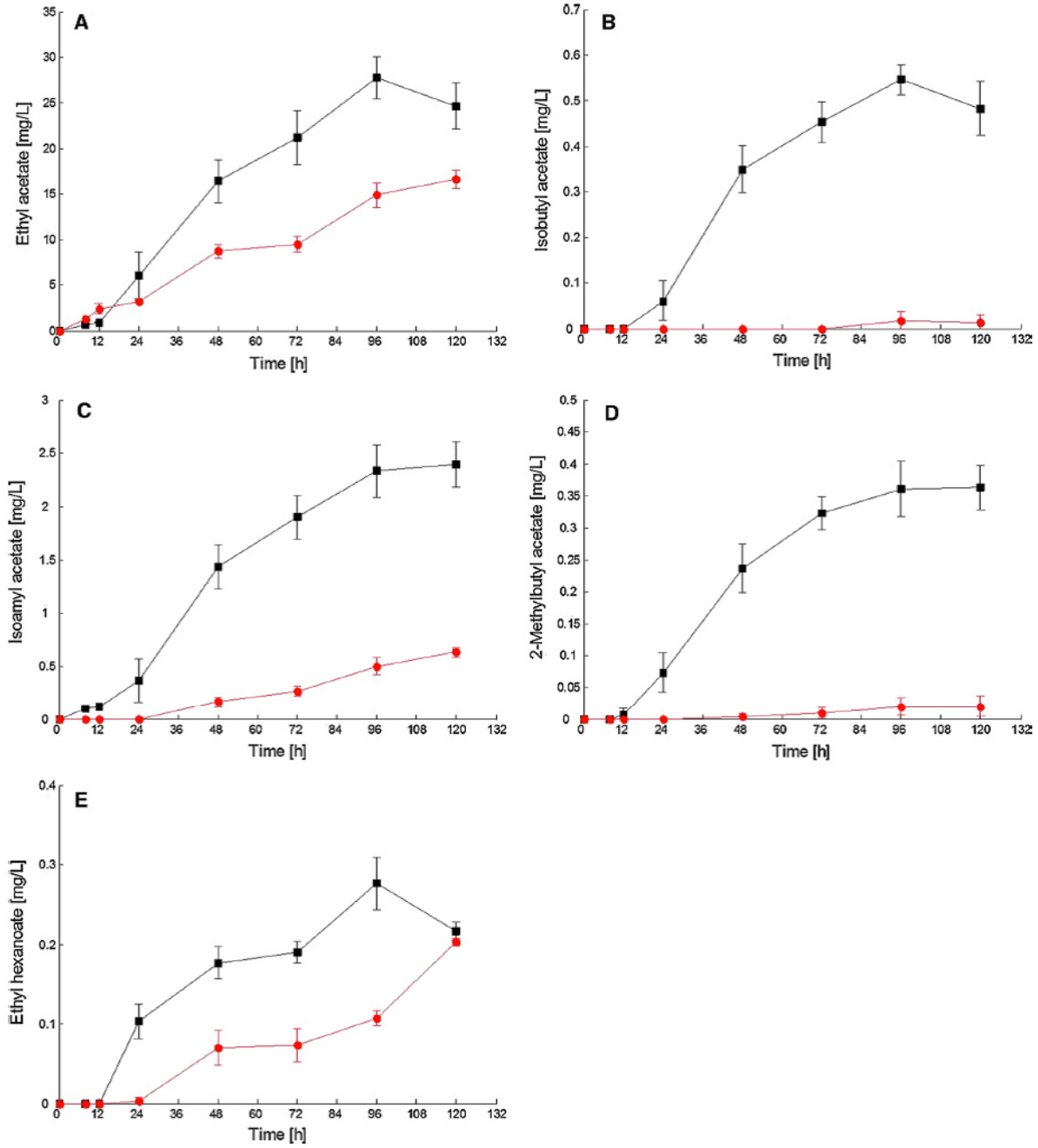

Fig. 3 Volatile esters synthesised in EBC tall-tube fermentations with the lager (*circle*) and the ale (*square*) brewing yeast strain. Production of ethyl acetate (**a**), isobutyl acetate (**b**), isoamyl acetate (**c**), 2-methylbutyl acetate (**d**), and ethyl hexanoate (**e**). Results are the averages of three independent fermentations. Error bars indicate ± SD. Lager yeast: 12°P 12°C, ale yeast: 12°P 20°C

production [56, 57]. Thus, most proteinogenic amino acids might be involved in aroma compound production. In a former study, proline was statistically evaluated as a significant amino acid in aroma compound profiling during yeast fermentation [41]. In this study, high proline uptake from wort was evaluated for S23. Its indirect role in aroma compound production could be explained by its degradation to glutamate which might be synthesised to glutamine

Table 2 Final aroma concentrations in fermentations performed with *S. pastorianus* strain S23 and *S. cerevisiae* strain S81 determined by HS-GC-FID

Aroma compound	*S. pastorianus* (S23) 12°P 12°C (mg/L) ± SD	*S. cerevisiae* (S81) 12°P 20°C (mg/L) ± SD	Threshold (mg/L)
Propanol	8.50 ± 0.91	12.23 ± 0.95	800[a]
Isobutanol	7.27 ± 0.25	66.43 ± 7.27	200[a]
Isoamyl alcohol	29.1 ± 1.23	52.53 ± 4.08	70[a]
2-Methyl butanol	8.70 ± 0.08	18.9 ± 2.08	65[b]
Ethyl acetate	16.67 ± 1.01	24.67 ± 2.57	30[a]
Isobutyl acetate	0.01 ± 0.02	0.48 ± 0.06	1.6[a]
Isoamyl acetate	0.63 ± 0.047	2.40 ± 0.216	1.2[a]
2-Methylbutyl acetate	0.02 ± 0.016	0.36 ± 0.035	1–2[b]
Ethyl hexanoate	0.20 ± 0.004	0.22 ± 0.012	0.21[a]

[a] Meilgaard [107]

[b] Krüger and Anger [108]

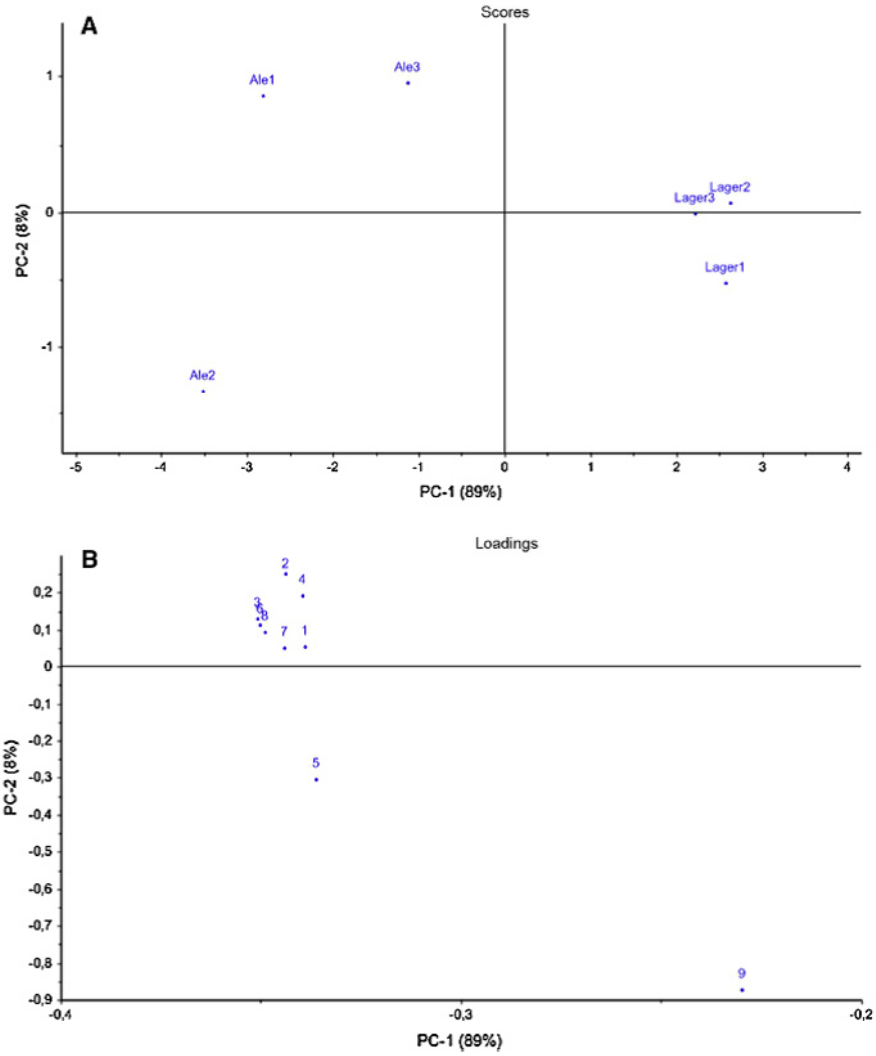

Fig. 4 Results of the principal component analysis carried out on the end concentrations of the higher alcohols and esters. Scores of the lager and ale beer samples formed by the first and second principal components. The numbers 1, 2, 3 refer to the three replicates (**a**). Loadings of the variables on the first and second principal components. The numbers refer to the aroma-active metabolites: 1-propanol, 2-isobutanol, 3-isoamyl alcohol, 4-2-methyl butanol, 5-ethyl acetate, 6-isobutyl acetate, 7-isoamyl acetate, 8-2-methylbutyl acetate, 9-ethyl hexanoate (**b**)

or transaminated to α-ketoglutarate; this can be subject to further transamination reactions and finally be followed by decarboxylation and reduction [58, 59]. However, under anaerobic yeast fermentation, little to no proline is degraded to glutamate, since these oxidative steps require an oxidase which needs molecular oxygen [60]. Certainly,

oxygen, even when present in low concentrations, can support proline catabolism which can take place during the first hours of fermentation [61]. The accumulation of proline has also been discussed as a stress protectant in yeast cells during alcoholic fermentation [62, 63]. Further investigation is clearly required before a reliable conclusion can be drawn. Nevertheless, the proline uptake under brewery fermentation conditions is obviously yeast strain dependent, caused by the differences in their genetic and physiological attributes.

Differential transcription of genes involved in amino acid uptake, amino acid metabolism and aroma compound production during yeast fermentation

An investigation of the transcriptional changes occurring during ale and lager yeast fermentation was carried out to evaluate the amino acid assimilation and its associated flavour formation under specific brewery conditions. As shown in Tables 3 and 4, the genes are grouped according to their involvement in amino acid metabolism and the aroma compound production. For the lager yeast, we found 25 up-regulated genes (Table 3) and 21 down-regulated genes (Table 4), whereas for the ale yeast, 22 genes were found to be up-regulated (Table 3) and 35 genes were down-regulated during fermentation (Table 4). For almost all up-regulated genes, an increase in transcription rate was evaluated right after pitching; except for *HIS2*, *GZF3* and *DAL80* with respect to the lager yeast, and *AGP1*, *BAP3* and *ARG4* with respect to the ale yeast strain (Table 3). Regarding the group of the down-regulated genes, all genes were down-regulated after 8 h of pitching, except for *MEP1* and *AUA1* in the case of the lager yeast. However, in the case of the ale yeast strain, four genes (*MHT1*, *ARG80*, *PPH3*, *DIP5*) were first down-regulated after 24 h and three genes (*AAD6*, *ARO10*, *ARG3*) only after more than 24 h (Table 4).

Expression of genes involved in amino acid uptake

The uptake of amino acids is determined by the activity of different yeast transporters [22]. In this study, with respect to the lager yeast strain S23, we evaluate 4 amino acid permeases with more specific substrate specificity (*TAT2, ALP1*, *PUT4* and *AGP2*), one ammonium permease (*MEP1*) which has high ammonium transmembrane transporter activity and two associated genes *AUA1* which is involved in ammonia regulation of amino acid transport in *S. cerevisiae* and *FUN34*, encoding a putative transmembrane protein involved in the export of ammonia (Tables 3, 4). According to Regenberg et al. [22], the tryptophan permease (Tat2p) has high-affinity tryptophan transmembrane transporter activity but can also transport tyrosine and phenylalanine into the yeast cells; Put4p can transport alanine, glycine and proline, while Alp1p is specific for arginine and Agp2p can transport isoleucine, leucine and valine [52]. These genes became more active as amino acid concentrations in wort became limited, except for *TAT2* which was already up-regulated right after pitching (Table 3). This could also be confirmed for most amino acid permeases regarding S81. Genes encoding permeases with more specific substrate affinity (*ALP1*, *AGP2*, *AGP3*, *MMP1*, *PUT4* and *MUP3*) were down-regulated at the beginning of fermentation and more expressed at the end of fermentation (Table 4), with a sevenfold change observed with *ALP1*. This explains the slow absorption of arginine during the first phase of fermentation. According to Rouillon et al. [64], Mmp1p has high-affinity S-methylmethionine transmembrane transporter activity; while Mup3p is specific for methionine uptake [65] and Agp3p acts as a permease for leucine uptake [66]. Besides, most genes encoding permeases with broad substrate specificity (*BAP3*, *GNP1* and *AGP1*) were up-regulated right after pitching (Table 3). It had been predicted that these transporters are responsible for the early uptake of glutamine, asparagine and threonine [16, 22] due to their high activity during early fermentation, which has been confirmed in this study. *BAP3*, which is the closest homologue to *BAP2*, encodes a transporter of branched-chain and other amino acids and was highly up-regulated at the end of fermentation when many amino acids were depleted (Tables 1, 3). At this time (72 h), there was still a great amount of leucine in the medium to induce *BAP3* expression (Table 1) [22]. In this study, more genes involved in amino acid and ammonia transport were differentially expressed with respect to the ale yeast fermentation compared to the lager yeast fermentation. The differences in amino acid uptake in consideration of both yeast strains (Table 1) could be due to different transcription rates of the genes encoding the several amino acid transporters. Therefore, the differences in amino acid uptake could be related to the diverse concentrations of higher alcohols produced by the two strains. Regarding the uptake of proline, an increased assimilation could first be detected at the end of fermentation in the case of the ale yeast, whereas after a few hours lag, the lager yeast showed a continuous uptake during the first 72 h. *PUT4*, encoding a specific proline permease, is repressed under ammonia conditions or by other better nitrogen compounds such as glutamine and asparagine [67–69], and was down-regulated during the first stage of fermentation (Table 4). Gibson et al. [52] proposed that the activity of this gene is not strongly influenced by the presence of oxygen during industrial fermentation. Furthermore, we suggest that the quantity of Put4 protein was not decreasing to such an extent that no proline transport would be possible. However, the remaining carriers, mainly in the lager strain, might be sufficient to allow proline uptake.

Table 3 Genes up-regulated in *S. pastorianus* and *S. cerevisiae* during fermentation which are involved in amino acid metabolism and its associated flavour formation

Gene	Fold-change (timed interval after pitching)							Description of gene product[a]
	8 h	12 h	24 h	48 h	72 h	96 h	120 h	
S. pastorianus (S23)								
								Transcription factors and associated molecules
DAL80	1,26	1,10	1,04	1,04	1,65	1,70	1,73	Negative regulator of genes in multiple nitrogen degradation pathways
GZF3	1,92	1,96	1,94	2,03	2,05	2,07	1,76	GATA zinc-finger protein
								Amino acid transport
TAT2	1,70	1,64	1,62	1,55	1,57	1,68	1,57	Tryptophan amino acid transporter
								Transaminaeses and flavour metabolism
ARO8	1,57	1,61	1,56	1,47	1,40	1,41	1,34	Aromatic aminotransferase
BAT1	1,63	1,63	1,55	1,50	1,69	1,79	1,60	Mitochondrial branched-chain amino acid aminotransferase
ATF2	2,60	2,64	2,49	2,03	2,01	2,10	1,77	Alcohol acetyltransferase
HOM2	1,86	1,85	1,78	1,68	1,64	1,68	1,56	Aspartic beta semi-aldehyde dehydrogenase
								Branched-chain family amino acid metabolism
ILV1	1,76	1,72	1,67	1,55	1,47	1,51	1,40	Threonine deaminase
ILV3	1,57	1,58	1,49	1,46	1,58	1,66	1,56	Dihydroxyacid dehydratase
								Aromatic amino acid family metabolism
ARO4	1,94	1,92	1,82	1,66	1,55	1,58	1,45	3-deoxy-D-arabino-heptulosonate-7-phosphate synthase
								Sulphur amino acid metabolism
SAM4	1,54	1,54	1,44	1,36	1,23	1,22	1,13	*S*-adenosylmethionine-homocysteine methyltransferase
								Lysine metabolism
LYS2	1,62	1,67	1,76	1,57	1,47	1,52	1,41	Alpha-aminoadipate reductase
LYS9	1,48	1,62	1,73	1,50	1,31	1,23	1,17	Saccharopine dehydrogenase
LYS12	1,49	1,53	1,64	1,46	1,36	1,41	1,34	Homo-isocitrate dehydrogenase
								Histidine metabolism
HIS1	1,74	1,69	1,58	1,48	1,36	1,38	1,25	ATP phosphoribosyltransferase
HIS2	1,37	1,42	1,54	1,53	1,44	1,39	1,36	Histidinolphosphatase
HIS5	1,71	1,66	1,66	1,64	1,56	1,53	1,40	Histidinol-phosphate aminotransferase
HIS7	2,09	2,03	1,96	1,94	1,89	1,95	1,72	Imidazole glycerol phosphate synthase
								Tryptophan metabolism
TRP2	1,72	1,74	1,66	1,56	1,46	1,44	1,29	Anthranilate synthase Component I
								Asparagine metabolism
ASP3-1	1,56	1,53	1,29	1,19	1,19	1,46	1,15	Cell-wall L-asparaginase II
								Leucine metabolism
LEU9	1,98	1,93	1,84	1,58	1,47	1,63	1,46	Alpha-isopropylmalate synthase II
								Glutamine metabolism
GLN1	1,57	1,61	1,63	1,52	1,47	1,53	1,42	Glutamine synthetase
								Arginine metabolism
ECM40	1,73	1,76	1,71	1,71	1,68	1,61	1,54	Mitochondrial ornithine acetyltransferase
								Serine and homoserine metabolism
SER2	2,21	2,04	1,93	1,70	1,57	1,58	1,41	Phosphoserine phosphatase
THR1	1,59	1,58	1,51	1,43	1,34	1,35	1,25	Homoserine kinase
S. cerevisiae (S81)								
								Transcription factors and associated molecules
MET18	2,00	1,87	1,72	1,67	1,65	1,33	1,24	DNA repair and TFIIH regulator
								Amino acid transport
BAP3	1,83	2,01	1,88	2,04	2,10	2,17	2,30	Branched-chain amino acid permease
GNP1	2,13	2,17	2,17	2,04	2,08	1,85	1,42	High-affinity glutamine permease

Table 3 continued

Gene	Fold-change (timed interval after pitching)							Description of gene product[a]
	8 h	12 h	24 h	48 h	72 h	96 h	120 h	
AGP1	1,43	1,34	1,37	1,42	1,51	1,55	1,81	Low-affinity amino acid permease
								Transaminaeses and flavour metabolism
ARO8	1,90	1,92	1,92	1,62	1,67	1,37	1,20	Aromatic aminotransferase
BAT1	1,66	1,67	1,73	1,82	1,76	1,48	1,23	Mitochondrial branched-chain amino acid aminotransferase
AAT1	2,27	2,17	1,76	1,66	1,73	1,33	1,04	Mitochondrial aspartate aminotransferase
HOM2	1,94	1,93	1,83	1,72	1,74	1,50	1,36	Aspartic beta semi-aldehyde dehydrogenase
								Branched-chain family amino acid metabolism
ILV1	1,53	1,54	1,51	1,40	1,47	1,15	0,86	Threonine deaminase
								Aromatic amino acid family metabolism
ARO4	1,86	1,79	1,54	1,47	1,47	1,22	1,20	3-deoxy-D-arabino-heptulosonate-7-phosphate synthase
								Sulphur amino acid metabolism
SAM4	1,76	1,72	1,40	1,25	1,22	1,01	0,99	AdoMet-homocysteine methyltransferase
								Lysine metabolism
LYS2	1,70	1,70	1,76	1,70	1,69	1,44	1,20	Alpha-aminoadipate reductase
LYS9	1,73	1,72	1,81	1,55	1,56	1,29	1,24	Saccharopine dehydrogenase
								Histidine metabolism
HIS1	1,62	1,60	1,40	1,25	1,25	1,02	0,93	ATP phosphoribosyltransferase
HIS5	1,58	1,58	1,59	1,40	1,50	1,27	1,18	Histidinol-phosphate aminotransferase
HIS6	2,18	1,99	1,74	1,62	1,63	1,53	1,43	Phosphoribosyl-5-amino-1-phosphoribosyl-4-imidazolecarboxamide isomerase
								Tryptophan metabolism
TRP2	1,59	1,54	1,52	1,29	1,31	1,19	1,23	Anthranilate synthase Component I
								Glutamine metabolism
GLN1	1,48	1,55	1,52	1,40	1,48	1,15	1,16	Glutamine synthetase
								Serine metabolism
SER2	1,78	1,79	1,51	1,41	1,43	1,16	- 1,01	Phosphoserine phosphatase
								Phenylalanine metabolism
PHA2	1,95	1,79	1,66	1,49	1,52	1,31	1,09	Prephenate dehydratase
								Arginine metabolism
ARG2	1,81	1,85	1,86	1,74	1,78	1,63	1,54	Acetylglutamate synthase
ARG4	1,34	1,39	1,52	1,52	1,58	1,54	1,42	Argininosuccinate lyase

[a] Descriptions according to those of *Saccharomyces* Genome Database (http://www.yeastgenome.org). Data represent the fold-changes of 25 genes of *S. pastorianus* strain S23, and 22 genes of *S. cerecisiae* strain S81. All genes presented show a statically significant change in expression compared with the reference sample, as determined by one-way ANOVA ($P < 0.05$). Genes were separated into functional categories

However, proline uptake could be supported by other amino acid transporters such as *AGP1*, which was found to be up-regulated [52, 67]. Considerable research to expose the reasons for the differences between ale and lager yeast strains has been conducted, and a number of typical differences between these yeast strains have been established [70, 71].

Activity of genes involved in amino acid metabolism

The majority of genes involved in amino acid metabolism were up-regulated during lager as well as ale yeast fermentations (Table 3). The *GLN1* gene was found to be active at an early stage of fermentation (maximum expression at 24 h) for the lager and the ale yeast. The product of the gene, glutamine, provides 15 % of the total cellular nitrogen [72]. The relatively early transcription of this gene might have been related to the rapid decrease of glutamine up to 99 % at 48 h from synthetic wort. The *GLN1* gene is involved in the synthesis of glutamine, and its expression is repressed during growth on glutamine [73, 74]. With regard to S81, the transcription level of *GLN1* did not exhibit highest values triggered by the fact that at 72 h there was still little amount of glutamine (0.004 mmol/L) left. Genes participating in lysine metabolism such as *LYS2* and *LYS9* as well as *LYS12* for the lager strain were most

Table 4 Genes down-regulated in *S. pastorianus* and *S. cerevisiae* during fermentation which are involved in amino acid metabolism and its associated flavour formation

Gene	Fold-change (timed interval after pitching)							Description of gene product[a]
	8 h	12 h	24 h	48 h	72 h	96 h	120 h	
S. pastorianus (S23)								
								Transcription factors and associated molecules
MET32	−1,71	−1,90	−1,22	−1,39	−1,44	−1,30	−1,25	Zinc-finger DNA-binding protein
STP2	−1,63	−1,55	−1,49	−1,39	−1,24	−1,24	−1,15	Transcription factor
								Amino acid transport
ALP1	−1,62	−2,26	−1,48	−1,44	1,06	1,34	1,38	Arginine transporter
AGP2	−1,93	−1,77	−1,54	−1,43	−1,30	−1,31	−1,24	High-affinity glutamine permease
PUT4	−1,75	−2,00	−1,54	−1,44	−1,05	1,22	1,12	Proline permease
AUA1	−1,49	−1,66	−1,52	−1,58	−1,58	−1,28	−1,42	Protein required for the negative regulation by ammonia of Gap1p
								Ammonia transport
MEP1	−1,29	−1,51	−1,50	−1,44	−1,48	−1,43	−1,34	Ammonium permease
FUN34	−2,45	−2,37	−2,03	−1,77	−1,57	−1,47	−1,27	Putative transmembrane protein
								Transaminases and flavour metabolism
AGX1	−2,00	−1,71	−1,44	−1,26	−1,09	−1,08	−1,05	Alanine: glyoxylate aminotransferase
YAT2	−1,52	−1,64	−1,52	−1,21	1,01	1,01	1,11	Carnitine acetyltransferase
EHT1	−1,51	−1,68	−1,20	−1,15	−1,16	−1,04	1,01	Acyl-coenzyme A:ethanol O-acyltransferase
ALD3	−2,15	−2,12	−1,87	−1,37	−1,19	−1,22	−1,10	Cytoplasmic aldehyde dehydrogenase
ARO10	−2,68	−2,49	−2,32	−2,46	−2,56	−2,87	−2,91	Phenylpyruvate decarboxylase
								Sulphur amino acid metabolism
MHT1	−1,55	−1,55	−1,43	−1,43	−1,31	−1,36	−1,28	*S*-Methylmethionine Homocysteine methyltransferase
								Glutamate and proline metabolism
GDH3	−2,44	−2,44	−2,30	−1,95	−1,52	−1,41	−1,17	NADP(+)-dependent glutamate dehydrogenase
CIT2	−1,56	−1,70	−1,50	−1,50	−1,39	−1,29	−1,28	Non-mitochondrial citrate synthase
IDP2	−2,44	−2,37	−1,86	−1,70	−1,47	−1,39	−1,28	Cytosolic NADP-specific isocitrate dehydrogenase
PUT1	−1,98	−2,27	−2,15	−2,05	−2,04	−1,75	−1,71	Proline oxidase
								Methionine metabolism
MET2	−1,62	−1,72	−1,13	−1,19	−1,21	−1,08	−1,19	L-homoserine-*O*-acetyltransferase
STR3	−1,80	−1,90	−1,67	−1,55	−1,45	−1,47	−1,39	Peroxisomal cystathionine beta-lyase
								Arginine metabolism
ARG82	−2,30	−2,38	−1,91	−1,75	−1,51	−1,53	−1,28	Inositol polyphosphate multikinase
S. cerevisiae (S81)								
								Transcription factors and associated molecules
DAL80	−3,68	−4,45	−4,41	−1,57	−1,45	−1,31	−1,39	Negative regulator of genes in multiple nitrogen degradation pathways
GAT1	−2,22	−2,16	−1,69	−1,53	−1,55	−1,29	−1,19	Transcriptional activator
ARG80	−1,75	−1,82	−2,00	−1,91	−2,03	−1,94	−1,75	Transcription factor involved in regulation of arginine-responsive genes
MET28	−2,18	−1,80	−1,55	−1,56	−1,52	−1,44	−1,30	Transcriptional activator
MET32	−1,98	−1,73	−1,86	−1,89	−1,84	−1,59	−1,34	Zinc-finger DNA-binding protein
PPH3	−1,15	−1,23	−1,51	−1,40	−1,47	−1,54	−1,54	Protein Phosphatase
								Amino acid transport
BAP2	−1,67	−1,91	−2,36	−1,49	−1,56	−1,25	−1,23	Branched-chain Amino acid Permease
GAP1	−1,63	−1,59	−1,50	−1,53	−1,52	−1,52	−1,55	General amino acid permease
ALP1	−4,82	−7,38	−5,52	−1,33	−1,66	−1,09	1,05	Arginine transporter
AGP2	−3,45	−3,71	−1,84	−1,63	−1,83	−1,33	−1,28	High-affinity glutamine permease
AGP3	−1,67	−1,67	−1,41	−1,41	−1,56	−1,43	−1,43	Low-affinity amino acid permease

Table 4 continued

Gene	Fold-change (timed interval after pitching)							Description of gene product[a]
	8 h	12 h	24 h	48 h	72 h	96 h	120 h	
MUP3	−1,61	−1,88	−1,69	−1,72	−1,54	−1,41	−1,20	Low-affinity methionine permease
MMP1	−1,91	−1,93	−1,94	−1,68	−1,77	−1,53	−1,45	High-affinity *S*-methylmethionine permease
DIP5	−1,04	−1,07	−1,49	−1,59	−2,05	−1,83	−1,32	Dicarboxylic amino acid permease
PUT4	−3,70	−3,98	−3,81	−2,43	−2,90	−1,77	−1,32	Proline permease
								Ammonia and urea transport
MEP1	−1,98	−2,00	−2,42	−2,01	−2,09	−1,91	−1,35	Ammonium permease
MEP2	−1,82	−1,91	−1,92	−1,79	−1,80	−1,64	−1,47	Ammonium permease
FUN34	−3,70	−3,90	−1,93	−1,46	−1,60	−1,22	−1,04	Putative transmembrane protein
DUR3	−1,67	−1,88	−1,45	−1,14	−1,26	−0,91	1,06	Plasma membrane transporter
								Transaminases and flavour metabolism
ALD4	−1,77	−1,91	−1,45	−1,24	−1,33	−1,10	−1,06	Aldehyde dehydrogenase
ARO10	−1,36	−1,20	−1,16	−1,46	−1,59	−1,97	−1,55	Phenylpyruvate decarboxylase
AAD4	−1,66	−1,58	−1,31	−1,49	−1,47	−1,38	−1,40	Putative aryl-alcohol dehydrogenase
AAD6	−1,43	−1,11	−1,16	−1,77	−1,80	−1,60	−1,68	Putative aryl-alcohol dehydrogenase
YAT1	−1,61	−1,48	−1,54	−1,48	−1,49	−1,41	−1,37	Outer mitochondrial carnitine acetyltransferase
								Sulphur amino acid metabolism
MHT1	−1,46	−1,37	−1,60	−1,55	−1,59	−1,56	−1,49	*S*-Methylmethionine Homocysteine methyltransferase
								Lysine metabolism
LYS20	−1,60	−1,81	−1,74	−1,48	−1,54	−1,54	−1,27	Homocitrate synthase
								Glutamate and proline metabolism
GDH3	−3,06	−3,01	−2,56	−1,83	−1,96	−1,45	−1,27	NADP(+)-dependent glutamate dehydrogenase
CIT2	−1,64	−1,62	−1,31	−1,16	−1,13	−1,11	−1,10	Non-mitochondrial citrate synthase
PUT1	−2,18	−2,54	−2,29	−2,12	−2,27	−1,57	−1,26	Proline oxidase
PUT2	−1,64	−1,71	−1,35	−1,32	−1,32	−1,16	−1,12	Delta-1-pyrroline-5-carboxylate dehydrogenase
								Threonine metabolism
ICL2	−2,87	−2,64	−1,85	−1,56	−1,52	−1,39	−1,06	2-methylisocitrate lyase of the mitochondrial matrix
								Methionine metabolism
MET2	−1,80	−1,52	−1,34	−1,25	−1,21	−1,17	−1,12	L-homoserine-*O*-acetyltransferase
STR3	−1,81	−2,09	−1,84	−1,86	−1,89	−1,56	−1,18	Peroxisomal cystathionine beta-lyase
								Arginine metabolism
ARG3	−1,79	−1,60	−1,68	−1,70	−1,77	−1,90	−2,08	Ornithine carbamoyltransferase
ARG82	−1,79	−2,07	−1,58	−1,42	−1,49	−1,08	1,09	Inositol polyphosphate multikinase

[a] Description following those of *Saccharomyces* Genome Database (http://www.yeastgenome.org). Data represent the fold-changes of 21 genes of *S. pastorianus* strain S23, and 35 genes of *Saccharomyces cerecisiae* strain S81. All genes presented show a statically significant change in expression compared with the reference sample, as determined by one-way ANOVA ($P < 0.05$). Genes were separated into functional categories

highly expressed after 24 h (Table 3), while lysine was still taken up from wort and depleted by as much as 99.19 % in the case of the lager strain (Table 1). No up-regulation was evaluated for *LYS20* (Table 4), which encodes a homocitrate synthase and catalyses the first step in lysine biosynthesis. Regulation of the lysine biosynthetic pathway in *S. cerevisiae* is an interaction between general amino acid control [75], feedback inhibition of homocitrate synthase activity by lysine [76, 77] and induction of Lys14p by alpha-aminoadipate semialdehyde [78]. With respect to the BCAA encoding genes—*ILV1* for both yeast strains and *ILV3* only for the lager strain—these were differentially transcribed during fermentation. *ILV1*, a threonine deaminase, catalyses the first step in isoleucine biosynthesis and is attached to the regulation of the isoleucine valine pathway, whose expression is under general amino acid control [79]. By contrast, *ILV3* encodes a dihydroxyacid dehydratase which catalyses the third step in the common pathway leading to biosynthesis of BCAA [80]. The evaluated up-regulations found in this study are in contrast to the previously described general amino acid control of these genes. Because no increase in the BCAAs was detected at any time during fermentation, we suggest that post-translational factors might be largely regulating this process. It has been reported that

amino acid metabolic pathways other than the BCAA pathway also play significant roles in the formation of volatile flavours [55]. Genes involved in methionine metabolism, such as *MET2*, which catalyses the first step of the de novo synthesis of methionine biosynthetic pathway from homocysteine [81, 82], were down-regulated during fermentation with respect to S81 and S23 (Table 4). The *MET2* gene is an important control point for amino acid metabolism [83], whose transcription is repressed by the presence of methionine [84], which was also found in this study. Further genes down-regulated during lager and ale yeast fermentation are involved in glutamate and proline metabolism (Table 4). *GDH3*, an NADP(+)-dependent glutamate dehydrogenase, plays an important physiological role in glutamate biosynthesis. Its expression is regulated by nitrogen and carbon sources. *GDH3* was most highly expressed at the end of fermentation, when glutamate becomes limited in fermentation media, which is in accordance with previous results [85]. The repressed expression of *GDH3* during the early stage of fermentation could also be supported by glucose, while the higher expression at the end of fermentation could also be due to the higher ethanol content [86]. In contrast to the lager yeast, *GDH3* was less expressed during the first 24 h of ale yeast fermentation (Table 4). The *PUT1* gene was also found to be down-regulated (Table 4) during lager and ale fermentations. This proline oxidase, which requires oxygen, is the first enzyme in the reaction cascade of proline reduction to glutamate and is subject to nitrogen catabolite repression (NCR) [60, 68, 87]. In this study, *PUT1* was already down-regulated after 8 h, especially during ale fermentation (Table 4). Table 1 shows that proline was assimilated by the ale and the lager yeast strain. Poole et al. [21] have reported that proline uptake should be repressed at the beginning of fermentation due to preferred nitrogen sources such as ammonia via NCR. Its oxygen-dependent activity was not clearly reflected in the transcription profile of *PUT1* in this study. The transcription rate increased slightly, but not significantly, at the end of fermentation, when no more oxygen should remain in the reactors. Nevertheless, the *PUT1* gene showed maximum transcription rate at the beginning of the fermentations (time point 0 h, data not shown). In the same manner as other amino acids, proline metabolism is regulated not only by the level of gene expression but also by that of enzymatic activity [88]. The regulation of proline reduction during fermentation and its intracellular utilisation is a matter of controversial discussion, and the absolute detailed mechanism of the nitrogen regulation still remains unclear [88, 89].

Expression of genes participating in aroma biosynthesis

Of those genes whose products are involved in flavour metabolism, *ARO8* showed the highest transcription activity right after pitching with respect to both yeast strains (Table 3). *ARO8* encodes the aromatic amino acid aminotransferase, which is involved in the Ehrlich pathway [26, 90]. The *BAT1* gene, encoding a BCAATase, participates in higher alcohol synthesis and was also found to be up-regulated at the beginning of fermentation (Table 3). Lower transcription levels in the S23 samples were found for the two amino acid transaminases *ARO8* and *BAT1*, catalysing the transfer of amino groups between amino acids and α-keto acids along with lower concentrations of isobutanol, isoamyl alcohol and 2-methyl butanol. On closer inspection of the ale yeast data, the *BAP2* transporter was shown to be down-regulated during the first 24 h of fermentation (Table 3), which might explain the decelerated uptake of BCAAs such as isoleucine, and possibly also the low uptake of valine in this study. Other down-regulated genes involved in the biosynthesis of higher alcohols were found to be strongly expressed towards the end of fermentation, for example the aldehyde dehydrogenases *ALD3* and *ALD4* (Table 3) [26]. Under glucose conditions, the amino acids valine, leucine, isoleucine, methionine, tyrosine, tryptophane and phenylalanine are almost reduced to their corresponding higher alcohol, whereas the oxidation to fusel acids plays a minor role [27, 28, 91]. *ARO10*, encoding a phenylpyruvate decarboxylase, was down-regulated at the end of fermentation during ale yeast fermentation. *ARO10* is discussed as an alternative gene for decarboxylases [92], whereas *PDC1*, *PDC5* and *PDC6* are discussed as the main genes encoding the pyruvate decarboxylases [93, 94]. These genes were highly expressed during the whole fermentation process, wherefore no fold-change during the fermentation process was evaluated (data not shown). A recent study showed the contribution of Aro10p as the key enzyme in the production of higher alcohols, suggesting *ARO10* as the principal contributor to the flavour-related decarboxylation reactions during the fermentation of alcoholic beverages [95, 96]. Consequently, the low production of higher alcohols could be related to the enhanced down-regulation of *ARO10* during lager yeast fermentation. Likewise, *AAD6*, encoding a putative aryl-alcohol dehydrogenase, displayed the highest transcription rates at the beginning of fermentation and was down-regulated after 48 h with respect to the ale yeast. Dickinson et al. [91] reported that neither *AAD6* nor the other *AAD* genes in *S. cerevisiae* seem to play a role in complex alcohol formation from amino acids. *HOM2*, encoding an aspartic beta semi-aldehyde dehydrogenase which catalysis the second step in the common pathway for methionine and threonine biosynthesis [97], was found to be up-regulated for S81 and S23. According to Styger et al. [98, 99], *HOM2* plays a direct role in the Ehrlich pathway as well as having a significant impact on higher alcohol production.

In addition, two genes involved in ester production (*ATF2* and *EHT1*) were evaluated in this study. *ATF2*, encoding alcohol acetyltransferase II, which is attached to the synthesis of acetate esters and catalyses the condensation reaction between a higher alcohol and acetyl-CoA in *S. cerevisiae*, was up-regulated in the period of 48 h to 96 h with respect to lager yeast fermentation [33, 100, 101]. Previous studies reported that *ATF2* seems to play a minor role compared to *ATF1*, the latter encoding the alcohol acetyltransferase I [39] whose overexpression has been found to affect the flavour profiles in fermented beverages [102]. The evaluated up-regulation of *ATF2* in this study indicates that *ATF2* might have an influence on acetate ester production during fermentation. Based on the up-regulation of *ATF2* concerning the lager yeast, it seems that the substrate components (higher alcohols) could be the rate limiting step for acetate ester production, when comparing the final concentrations of aroma-active compounds with the ale yeast strain in this study. *EHT1*, encoding an acyl-CoA:ethanol *O*-acyltransferase catalysing the condensation reaction between an acyl-CoA component and ethanol to form ethyl esters, was down-regulated (Fig. 4b). The concentrations of ethyl hexanoate were found to be within the normal range, suggesting that the enzyme activity does not appear to be the limiting factor for ethyl ester production. Saerens et al. [103] proved that there is a strong negative consent between *EHT1* expression level and ethyl ester production. Ester levels are known to be influenced by a number of factors [56, 104], and it is known that beer ester concentrations as well as higher alcohols vary depending on the yeast involved [54, 105].

Conclusion

Only a limited number of studies exist which have involved the analysis of genes with a function in aroma compound production and amino acid metabolism during brewery ale and lager fermentations. Previous studies have been carried out in pilot-scale or industrial-scale fermentations that might make it difficult to predict the transcription profile of any given yeast strain as well as its aroma and flavour profile. This results in lower reproducibility due to taking samples from one vessel only [52, 106]. Also, the most frequently used industrial brewing ale and lager yeast strains in German beer production have not been compared in global transcriptome analyses specially focused on flavour formation. For this study, small-scale fermentations were arranged in triplicates for the *S. cerevisiae* strain S81 and the *S. pastorianus* strain S23, in order to measure changes in the transcription of genes associated with flavour and amino acid metabolism over a specific time period during fermentation. In conclusion, the experiments revealed similarities as well as disparities in gene expression pattern when comparing both yeast strains. In particular, the differences in sequential amino acid uptake suggest strain-dependent amino acid assimilation, so that a reclassification of groups of amino acid uptake would be meaningful. This strain-dependent amino acid uptake could be one factor explaining the different concentrations of aroma-active substances when using various yeast strains. However, a lot of other parameters such as oxygen availability, sugar concentrations, biomass production, fermentation temperature and amino acid concentrations, as well as the size and shape of the fermentation vessel must also be taken into account for their influence on flavour biosynthesis. The results in this study indicate that the differences in amino acid uptake with respect to both yeast strains could be due to the different transcription rates of genes encoding amino acid transporters, which could be related to the various concentrations of higher alcohols. In addition, the different expression levels of specific genes involved in the biosynthesis of aroma compounds could contribute to the different flavour compound profiles which might be influenced by the genetic background of the yeast strains. This suggests that an analysis of gene expression level may help to predict and affect the resulting flavour profile. Additional research is needed, especially to improve the understanding of the inducibility of amino acids on gene-regulating mechanisms and biosynthetic pathways of aroma-active substances during brewery fermentation. These findings might help to generate defined amounts of sensory metabolites through the substrate composition in wort in order to regulate those specific sensory effects in fermented beverages which are in accordance with specific consumer preferences.

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Conflict of interest None.

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2.5 Effect of amino acid supply on the transcription of flavour-related genes and aroma compound production during lager yeast fermentation

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Effect of amino acid supply on the transcription of flavour-related genes and aroma compound production during lager yeast fermentation

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ABSTRACT

The objective of this study was to investigate the effect of adding selected amino acids (proline, leucine, cysteine, valine, glutamine and isoleucine) to synthetic wort on the generation of aroma compounds with respect to the transcription levels of specific genes involved in their biosynthetic pathway under brewery fermentation. The results showed that the changes in the selected amino acid levels had no eminent impact on the general course of fermentation. Addition of leucine increased the production of isoamyl alcohol and isoamyl acetate and 2-methylbutyl acetate. Adding valine and isoleucine increased the production of isobutanol and 2-methyl butanol, respectively. Overall, total higher alcohol production increased by amino acid supplementation; this effect could be associated with the upregulation of pyruvate decarboxylases and phenylpyruvate decarboxylase. Amino acid supplementation resulted in a reduction in the final total concentration of esters, especially ethyl acetate. This reduction might have been caused by the downregulation of the genes participating in ester biosynthesis. Our results demonstrate that during lager yeast fermentation, the production of aroma-active compounds can be significantly affected by changing the supply of even one amino acid.

1. Introduction

The composition of wort is affected by the nature of raw materials, especially the brewing water, the types and addition rate of hops and the blend and types of malt. Wort used for beer production contains approximately 4%–5% of nitrogenous material, including amino acids, small peptides and proteins (Boulton & Quain, 2001; Moll, Duteurte, Scion, & Lehuede, 1978). The relative proportions of these nitrogenous components depend on the composition of the grist and the conditions of wort production. The nitrogen content in brewing wort can vary between 700 and 1000 mg/L and is distributed among proteins (20%), polypeptides (22%) and peptides and free amino acids (58%) (Clapperton, 1971). Amino acids are the main source of assimilable nitrogen (O'Connor-Cox & Ingledew, 1989). The amino acids are absorbed by yeast in a sequential order (Henschke & Jiranek, 1993; Jones & Pierce, 1964) by a number of amino acid transporters located in the cell membrane. The amino acid composition of wort affects the synthesis of aroma-active metabolites in fermented beverages (Sablayrolles & Ball, 1995). Amino acids assimilated via the Ehrlich pathway are transaminated by mitochondrial and cytosolic branched-chain amino acid aminotransferases (Eden, Van Nedervelde, Drukker, Benvenisty, & Debourg, 2001). The resulting α-keto acid is decarboxylated to form an aldehyde and reduced to the corresponding higher alcohol in the catabolic route of fusel alcohol production (Hazelwood, Daran, Van Maris, Pronk, & Dickinson, 2008). Simultaneously, the anabolic pathway of higher alcohol formation receives the α-keto acids from carbohydrate metabolism (Chen, 1978). The principal sensory-active higher alcohols responsible for beer aroma are propanol, isobutanol, isoamyl alcohol, 2-methyl butanol and phenyl ethanol (Meilgaard, 1975). Esters are also very important as they are responsible for the fruity character of yeast-fermented beverages. The acetate esters ethyl acetate, isoamyl acetate, isobutyl acetate, phenyl ethyl acetate, 2-methylbutyl acetate and the ethyl esters of C6 and C8 short-chain fatty acids, ethyl hexanoate and octanoate are of greatest importance (Trelea, Titica, & Corrieu, 2004; Verstrepen et al., 2003a). The synthesis of acetate esters depends on the concentration of their corresponding higher alcohols except ethyl acetate, whose

Abbreviations: PLS, Partial least square.

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synthesis depends on ethanol concentration. Acetate esters are the product of enzyme-catalysed condensation reaction between acetyl-CoA and ethanol or a higher alcohol. This reaction is catalysed by alcohol acetyltransferases (Malcorps & Dufour, 1992; Yoshioka & Hashimoto, 1981). The ethyl esters are formed, with the participation of acyl-CoA synthetase, by ethanol and acyl-CoA. These compounds are produced from the fatty acid metabolites and free coenzyme A (Laposata, 1998; Saerens et al., 2006). Although the esters are only present in trace quantities, they can affect the flavour of fermented beverages. Hence, minor changes in their concentration may have dramatic effects on flavour perception (Verstrepen et al., 2003a). It has been shown that nitrogen-compound composition of the medium affects the fermentation kinetics as well as the concentrations of aroma-active compounds (Bell & Henschke, 2008; Carrau et al., 2008; Wang, Bohlscheid, & Edwards, 2003). Hernández-Orte et al. have reported a rise in the isobutanol concentration in wine produced with increased amounts of amino acids (Hernández-Orte, Ibarz, Cacho, & Ferreira, 2005). Numerous studies have investigated the effect of the nitrogen source and its availability on the final content of sensory-active metabolites (Torrea, Fraile, Grade, & Ancín, 2003; Ugliano et al., 2009). However, less is known how single amino acid concentrations affect the expression of the genes involved in the biosynthesis of aroma-active metabolites in industrial beer production.

In this study, the effect of single amino acid supplementation on the transcription levels of genes involved in the biosynthesis of aroma-active higher alcohols and esters in lager yeast was investigated. This effect was studied in synthetic wort to which large amounts of leucine, isoleucine, valine, proline, glutamine or cysteine were added. The choice of the six amino acids was based on previous work conducted in our laboratory. According to PLS regression analysis, leucine, isoleucine, valine, proline, glutamine and cysteine are the amino acids that have the largest impact on the aroma compound profile in the fermentation process of *Saccharomyces pastorianus* (Procopio, Krause, Hofmann, & Becker, 2013). The amount of added amino acids was calculated by taking into account the composition of the medium and the maximum concentrations of the different amino acids in the wort (Krüger & Anger, 1900). The transcription levels of the relevant genes were analysed by qPCR in the fermentation samples with different initial amino acid concentrations. These results should help in understanding the contribution of specific genes to the production of desired concentrations of aroma-active compounds during beer fermentation.

2. Material and methods

2.1. Yeast strain and fermentation conditions

The industrial yeast strain used in this study was *S. pastorianus* (TUM 34/70; Forschungszentrum Weihenstephan für Brau-und Lebensmittelqualität). Yeast pre-cultures were shaken overnight at 28 °C in test tubes with 10 mL of unhopped malt extract medium (Weyermann, 12 °Plato). After 16 h of growth, the culture was used to inoculate 150 mL of unhopped malt extract medium in 180 mL glass bottles with open swing stoppers covered with Parafilm. The second pre-culture was shaken at 28 °C for 48 h followed by inoculation in 2 L of medium in a Duran bottle and shaken at 28 °C for further 48 h under semi-anaerobic conditions. The cells were cropped, washed with sterile distilled water and pitched into the synthetic wort at a ratio of 15×10^6 viable cells/mL. The synthetic medium was used to improve reproducibility; it has been used to identify significant amino acids in aroma-compound profiling of TUM 34/70 (Procopio et al., 2013). The standard fermentation medium contained the following (g/L): yeast nitrogen base without amino acids (Difco), 6.7; K_2HPO_4, 1.3; glucose, 12; maltose, 74; maltotriose, 17; fructose, 2.5; sucrose, 4; glycine, 0.04; alanine, 0.12; valine, 0.13; leucine, 0.17; isoleucine, 0.08; serine, 0.07; threonine, 0.08; asparagine, 0.16; glutamine, 0.11; aspartic acid, 0.9; glutamic acid, 0.1; cysteine, 0.01; methionine, 0.02; lysine, 0.14; arginine, 0.15; histidine, 0.05; phenylalanine, 0.15; tyrosine, 0.12; tryptophan, 0.02 and proline, 0.36. The pH was adjusted to 5.4 with 20% lactic acid (Sacher, 2006). Specific gravity of the synthetic wort was 12 °P. Amino acids were added at double their standard concentrations. The final concentrations were as follows: leucine (Leu), 0.34 g/L; isoleucine (Ile), 0.16 g/L; valine (Val), 0.26 g/L; proline (Pro), 0.72 g/L; cysteine (Cys), 0.02 g/L and glutamine (Gln), 0.22 g/L. Previous experiments have shown that just a 1.5-fold increase in amino acid concentration has a considerable impact on lager yeast fermentation (Procopio et al., 2013). Fermentations were conducted in 2-L EBC tubes in triplicates; static fermentation was conducted at 12 °C. Samples were taken at intervals for up to 120 h after pitching. The synthetic wort and the cells were separated by centrifugation. At the beginning of fermentation and after 120 h, the cell pellets were flash-frozen in liquid nitrogen and stored at −80 °C for RNA extraction.

2.2. Fermentation analysis

The course of fermentation and yeast growth were monitored. Samples were periodically withdrawn and the yeast and fermenting wort separated by centrifugation. Before centrifugation, cell suspensions were diluted to an appropriate volume. The cell density was calculated using a counting chamber (Neubauer improved, BRAND GMHB + CO KG) and a standard light microscope (Zeiss) at 200× magnification. The density of the extract and alcohol content of the fermenting media were measured with the DMA 4500 density analyser and Alcoholyser Plus (Anton Paar, Graz, Austria).

2.3. Headspace gas chromatography analysis

Headspace gas chromatography coupled with flame ionization detection was used for the quantification of higher alcohols and esters in the fermentation products. Samples were collected in 15 mL precooled glass tubes, which were immediately closed and cooled on ice. The samples were analysed with a calibrated Hewlett–Packard 6890 gas chromatograph system equipped with a headspace sampler (HP 7694; Hewlett–Packard, Waldbronn, Germany) and an HP-5 column (crosslinked 5% Ph–95% Me–Si; length, 50 m; inside diameter, 0.32 mm; layer thickness, 0.52 μm; Waldbronn, Germany). Samples were heated for 20 min at 65 °C in the headspace autosampler. The injection block and flame ionization detector were kept at 150 and 250 °C, respectively. Helium was used as the carrier gas. The oven temperature was held at 50 °C for 11 min, then increased to 120 °C at a rate of 10 °C per min, held for 5 min, then increased to 220 °C at a rate of 20 °C per min and finally held at 220 °C. Analyses were performed in duplicate, and the results were analysed with Agilent Technologies Chemstation Rev. A.10.01 software. The results were statistically evaluated using one-way ANOVA ($p < 0.05$) followed by Tukey's test to determine the significance of differences between the supplemented and standard fermentations.

2.4. Gene expression analysis

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) and further purified using RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription (RT) was conducted following the RevertAid Reverse Transcriptase protocol with oligo(dT) and random hexamer primers (Fermentas GmbH, St. Leon-Rot, Germany). The quantitative analysis was performed by RT-qPCR. The

TAF10, *UBC6* and *TCF1* (Teste, Duquenne, Francois, & Parrou, 2009) genes were used as references in gene expression analysis (Table S1 and Fig. S1, Supplementary data). Some primers were designed according to Saerens et al. (Saerens, Verbelen, Vanbeneden, Thevelein, & Delvaux, 2008a) and Vidal et al. (Vidal et al., 2013). The primers are available upon request. mRNA quantification was conducted using SYBR Green PCR Master Mix kit (Peqlab Biotechnologie GmbH, Erlangen, Germany) in the LightCycler 480 (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. The following conditions were used for the amplification: initial denaturation for 60 s at 95 °C, amplification by 45 cycles of 10 s at 95 °C, 25 s at the optimal annealing temperature for each primer pair and 10 s at 72 °C elongation temperature. For the relative quantification of gene expression, the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) was used. The values represent the average of three independent replicates. The results were statistically evaluated using one-way ANOVA ($p < 0.05$) followed by Tukey's test.

3. Results and discussion

3.1. Effect of amino acid supplementation on the course of fermentation

S. pastorianus TUM 34/70 was used to ferment synthetic wort supplemented with different concentrations of amino acids under industrial-like conditions specific for lager beer production, as for example the concentration of extract with 12°P, specific yeast biomass of 15 Mio/mL and cold fermentation temperature. Nitrogen demand depends on the conditions of fermentation, e.g. sugar content and inoculum size (Vidal et al., 2013). In this study, the amounts of amino acids to be added were calculated taking into account the medium (see Material and Methods: standard synthetic wort) and maximum concentrations of different amino acids in wort. The concentrations of supplemental amino acids were as follows: leucine, 0.34 g/L; isoleucine, 0.16 g/L; valine, 0.26 g/L; glutamine, 0.22 g/L; cysteine, 0.02 g/L and proline, 0.72 g/L (Krüger & Anger, 1900). The effect of amino acid supplements on the progress of the fermentation behaviour is shown in Fig. 1. The addition of amino acids, except for glutamine supplementation, proved a small significant increased residual extract in comparison with the standard fermentation (Table 1). This might be due to a small reduction in the biomass after amino acid addition (except for glutamine). It is generally accepted that the addition of yeast assimilable nitrogen results in an increase in biomass production (Henschke & Jiranek, 1993). This could not be confirmed in this study, suggesting that the addition of one amino acid has not the same effect on biomass production. Similar findings have been reported by Vidal et al. (Vidal et al., 2013), who suggested differences in whole fermentation process between different nitrogen situations. Nevertheless, the initial amino acid content had no significant impact on the synthesis of ethanol (Table 1). The exponential growth phase started at ca. 95% of the initial sugar content and ended when about 50% of the sugar had been consumed (data not shown). At that point, the biomass started to decrease because of flocculation and sedimentation of the yeast.

3.2. Effect of amino acid supplementation on flavour-active metabolites

The higher alcohols isobutanol, isoamyl alcohol and 2-methyl butanol were generated throughout the process of fermentation (Fig. 2), as was ethanol. In contrast, propanol is being generated exclusively as a consequence of nitrogen metabolism (Mouret et al., 2014). The final concentrations of these aroma-active compounds

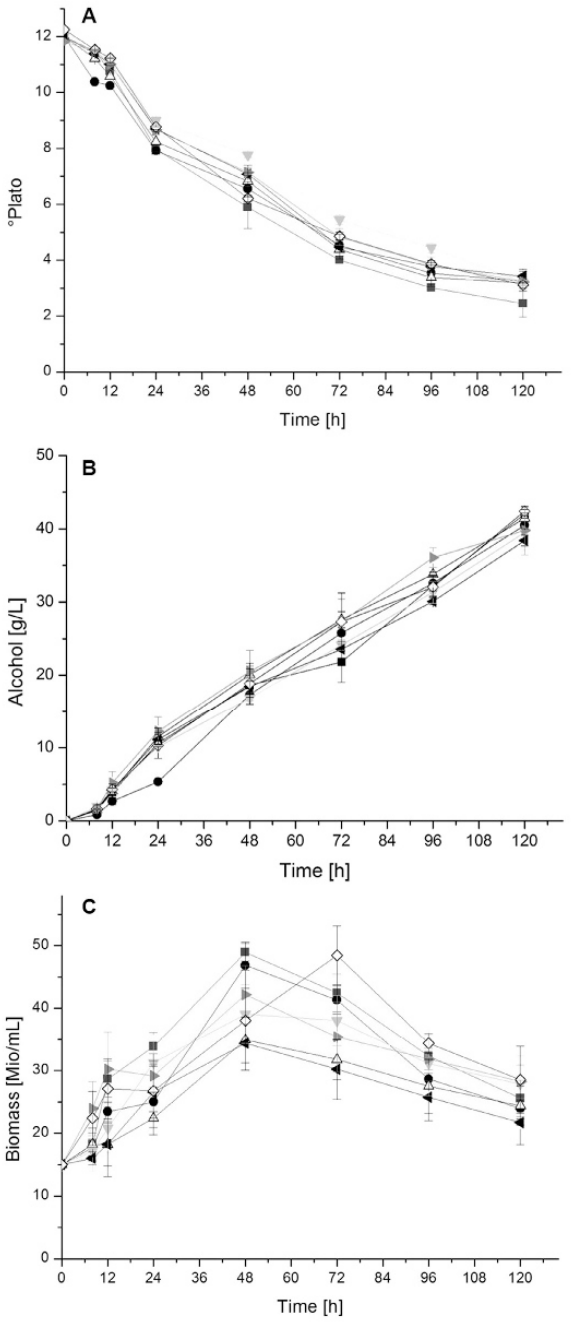

Fig. 1. Fermentation profile of EBC tall-tube fermentations with different amino acid additions. Course of sugar content expressed as °Plato (A), alcohol formation (B) and cell number development (C) throughout the fermentations. Results are the averages of three independent fermentations. Error bars indicate ± SD. (■) standard amino acid concentration; (●) proline addition; (△) cysteine addition; (▽) valine addition; (◂) leucine addition; (▸) isoleucine addition; (◊) glutamine addition.

Table 1
Fermentation parameters obtained in the fermentation of the standard and amino acid supplementations.

	Standard	Proline	Cysteine	Valine	Leucine	Isoleucine	Glutamine
Residual extract (°P)	2.45 ± 0.49	3.25 ± 0.35[a]	3.2 ± 0.03[a]	3.19 ± 0.07[a]	3.43 ± 0.26[a]	3.22 ± 0.12[a]	3.11 ± 0.1
Degree of alcohol (g/L)	42.05 ± 0.59	40.50 ± 0.47	41.44 ± 0.71	39.52 ± 2.42	38.42 ± 0.73	39.79 ± 3.28	42.42 ± 0.60

Values are expressed as means of triplicates.
[a] Means statistical differently to control ($p < 0.05$).

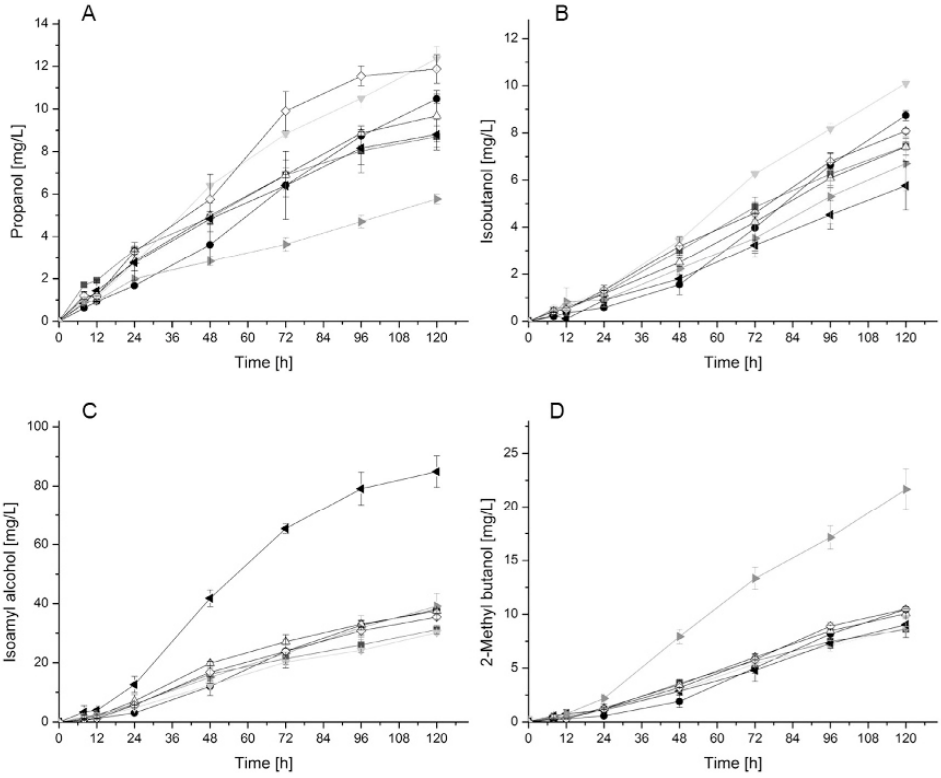

Fig. 2. Higher alcohols synthesized in EBC tall-tube fermentation with different amino acid additions. Production of propanol (A), isobutanol (B), isoamyl alcohol (C), and 2-methyl butanol (D). Results are the averages of three independent fermentations. Error bars indicate ± SD of three independent replicates. (■) standard amino acid concentration; (●) proline addition; (Δ) cysteine addition; (▼) valine addition; (◂) leucine addition; (▶) isoleucine addition; (◊) glutamine addition.

Table 2
Final aroma concentrations in fermentations performed with the lager yeast under different amino acid conditions determined by head-space GC-FID.

Aroma compound	Standard	Proline	Cysteine	Valine	Leucine	Isoleucine	Glutamine
Alcohols (mg/L) ± SD							
Propanol	8.70 ± 0.50	10.48 ± 0.22[a]	9.68 ± 1.20	12.37 ± 0.57[a]	8.80 ± 0.72	5.77 ± 0.25[a]	11.88 ± 0.68[a]
Isobutanol	7.44 ± 0.18	8.74 ± 0.21	7.42 ± 0.35	10.10 ± 0.17[a]	5.77 ± 1.03	6.70 ± 1.14	8.08 ± 0.11
Isoamyl alcohol	31.21 ± 1.42	38.00 ± 0.36	37.55 ± 0.77	30.17 ± 0.80	84.90 ± 5.31[a]	39.17 ± 4.32	35.47 ± 0.80
2-Methyl butanol	8.59 ± 0.04	10.46 ± 0.19	10.05 ± 0.42	8.73 ± 0.15	9.03 ± 1.19	21.67 ± 1.88[a]	10.46 ± 0.20
Total higher alcohols(mmol/L)	0.70	0.84	0.8	0.78	1.29	0.88	0.83
Esters (mg/L) ± SD							
Ethyl acetate	14.38 ± 0.09	10.32 ± 0.09[a]	8.27 ± 0.21[a]	7.70 ± 0.63[a]	9.80 ± 1.41[a]	11.90 ± 2.17	10.31 ± 1.04[a]
Isobutyl acetate	0.013 ± 0.002	0.017 ± 0.005	0.007 ± 0.00	0.016 ± 0.005	0.010 ± 0.00	0.007 ± 0.00	0.020 ± 0.005
Isoamyl acetate	0.695 ± 0.03	0.933 ± 0.047	0.837 ± 0.021	0.400 ± 0.00	1.633 ± 0.151[a]	0.567 ± 0.083	0.877 ± 0.042
2-Methylbutyl acetate	0.020 ± 0.01	0.083 ± 0.006[a]	0.073 ± 0.006	0.113 ± 0.046[a]	0.113 ± 0.015[a]	0.203 ± 0.023[a]	0.090 ± 0.00[a]
Ethyl hexanoate	0.221 ± 0.003	0.110 ± 0.010[a]	0.156 ± 0.018	0.113 ± 0.02	0.217 ± 0.025	0.227 ± 0.035	0.113 ± 0.006
Total esters (mmol/L)	0.170	0.126	0.102	0.092	0.126	0.143	0.125

Values are expressed as means of triplicates.
[a] Means statistical differently to control ($p < 0.05$).

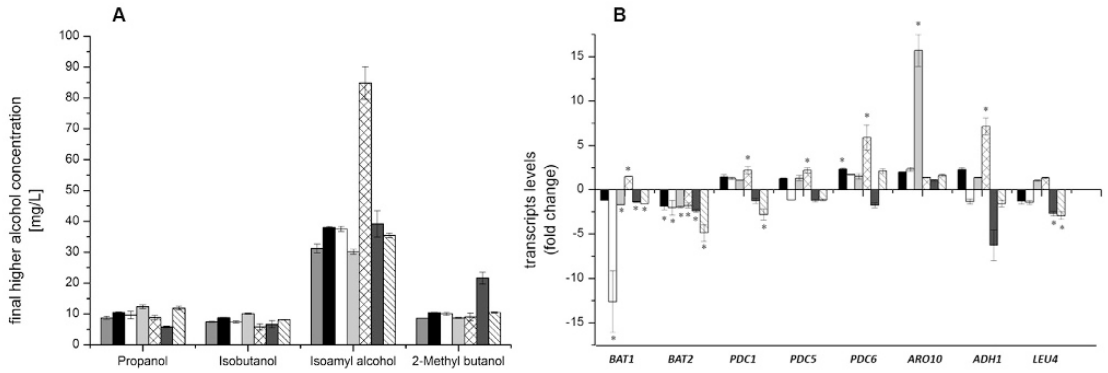

Fig. 3. Final higher alcohol concentrations in EBC tall-tube fermentations under different amino acid supply. Results are the averages of three independent fermentations. Error bars indicate ± SD (A). Relative transcription profile by the lager strain TUM 34/70 of genes involved in higher alcohol production. Results are the averages of three independent fermentations relative to the condition without supplementation of amino acids (*$p < 0.05$). Error bars indicate ± SD (B). (Grey bars) standard fermentation; (Black bars) proline addition; (white bars) cysteine addition; (light grey bars) valine addition; (double striped bars) leucine addition; (dark grey bars) isoleucine addition; (striped bars) glutamine addition.

are shown in Table 2 and Fig. 3A. In this study, isoamyl alcohol was the alcohol with the highest end-concentration in all fermentations. Addition of leucine caused a significant increase in isoamyl alcohol levels; this was not unexpected, considering the mechanisms involved in the Ehrlich pathway (Hazelwood et al., 2008). The increase in 2-methyl butanol and isobutanol levels resulting from isoleucine and valine supplementation, respectively, can be similarly explained. Amino acid addition significantly increased the production of the respective higher alcohols (Table 2). Previous studies have shown that with the addition of different amino acids, both *Saccharomyces* and non-*Saccharomyces* yeast produce increased amounts of respective higher alcohols. This is achieved by increasing their capacity to decarboxylate the transaminated α-keto acids and then reduce them to obtain the final alcohols (Etschmann, Bluemke, Sell, & Schrader, 2002; Hazelwood et al., 2008; Hernández-Orte et al., 2005). In this study, cysteine supplementations had no significant effect on fusel alcohol formation, which could be due to less added concentrations of cysteine. Cysteine, in general, is not very abundant in brewer's wort (Krüger & Anger, 1900). Furthermore, a global increase in propanol formation after the addition of different amino acids was evaluated (excluding isoleucine). The cultures with added proline, valine and glutamine produced significantly higher amounts of propanol than the control fermentation. These results are not in complete agreement with the data reported by Garde-Cerdan and Ancin-Azpilicueta (2008) and Hernández-Orte, Ibarz, Cacho, and Ferreira (2006), who have not found positive correlations between the formation of some higher alcohols and amino acid supplementation. However, these differences might have been caused by the fact that their studies used a mixture of amino acids. Our results show that besides the branched-chain amino acids, proline and glutamine supplementations enhance the formation of higher alcohols, especially the formation of propanol. In general, the addition of a single amino acid resulted in an increase in the total fusel alcohol concentration (Table 2). Changes in the formation of fusel alcohols caused by amino acid supplementation might be related to alterations in the intracellular pools of precursors and intermediates involved in their synthesis.

Beside the higher alcohols, the formation of esters was studied. All esters were synthesised relatively slowly during the exponential growth phase (Fig. 4). The final concentrations of the volatile esters are shown in Table 2 and Fig. 5A. The impact of amino acid addition on ester production varied depending on the ester. Most differences between various profiles of ester formation were very small. Nevertheless, the statistical analysis revealed a significant effect of amino acid supplementations on 2-methylbutyl acetate formation, except for cysteine addition. The addition of isoleucine, in particular, caused a large increase in the concentration of 2-methylbutyl acetate. It was also found that the addition of leucine caused an increase in isoamyl acetate production. However, adding valine did not affect isobutyl acetate formation. The increased production of 2-methylbutyl acetate and isoamyl acetate was probably caused by high levels of 2-methyl butanol, isoamyl alcohol and acetyl-CoA. These molecules are the necessary precursors for the formation of acetate esters by alcohol acetyltransferases (Viana, Gil, Genoves, Valles, & Manzanares, 2008). Amino acid supplementation caused a global decrease in ethyl acetate production in comparison with standard fermentation. The decreased production of ethyl acetate might have been caused by the competition for acetyl-CoA, which is also needed for the synthesis of other acetate esters. Similar findings have already been reported (Lee, Yu, Curran, & Liu, 2011). Cysteine, valine, glutamine and proline addition decreased the formation of ethyl hexanoate. The addition of proline, in particular, significantly lowered the concentration of ethyl hexanoate (Table 2). Saerens et al. (Saerens et al., 2008b) have reported that increasing the levels of nitrogen causes a larger increase in the formation of acetate esters than in the formation of ethyl esters. This result was partially confirmed in our study. In general, amino acid supplementation either enhances or decreases the formation of some esters. Unlike the total concentration of higher alcohols, the total ester concentration is not affected by amino acid supplementation because of the low concentration of ethyl acetate. This could be related to potential changes in intracellular acetyl-CoA levels.

3.3. Effect of amino acid supplementation on gene expression levels

The expression of 15 genes involved in the synthesis of flavour-active compounds was quantified in all fermentations (Figs. 3B and 5B). Under our fermentation conditions, we observed significant upregulation or downregulation of genes involved in the biosynthesis of higher alcohols, encoding transaminases (*BAT1* and *BAT2*), decarboxylases (*PDC1*, *PDC5*, *PDC6* and *ARO10*) and alcohol dehydrogenase (*ADH1*), (Fig. 3B). The expression of the genes for the

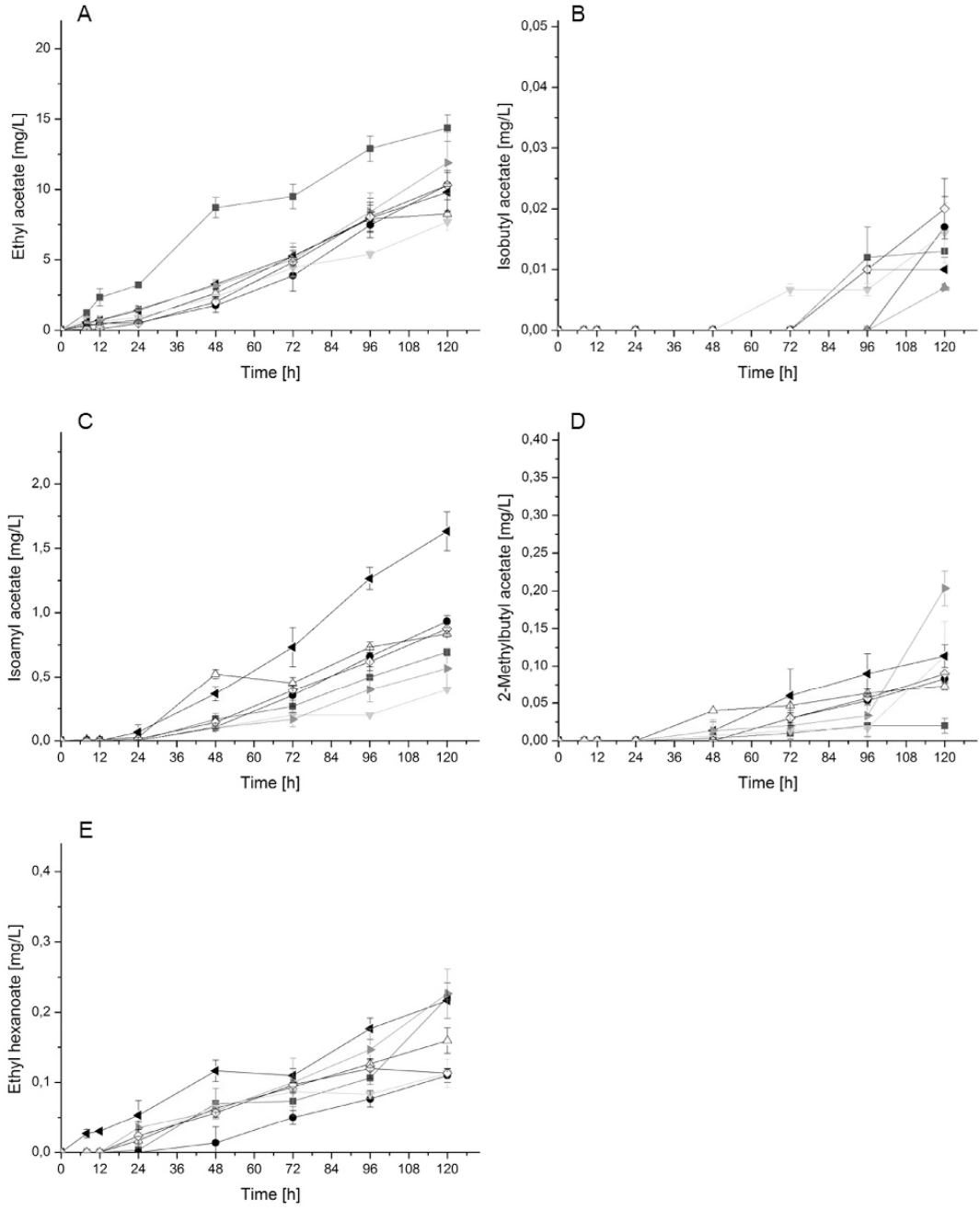

Fig. 4. Volatile esters synthesized in EBC tall-tube fermentations with different amino acid additions. Production of ethyl acetate (A), isobutyl acetate (B), isoamyl acetate (C), 2-methylbutyl acetate (D), and ethyl hexanoate (E). Results are the averages of three independent fermentations. Error bars indicate ± SD. (■) standard amino acid concentration; (●) proline addition; (△) cysteine addition; (▼) valine addition; (◂) leucine addition; (▸) isoleucine addition; (◊) glutamine addition.

branched-chain amino acid transaminases, *BAT1* and *BAT2*, was downregulated after amino acid supplementation, with the exception of *BAT1* expression after the addition of leucine. This result suggests that besides Bat1p and Bat2p, other transaminases might be involved in the biosynthesis of higher alcohols in amino acid-supplemented media. For instance, Aro8p and Aro9p, initially characterised as the aromatic amino acid aminotransferases I and II, might act as broad-substrate-specificity amino acid transaminases

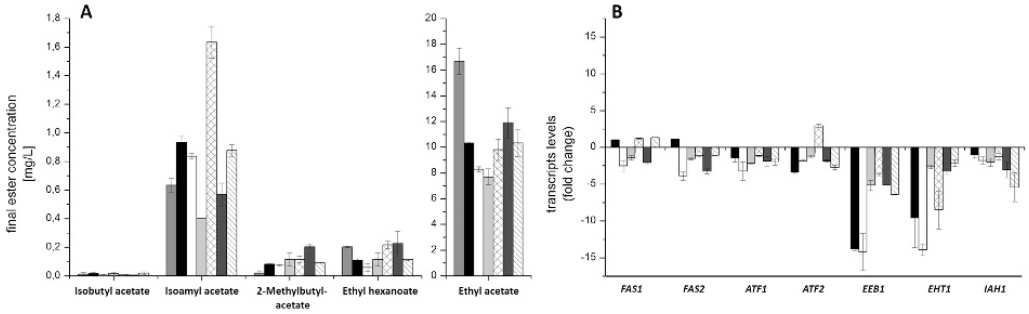

Fig. 5. Final ester concentrations in EBC tall-tube fermentations under different amino acid supply. Results are the averages of three independent fermentations. Error bars indicate ± SD (A). Relative transcription profile by the lager strain TUM 34/70 of genes involved in ester production. Results are the averages of three independent fermentations relative to the condition without supplementation of amino acids (*$p < 0.05$). Error bars indicate ± SD (B). (Grey bars) standard fermentation; (Black bars) proline addition; (white bars) cysteine addition; (light grey bars) valine addition; (double striped bars) leucine addition; (dark grey bars) isoleucine addition; (striped bars) glutamine addition.

in the Ehrlich pathway (Hazelwood et al., 2008; Iraqui, Vissers, Cartiaux, & Urrestarazu, 1998). Furthermore, the expressions of keto-acid decarboxylases *PDC1*, *PDC5* and *PDC6* and the alcohol dehydrogenase *ADH1* were mostly upregulated in the fermentations with various amino acid supplements. Under leucine supplementation in particular, the synthesis of higher alcohols is significantly dependent on these decarboxylases as well as on *ADH1*, which is in accordance with the highest total fusel alcohol concentrations found in leucine supplemented fermentations. The expression of phenylpyruvate decarboxylase *ARO10* was also upregulated after amino acid supplementation. In fermentations with high levels of valine, a 15-fold increase in *ARO10* expression was observed. A recent study by Romagnoli et al. (Romagnoli, Luttik, Kötter, Pronk, & Daran, 2012) has reported that *ARO10* is the key decarboxylase in fusel alcohol formation and strongly affects the aroma-related decarboxylation in lager yeast fermentations. Another important gene, *LEU4*, involved in the intermediate steps of the branched-chain amino acid metabolism has been analysed (Chang, Cunningham, Gatzek, Chen, & Kohlhaw, 1984). Amino acid supplementation resulted in a decrease of *LEU4* expression, except after addition of large amounts of valine and leucine. Especially after the addition of isoleucine and glutamine, the expression of *LEU4* gene was significantly downregulated, with a 2.5- and 3-fold change in transcription level, respectively. *LEU4* gene encodes α-isopropylmalate synthase, which is the main isozyme responsible for the first step in the leucine biosynthesis pathway. *LEU4* catalyses the conversion of α-ketoisovalerate, a common intermediate in leucine and valine biosynthesis, into α-isopropylmalate, which is further converted into α-ketoisocaproate, precursor of isoamyl alcohol (Drain & Schimmel, 1988; Oba, Nomiyama, Hirakawa, Tashiro, & Kuhara, 2005). Downregulation of *LEU4* under high nitrogen-compound concentrations has been explained by the direct inactivation of its transcription factor inducer Gcn4p (Vidal et al., 2013). *GCN4* has been originally characterised as a positive regulator of genes expressed during amino acid starvation; this suggests that after the addition of proline (0.72 g/L) *LEU4* should be strongly downregulated. The downregulation of *LEU4* by amino acid supplementation is not accompanied by a significant reduction in isoamyl alcohol levels. Further investigation is clearly needed before reliable conclusions can be drawn.

We observed significant upregulation and downregulation of the genes encoding fatty acid synthases (*FAS1* and *FAS2*), alcohol acetyltransferases (*ATF1* and *ATF2*), acyl-coenzymeA:ethanol O-acyltransferases (*ETH1* and *EEB1*) and the isoamyl acetate-hydrolysing esterase *IAH1* (Fig. 5B). The concentrations of acetyl-CoA and the appropriate fusel alcohols (ethanol in the case of ethyl acetate), as well as the activity of the enzymes involved in their formation, are very important for the formation of acetate esters. In most amino acid-supplemented fermentations, we observed a decrease in the expression of *ATF1* and *ATF2*. Only in the case of leucine supplementation, a significant upregulation of *ATF2* was observed (Fig. 5B). According to several studies (Lilly, Lambrechts, & Pretorius, 2000; Malcorps, Cheval, Jamil, & Dufour, 1991; Verstrepen et al., 2003b), the availability of substrates is not a major limiting factor but the expression levels of *ATF1* and *ATF2* are important. This result might also explain the reduction in total ester concentrations caused by amino acid supplementation, which is caused by the low ethyl acetate concentrations in the supplemented fermentations, in comparison with the control. Furthermore, we examined the transcription levels of *IAH1*, the gene encoding isoamyl acetate-hydrolysing esterase, in the fermentations supplemented with different amino acids. We found that the transcription of this gene was suppressed under those conditions.

Medium-chain fatty acids (MCFAs) are produced by the fatty acid synthase (FAS) complex (during the synthesis of long-chain fatty acids) and released during the early stage of fermentation. MCFAs are rate limiting in ethyl ester production and affect, among others, the levels of ethyl hexanoate. Furukawa et al. (Furukawa, Yamada, Mizoguchi, & Hana, 2003) have shown in overexpressing experiments, that *FAS1* and *FAS2* are the fatty acid synthetic genes that contribute to increased MCFA formation. Under our fermentation conditions, especially with cysteine and isoleucine supplementation, we observed a significant downregulation of the *FAS1* and *FAS2* genes and a decrease in the expression of *EHT1* and *EEB1* (Fig. 5B). This result could account for the decrease in ethyl hexanoate levels in cysteine-supplemented batches in comparison with the standard fermentation. Notably, proline supplementation induced a downregulation of the ethanol acyltransferases correlating with the significant reduced ethyl hexanoate concentration.

4. Conclusion

The supplementation of synthetic wort with the chosen amino acids did not strongly affect the sugar consumption or ethanol formation during lager yeast fermentation. Increasing the concentration of proline, valine and glutamine increased the amount of produced propanol. Fermentation batches supplemented with valine produced increased amounts of isobutanol and the batches

with added isoleucine formed more 2-methyl butanol than controls. Increasing the concentration of leucine had a substantial impact on the biosynthesis of isoamyl alcohol. Within the used range of supplementary amino acid concentrations, *S. pastorianus* produced greater amounts of total higher alcohols than those produced in control fermentations. The supplemented fermentations produced significantly more 2-methylbutyl acetate compared with the controls, except for cysteine-supplemented batches. Supplementation with leucine caused significantly increased production of isoamyl acetate. These acetates contribute to fresh and fruity aromas in the beer. The supplemented worts produced considerably less ethyl acetate, which is associated with the undesirable solvent-like aroma. However, the final concentration of total esters produced in amino acid-supplemented fermentation batches was reduced in comparison with controls. These results demonstrate that varying the supply of a single amino acid can change the final concentrations of some important aroma-contributing metabolites, affecting the aroma profile of lager beer. We demonstrated that the expression of genes participating in the biosynthesis of aroma-active compounds was affected by the addition of selected amino acids. Thus, amino acid supplementation might affect the flavour perception of lager beer.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.lwt.2015.03.007.

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3. Discussion

Brewing science, as an applied science covering a complex operation, has concentrated in the past on evaluating correlations between specific practical measures and their resulting outputs. Several studies have established the effect of individual parameters on final beer quality. Many of these studies have mostly failed to reconstitute the actual interrelationships, because of the complexity of the topic. With advances in science in recent decades, the knowledge of beer and brewing too has taken a large step forward. This is, in particular, true of studies of the influence of yeast. Now that the genome DNA sequence of *S. cerevisiae* has been reported by Mewes et al. (1997), the brewing industry can gain huge benefits from the next level of biological research. Studies combining metabolomic and transcriptomic data with classical brewing chemistry are presenting interesting and new opportunities for research in beverage and beer quality (Rossouw et al., 2008).

In particular, the perception of aroma is a dominant factor influencing beer quality (Pinho, Ferreira, & Santos, 2006). Fermentation characteristics of yeast, such as amino acid assimilation and metabolism of secondary sensory-active higher alcohols and esters, are based on physiological interactions, which encompass the transcription and regulation of aroma-associated genes and the accompanying enzyme availability/activity (Hernández-Orte, Ibarz, Cacho, & Ferreira, 2006; Vidal et al., 2013). In the present study, significant amino acids and their influence on the regulation of key genes in the biosynthetic pathway of higher alcohols and esters of an ale and lager yeast strain were identified, with the aim of generating a defined aroma compound profile.

In an initial experimental design (see Chapter 2.3), the total amino nitrogen content was modified by variation of amino acids added to synthetic wort, given that the amino acid pool has a major role in aroma profiling owing to its direct influence on higher alcohol production (Hazelwood et al., 2008; Sablayrolles & Ball, 1995). After fermentation of different amino acid combinations with *S. cerevisiae* strain S81 and *S. pastorianus* strain S23, several important higher alcohols and esters were analysed by HS-GC to determine specific aroma compound spectra. By multivariate data analysis, a fingerprint of amino acid importance in the detected aroma compound spectrum was created. On the one hand, the results confirmed for both strains the influence of BCAA content on their corresponding higher alcohols via catabolic pathways. In contrast, the importance of the amino acids to the aroma profile must be strain dependent, as shown in Figure 3 – 1. PLS regression was used as a tool to develop a fingerprint of amino acid importance with respect to aroma compound profile. Extracting informative variables in multivariate regression using PLS can be performed by analysis of

regression coefficients combined with VIP (Chong & Jun, 2005; Eriksson, Johansson, Kettaneh-Wold, & Wold, 2006). Each regression parameter and VIP value are represented on a grey scale according to its magnitude. Thus, amino acids corresponding to a value representing a black dot in both colour maps shown are seen to be those that are essential to the detected aroma compound profile.

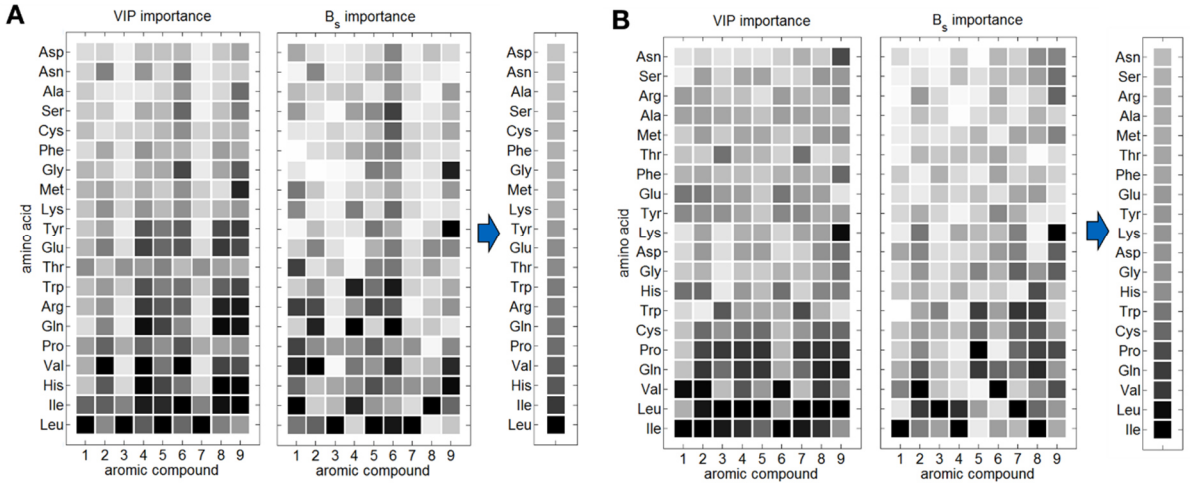

Figure 3 - 1: Statistical importance of each amino acid on the concentrations of aroma compounds of (A) ale yeast strain S81 and (B) lager yeast strain S23; x axis labels are 1 - propanol, 2 - isobutanol, 3 - isoamyl alcohol, 4 - 2-methyl butanol, 5 - ethyl acetate, 6 - isobutyl acetate, 7 - isoamyl acetate, 8 - 2-methyl butylacetate and 9 - ethyl hexanoate; (A,B - left) - variable importance in the projection (VIP) map; (A,B - right) - standardized regression coefficient map - amino acids were sorted by their absolute VIP and Bs values; (A,B – single column) - summed and normalized importance measure of both methods; black indicates high statistical importance on the resulting concentrations of aroma compounds.

These amino acids are leucine, isoleucine, valine, histidine, glutamine and, surprisingly, proline for S81 and leucine, isoleucine, valine, glutamine, cysteine and again proline for S23 (Fig. 3 – 1). The amino acids leucine, isoleucine and valine are the BCAAs taken up by yeast, being transaminated, decarboxylated and then reduced to the higher alcohols isobutanol, isoamyl acetate and 2-methyl butanol via the Ehrlich pathway (Hazelwood et al., 2008). The esters isoamyl acetate, isobutyl acetate and 2-methylbutyl acetate are synthesised via corresponding higher alcohol and acetyl-CoA (Malcorps & Dufour, 1992), so that increased concentrations of these six aroma-active metabolites appear in response to high levels of the corresponding amino acid in the medium, as well as vice versa. Cysteine may be involved in aroma compound production, given that cysteine can be catabolised to pyruvate (Hong, Balkrishnan, & Christie, 2007). This α-keto acid can appear in the transamination process

and/or can be further degraded to *n*-propanol, isobutanol, isoamyl alcohol and 2-methyl butanol following several biochemical pathways (Chen, 1978). There is also no direct pathway between histidine and a higher alcohol, suggesting that histidine may be transformed to α-ketoglutarate or glutamate via histidine metabolism (Walker, 1998). Both can be intermediates in transamination reactions in the Ehrlich pathway, whereby the participation and significance of histidine in fusel alcohol production can be explained (Pires et al., 2014). However, more research is necessary before a reliable conclusion can be drawn. The amino acid glutamine may be involved in aroma compound production by virtue of being an intermediate in the biosynthesis of the key amino acid glutamate (Pires et al., 2014; Walker, 1998), which occurs in most of the transamination reactions described above (Fig. 1 - 4). An interesting and unexpected finding was that proline contributed to the detected aroma compound profile of both yeast strains. The conventional view in brewing science described by Jones and Pierce is that proline uptake is very limited and should not occur under anaerobic fermentation (Jones & Pierce, 1964). So, how could proline take part in the metabolic pathways of fusel alcohol and ester production?

For further investigation, differentially expressed genes participating in amino acid and particularly proline metabolism were investigated during the course of fermentation. The global transcription profile of the ale and lager yeast strains was analysed by means of microarray technology. The Affymetrix system is the most valid representation of the changes undergone by the yeast transcriptome during the course of fermentation. Amino acid uptake was monitored, and volatile compounds were quantified under specific industrial brewing conditions using synthetic wort (see Chapter 2.4). Although proline is thought to be the least-preferred nitrogen source for many strains of *S. cerevisiae*, it is the most abundant source of nitrogen in wort (Jones & Pierce, 1964; Krüger & Anger, 1990). The unavailability of high-quality sources of nitrogen such as ammonia, asparagine or glutamine forces *S. cerevisiae* to degrade proline into glutamate via the proline utilisation pathway in the mitochondria (Brandriss & Magasanik, 1997). Proline catabolism is mediated by proline oxidase and delta 1-pyrroline-5-carboxylate dehydrogenase, which are encoded by *PUT1* and *PUT2*, respectively (Brandriss, 1983; S. S. Wang & Brandriss, 1987) (Fig. 3 – 2). The transcriptional upregulation of these genes occurs only in the presence of proline, but the presence of a good nitrogen source (Huang & Brandriss, 2000), which was available in synthetic wort, led to a downregulation of *PUT1* for the lager yeast strain and *PUT1* and *PUT2* for the ale yeast strain (Fig. 3 – 3).

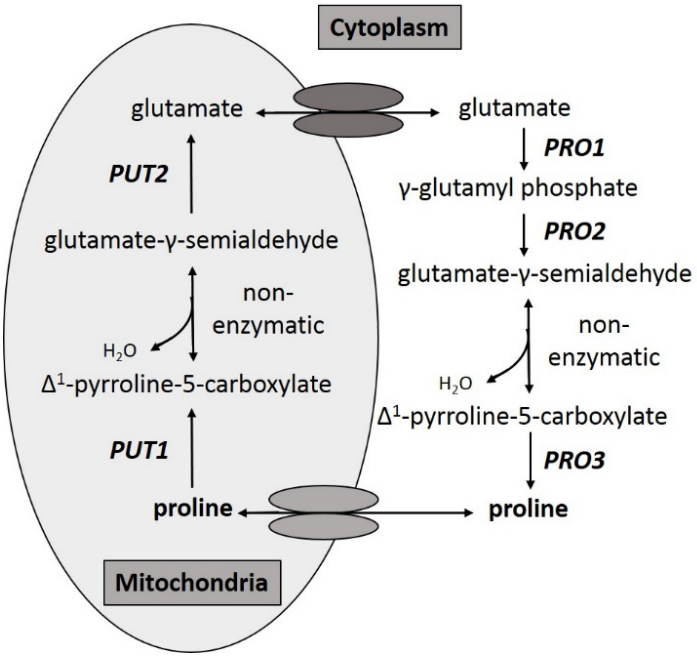

Figure 3 – 2: Simplified metabolic pathway of proline in *S. cerevisiae* and the genes involved. Proline is converted to glutamate within mitochondria in two steps by the enzymes proline oxidase and delta 1-pyrroline-5-carboxylate dehydrogenase. Yeast cells can also synthesise proline from glutamate in the cytoplasm. The proline transport mechanism between the cytoplasm and the mitochondria remains unclear.

In an investigation of amino acid uptake (Chapter 2.4), four amino acid permeases with more specific substrate specificity (*TAT2, ALP1*, *PUT4* and *AGP2*) were evaluated regarding the lager yeast strain S23. These genes became more active as amino acid concentrations in synthetic wort became limited, except for *TAT2*, encoding a tryptophan amino acid transporter. This could also be confirmed for most amino acid permeases of S81. Genes encoding permeases with more specific substrate affinity (*ALP1*, *AGP2*, *AGP3*, *MMP1*, *PUT4* and *MUP3*) were downregulated at the beginning of fermentation and more highly expressed at the end of fermentation (Fig. 3 – 3). This finding indicates that the general amino acid permeases are more active at the beginning of fermentation. The most important gene for proline uptake is *PUT4*, encoding a specific proline permease, which is repressed under high-ammonia conditions or by better nitrogen compounds such as glutamine and asparagine (Andréasson, Neve, & Ljungdahl, 2004; Ter Schure, Van Riel, & Verrips, 2000). *PUT4* was downregulated during the first stage of fermentation and became more active at the end of fermentation (Fig. 3 – 3).

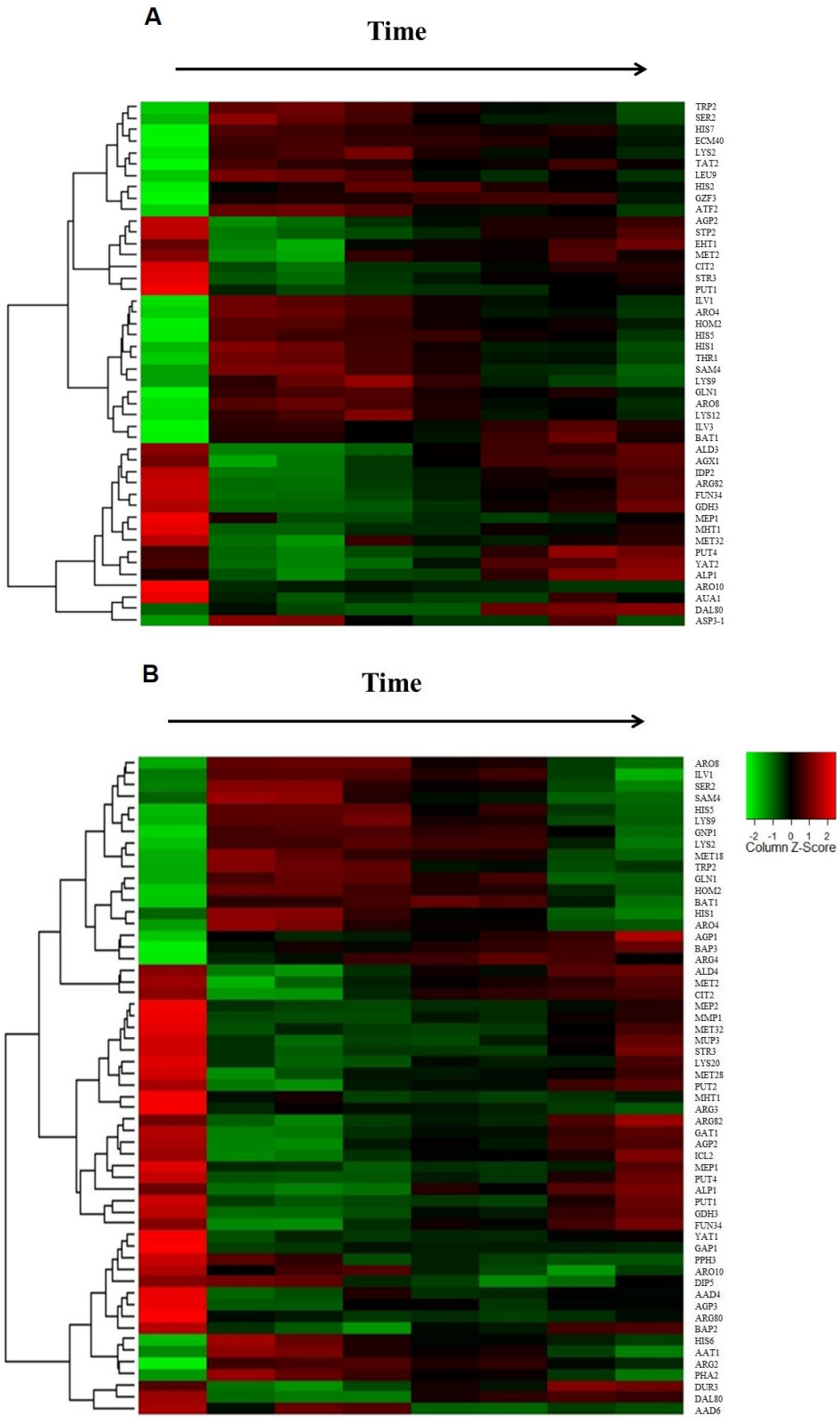

Figure 3 - 3: Hierarchical cluster of DNA microarray data from time courses of *S. pastorianus* strain S23 and *S. cerevisiae* strain S81 during brewery fermentation. Samples were taken at 0, 8, 12, 24, 48, 72, 96, 120 h after pitching. Genes involved in amino acid metabolism and its associated flavour formation were selected for this analysis if their expression level deviated from time 0 by at least a factor of 1.5 in at least two time points ($p < 0.05$). The dendrogram and coloured image were produced

by R packages. The colour scale ranges from saturated green for log ratios -2.0 and below to saturated red for log ratios 2.0 and above. Each gene is represented by a single row of coloured boxes; each time point by a single column. A and B: Clustering of 46 lager yeast genes and 57 ale yeast genes which are differentially expressed during fermentation.

GAP1, encoding the general amino acid permease, also responsible for proline uptake, showed no change in transcriptional activity. Nevertheless, the quantity of Put4 protein perhaps did not decrease to such an extent that no proline transport was possible. The remaining carriers, mainly in the lager strain, where a higher uptake of proline was observed, may have been sufficient to allow proline uptake. Figure 3 – 4 shows that proline uptake occurred during cell growth when oxygen was still available and was excreted at the end of fermentation. Oxygen can support proline catabolism even at very low concentrations (Gibson et al., 2009). Besides proline, a reduction in the concentration of all amino acids was observed during fermentation. Sequential uptake could not be accurately determined because several amino acids were reduced to negligible levels relatively early during fermentation (see Chapter 2.4). Further, amino acid uptake by S81 and S23 did not follow a defined course and the sequence differed, possibly owing to the different genetic backgrounds of lager and ale yeast. As already mentioned, proline was assimilated by the two strains. Certainly, the amount of proline uptake and the chronological sequence differ between the strains. S23 showed 52.86% but S81 only 24.70% uptake (Fig. 3 – 4 and Chapter 2.4).

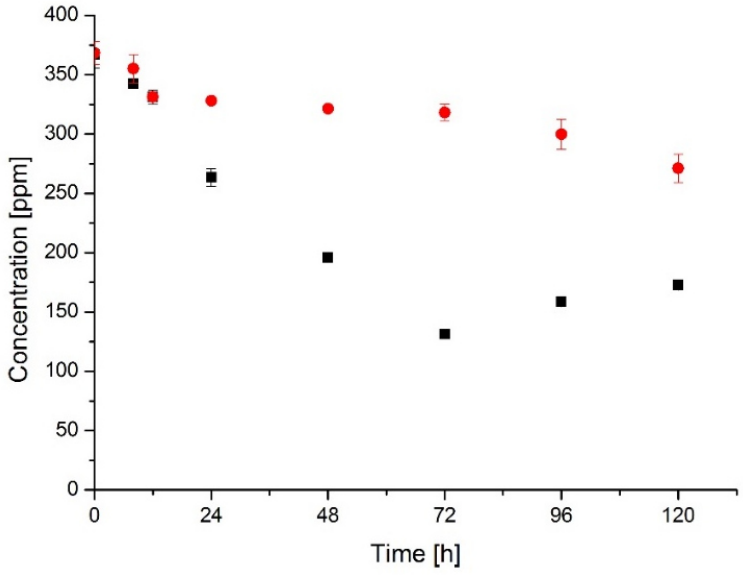

Figure 3 - 4: Proline uptake during brewery fermentation of lager yeast strain S23 (black square) and ale yeast strain S81 (red circle) in EBC tall-tubes. Error bars indicate ± SD. Lager yeast: 12°P 12°C, ale yeast: 12°P 20°C.

Given that proline is not converted to a higher alcohol by the Ehrlich pathway, its significance in aroma formation could be explained by its degradation to glutamate (Fig. 3 – 2), which may be converted to glutamine or transaminated to α-ketoglutarate; this product can be subjected to further transamination reactions, finally followed by decarboxylation and reduction (Prusiner & Stadtman, 1973; Walker, 1998). The accumulation of proline has also been discussed as a stress protectant in yeast cells during alcoholic fermentation (Kaino & Takagi, 2008; Takagi, Takaoka, Kawaguchi, & Kubo, 2005a). Proline contributed to improvement in the freezing tolerance of *S. cerevisiae* (Takagi, Takaoka, Kawaguchi, & Kubo, 2005b). Yeast cells accumulate proline in the vacuole, suggesting that there is a system for the transport of proline to the vacuole, and that this vacuolar accumulation may be important in freezing resistance (Morita, Nakamori, & Takagi, 2002).

The disruption of the *PRO1* gene, encoding gamma-glutamyl kinase, which catalyses the first step in proline biosynthesis, caused hypersensitivity to freeze–thaw damage (Ando, Nakamura, Murata, Takagi, & Shima, 2007). *Saccharomyces* yeasts with high freezing tolerance tended to accumulate higher levels of intracellular proline (Morita, Nakamori, & Takagi, 2003). This tendency could also explain the higher proline uptake in the lager yeast cells, given that the fermentation of lager beers requires a lower temperature than that of ales. These temperatures lead to the clean taste and the lack of fruity esters. Fermentation of brewery wort at such a low temperature is possible only owing to the cryotolerant nature of *S. pastorianus* (Gibson, Storgårds, Krogerus, & Vidgren, 2013). The Patagonian species characterised as *S. eubayanus* in 2011 gives lager yeast its interesting cold tolerance and is responsible for the characteristic flavour of lager beer (Libkind et al., 2011). However, a Tibetan population of *S. eubayanus* more closely related to *S. pastorianus* than the strain from Patagonia has been found. The genome sequence identity of this Tibetan strain with the non-ale moiety of *S. pastorianus* Weihenstephan 34/70 was 99.82% (Bing, Han, Liu, Wang, & Bai, 2014).

Given that amino acids have high impact on final beer quality, the effect of single-amino acid supplementation on the transcription levels of genes involved in the biosynthesis of aroma-active higher alcohols and esters in yeast, which have partly been confirmed in Chapter 2.4, needed to be further characterised. The difference in growth conditions, with EBC-tall tubes of just 2 L of medium for the screening compared with high-volume pilot plants, may explain why certain genes were not identified in our Affymetrix screening. We accordingly decided to take into account further genes such as the pyruvate dexarboxylase genes *PDC1*, *PDC5* and *PDC6*, postulated to participate in this reaction (Ter Schure, Flikweert, Van Dijken, Pronk, & Verrips, 1998; Yoshimoto et al., 2001).

According to PLS regression analysis in Chapter 2.3, leucine, isoleucine, valine, proline, glutamine and cysteine are the amino acids that have the main influence on the aroma compound profile in the fermentation process of *S. pastorianus* strain S23. An addition of a doubled amount of the key amino acids, corresponding to the maximum concentrations in wort according to Krüger and Anger, causes minor or major differences in the aroma compound profile according to the amino acid added. Isoamyl alcohol showed the highest end concentrations in all fermentations. In particular, leucine addition was significant, due to the mechanisms involved in the Ehrlich pathway (Hazelwood et al., 2008). Amino acid addition significantly increased the production of the respective higher alcohols (Fig. 3 – 5A). Furthermore, a global increase in propanol formation occurred after the addition of several amino acids (excluding isoleucine). Besides the BCAAs, proline and glutamine supplementations enhance the formation of higher alcohols, particularly propanol. Propanol is generated exclusively as a consequence of nitrogen metabolism (Mouret et al., 2014). In general, there is a clear relationship between amino acid addition and the formation of higher alcohols (Fig. 3 – 5A). The effect of amino acid supplementation is explained by the increasing ability of the yeast to decarboxylate the transaminated α-keto acids followed by their reduction to higher alcohols (Hazelwood et al., 2008; Hernández-Orte et al., 2005). Thus, changes in the formation of fusel alcohols caused by amino acid supplementation are associated with changes in the intracellular pools of precursors and intermediates involved in their synthesis.

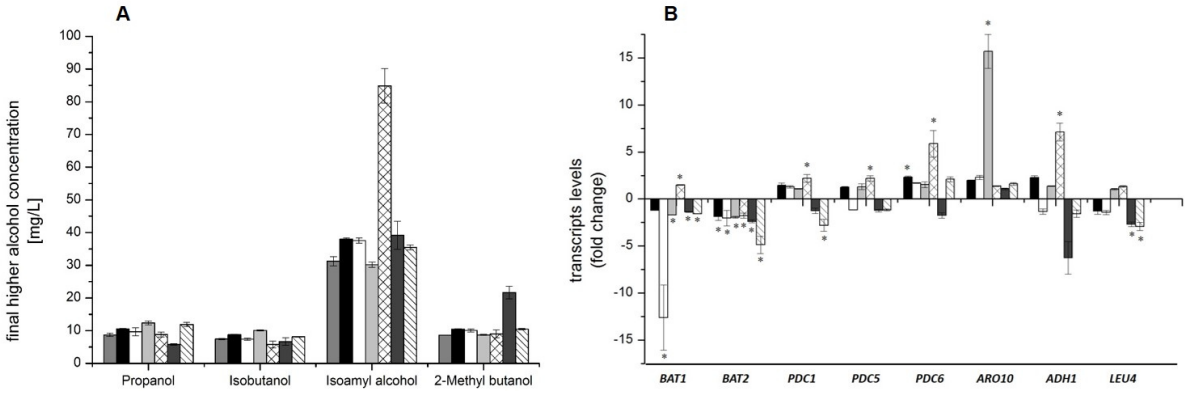

Figure 3 - 5: Final higher alcohol concentrations in EBC tall-tube fermentations under different amino acid supply. Results are the averages of three independent fermentations. Error bars indicate ± SD (A). Relative transcription profile by the lager strain S23 of genes involved in higher alcohol production. Results are the averages of three independent fermentations relative to the condition without supplementation of amino acids (* $p < 0.05$). Error bars indicate ± SD (B). (Grey bars) standard fermentation; (Black bars) proline addition; (white bars) cysteine addition; (light grey bars) valine

addition; (double striped bars) leucine addition; (dark grey bars) isoleucine addition; (striped bars) glutamine addition.

Surprisingly, the BCAA transaminases *BAT1* and *BAT2* catalysing the first step in the Ehrlich pathway were downregulated with the amino acid supplementations, with the exception of *BAT1* expression under leucine-supplemented conditions (Fig. 3 – 5B). To determine whether the end concentration of the higher alcohols was correlated with the expression levels of the main genes involved in the biosynthetic pathway, a Pearson correlation was calculated (Table 3 – 1). The positive correlation between *BAT1* and isoamyl alcohol as well as the significant increase of isoamyl alcohol after leucine addition (Fig. 3 – 5A) should confirm the inducibility of *BAT1* by leucine.

The addition of the other amino acids, except for cysteine, also significantly increased the concentration of some of the remaining higher alcohols. However, no further correlation between a higher alcohol, except for isobutanol, and the expression of the analysed genes could be identified. Thus, other transaminases like *ARO8* and *ARO9* must also be involved in the first step of fusel alcohol formation. Aro8p and Aro9p were initially characterised as the aromatic amino acid aminotransferases I and II, respectively (Iraqui et al., 1998). These aminotransferases may act as broad substrate-specificity amino acid transaminases in the Ehrlich pathway (Hazelwood et al., 2008). Furthermore, under the different amino acid-supplementation conditions, the pyruvate decarboxylases *PDC1*, *PDC5*, *PDC6* and the alcohol dehydrogenases *ADH1* were mostly upregulated. In particular under leucine-supplementation conditions, the synthesis of higher alcohols is significantly dependent on these decarboxylases as well as on *ADH1*, a finding in accordance with the highest total fusel alcohol concentrations, particularly in view of the high isoamyl alcohol concentrations following leucine addition (Fig. 3 – 5). These results could be further confirmed by the positive correlations found between isoamyl alcohol and most genes involved in the biosynthetic pathway of fusel alcohol production (Table 3 – 1).

Fermentations with particularly high levels of valine yielded a 15-fold increase of the expression of the *ARO10* gene, encoding the phenylpruvate decarboxylase, confirming the strong impact of *ARO10* on the aroma-associated decarboxylation of yeast fermentations, already reported by Romagnoli et al. (Romagnoli et al., 2012). The expression level of *ARO10* was also be correlated with the end concentration of isobutanol. This positive correlation as well as the significant increase of isobutanol with valine addition (Fig. 3 – 5A and Chapter 2.5) indicates that *ARO10* is inducible by valine, ensuring high isobutanol concentrations.

Table 3 – 1: Correlation between gene expression and flavour compound production.

Para-meter	Propanol	Iso-butanol	Isoamyl alcohol	2-Methyl butanol	Ethyl acetate	Isobutyl acetate	Isoamyl acetate	2-Methylbutyl acetate	Ethyl hexanoate
Group A									
BAT1	-0,19	-0,46	**0,77**	-0,19					
BAT2	-0,19	-0,04	-0,06	-0,37					
PDC1	-0,18	-0,39	**0,82**	-0,26					
PDC5	0,04	-0,39	**0,92**	-0,39					
PDC6	0,15	-0,40	**0,91**	-0,42					
ARO10	0,58	**0,76**	-0,29	-0,27					
ADH1	-0,03	-0,47	**0,94**	-0,35					
LEU4	0,10	-0,06	0,51	-0,61					
Group B									
ATF1					0,74	0,02	0,37	-0,37	
ATF2					-0,01	-0,37	0,75	-0,0003	
IAH1					0,46	-0,22	0,25	-0,60	
Group C									
FAS1									-0,17
FAS2									-0,16
EEB1									0,53
EHT1									0,22

Calculation of the Pearson product moment correlation coefficient to define the extent of a linear relationship between gene expression and the final concentration of a flavour compound in all six conditions tested

The production of the calculated esters was also variable in the response to amino acid supply (Fig. 3 – 6A). It is obvious that isoleucine supplementation led to the highest 2-methylbutyl acetate concentrations in the final product, a result directly related to the highest 2-methyl butanol formation (Hazelwood et al., 2008). The same observation was found for leucine supplementation and the formation of isoamyl acetate but not for isobutyl acetate production under valine supply.

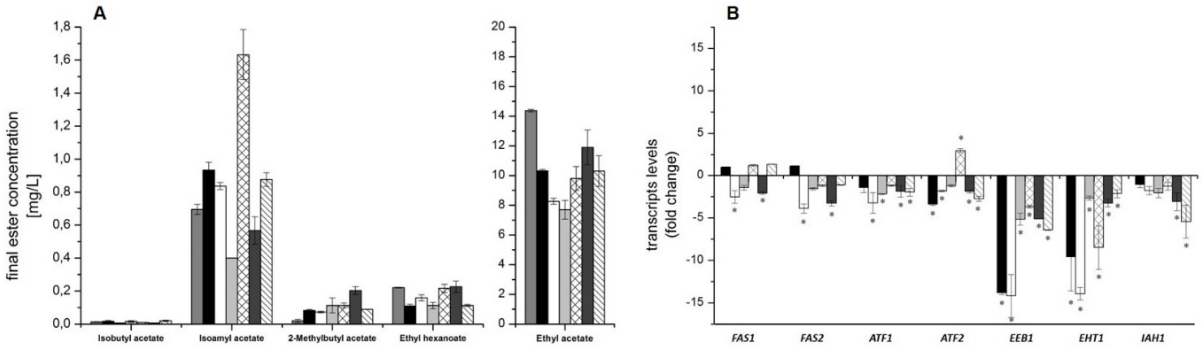

Figure 3 - 6: Final ester concentrations in EBC tall-tube fermentations under different amino acid supply. Results are the averages of three independent fermentations. Error bars indicate ± SD (A).

Relative transcription profile by the lager strain S23 of genes involved in ester production. Results are the averages of three independent fermentations relative to the condition without supplementation of amino acids (* $p < 0.05$). Error bars indicate ± SD (B). (Grey bars) standard fermentation; (Black bars) proline addition; (white bars) cysteine addition; (light grey bars) valine addition; (double striped bars) leucine addition; (dark grey bars) isoleucine addition; (striped bars) glutamine addition.

Surprisingly, all amino acid supplementation conditions provoked a decrease in expression of *ATF1* and *ATF2*, except for leucine supply, for which a significant upregulation of *ATF2* was observed (Fig. 3 – 6B). According to other authors, the *ATF1* gene is more deeply involved in acetate ester formation than *ATF2* (Saerens, Verbelen, et al., 2008a; Verstrepen, Derdelinckx, Dufour, Winderickx, Thevelein, et al., 2003a). This difference could not be confirmed in either Chapter 2.4 or 2.5. It should be remembered that the fermentations in this study were batch processes, and that the aim was to focus on genes that would exert strong effects in such conditions. It is also interesting to note that differences in the growth conditions of the yeast cell could favour certain gene products over others playing a part in aroma compound metabolism (Styger, 2011). Besides the activity of the enzymes involved in acetate ester formation, the concentrations of the two substrates, acetyl-CoA and the corresponding fusel alcohol, are also of interest. However, according to several authors (Lilly et al., 2000; Malcorps et al., 1991; Verstrepen, Van Laere, et al., 2003b), the availability of substrates is not the major limiting factor but rather the expression level of *ATF1* and *ATF2*. Still, the quantity of those enzymes perhaps did not decrease to such an extent that no acetate ester synthesis would be possible. Furthermore, the significant downregulation of the *FAS1* and *FAS2* genes and the decrease in the expression level of *EHT1* and *EEB1* could account for the decrease in ethyl hexanoate levels. Notably, proline supplementation induced a downregulation of the ethanol acyltransferases, which is expected to be associated with the significantly reduced ethyl hexanoate concentration, but the expression level of the AEATase genes proved no correlation with the end concentration of ethyl hexanoate (Table 3 – 1).

From the above results, it could be confirmed that of those genes that were further investigated, *BAT1*, *PDC1*, *PDC5*, *PDC6*, *ARO10* and *ADH1* play a direct role in the Ehrlich pathway, especially with focus on isoamyl alcohol. However, not all of the analysed genes showed clear associations with the various aroma-active metabolites. Some of the genes may act as promiscuous enzymes under these special fermentation conditions. It has long been scientifically recognised that the lock–key theory of enzymes and the reactions that they catalyse are not as explicit as first believed. It is possible that enzymes show different catalytic activity in environmental conditions different from their natural state. Further, some enzymes

have a broad range of substrates that they can catalyse, and some enzymes are able to catalyse distinctly different chemical reactions (Hult & Berglund, 2007).

Alternatively, amino acids have not such a large influence on the tested genes as previously thought. Even if several expression data displayed significant differences compared with standard amino acid conditions, the up- and downregulations of most of the tested genes were much lower than expected. It appears that amino acids contribute to aroma-active metabolites more by substrate availability than by regulatory effects on gene expression, except for leucine. In particular for the AATases, it is clearly shown that amino acids have not the same inducibility of *ATF1* and *ATF2* as has high-gravity brewing. *ATF1* is highly induced by glucose as a target of the Ras/cAMP/PKA signalling pathway (Verstrepen, Derdelinckx, Dufour, Winderickx, Pretorius, et al., 2003d) explaining increases in *ATF1* expression at higher wort density and further increasing the concentration of acetate esters (Saerens, Verbelen, et al., 2008a).

Furthermore, an increase in acetate esters according to high sugar levels in wort can originate in the anabolism of higher alcohol production. Figures 3 -7 clearly show that also sugars are contributing to the aroma-active metabolites of *S. pastorianus* strain S23 and *S. cerevisiae* strain S81. Again, PLS regression was used as a tool to develop a fingerprint of sugar importance with respect to aroma compound profile. These sugars were maltotriose and glucose for S81 and fructose and glucose for S23 (Fig. 3 – 7). During carbohydrate metabolism, pyruvate and α-keto acids are generated via the biosynthesis of amino acids and are degraded to higher alcohols, also followed by decarboxylation and reduction reactions (Chen, 1978). Thus, many fermentation volatiles important to beer aroma arise from yeast metabolism of sugars (Chen, 1978; Nisbet et al., 2014). It has recently been shown (Nisbet et al., 2014) that during wine fermentation, most volatiles are derived mainly from hexoses such as glucose and fructose. These volatiles include more than 75% of the higher alcohols associated with amino acid metabolism and their corresponding esters. It thus appears that the anabolic pathway is the major contributor of higher alcohol production, at least in wine fermentation.

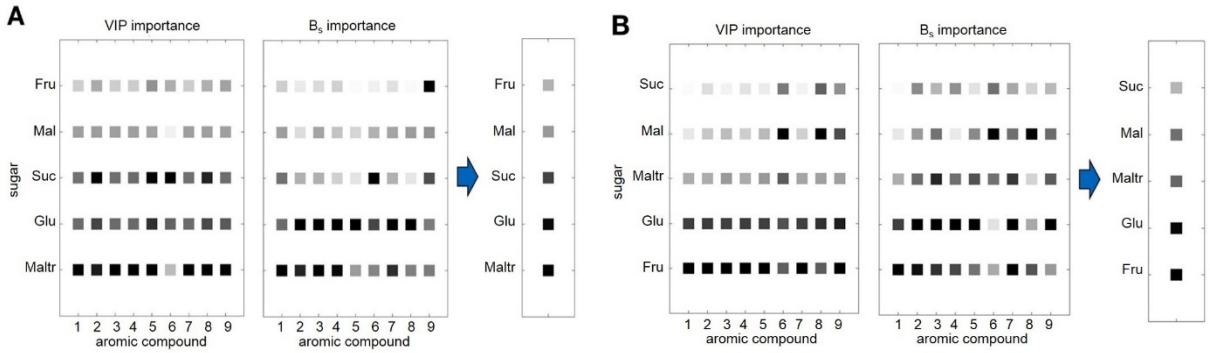

Figure 3 – 7: Statistical importance of each sugar on the concentrations of aroma compounds of (A) ale yeast strain S81 and (B) lager yeast strain S23; x axis labels are 1 - propanol, 2 - isobutanol, 3 - isoamyl alcohol, 4 - 2-methyl butanol, 5 - ethyl acetate, 6 - isobutyl acetate, 7 - isoamyl acetate, 8 - 2-methyl butylacetate and 9 - ethyl hexanoate; (A,B - left) - variable importance in the projection (VIP) map; (A,B - right) - standardized regression coefficient map - sugars were sorted by their absolute VIP and Bs values; (A,B – single column) - summed and normalized importance measure of both methods; black indicates high statistical importance on the resulting concentrations of aroma compounds.

The prediction of specific aroma compound profiles by analysis of gene expression levels is clearly not simple, and several explanations for this complexity have been offered above. Further, under our special fermentation conditions, aroma compound production may be partly regulated at the posttranscriptional level. Similar findings and/or hypotheses have been reported (Molina et al., 2007). In particular, carbonylations are consequences of oxidative stress and target a modified protein for degradation (Grimsrud, Xie, Griffin, & Bernlohr, 2008). Protein concentrations depend not only on the mRNA level but also on translation and degradation rate. Huang et al. (Huang et al., 2011) tried to predict mRNA translation rate using a computational model with prediction accuracies of about 70%. However, it is a great challenge to construct such a translation rate prediction model, taking into account some of the following features: codon usage frequency, number of RNA binding proteins known to bind its mRNA product, coding sequence length, protein abundance and 5′ UTR free energy. These features might provide useful information for understanding the mechanisms of translation and dynamic proteome (Huang et al., 2011).

In summary, amino acids clearly influence the aroma spectrum of beer. Leucine, isoleucine, valine, glutamine, cysteine and proline for *S. pastorianus* strain S23 and leucine, isoleucine, valine, histidine, glutamine and proline for *S. cerevisiae* strain S81 have been identified as the amino acids most important for individual measured aroma compound profiles. Differences

in sequential amino acid uptake have been shown to be probably strain dependent, owing to their different genetic makeups. There is also strong evidence that this strain dependent amino acid uptake explains the different concentrations of aroma-active substances in beer among various yeast strains. Overall, this work provides fundamental and novel information about the effects of single amino acid supply on the final concentrations of important aroma-contributing metabolites. This is partly triggered by the expression levels of genes participating in the biosynthesis of aroma-active compounds, which are affected by the addition of the significant amino acids. This information may help to improve understanding of the importance of specific aroma biosynthesis genes to the production of defined concentrations of some higher alcohols and esters during brewery fermentation. However, depending on the influence of sugars on higher alcohol production, it is desirable to find a balance between the anabolic and catabolic pathway to predict specific aroma profiles in yeast fermented beverages.

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