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Genetic and molecular basis of the aroma production in *S. kudriavzevii*, *S. uvarum* and *S. cerevisiae*

PhD thesis

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INFORMAN

Que la presente memoria "Genetic and molecular basis of the aroma production in *S. kudriavzevii*, *S. uvarum* and *S. cerevisiae*" constituye la tesis doctoral de Don Jiří Stříbný para optar al grado de Doctor por la Universidad de Valencia. Asimismo, certifican haber dirigido y supervisado tanto los distintos aspectos del trabajo como su redacción.

Y para que conste a los efectos oportunos, firman el presente informe en Valencia a 3 de Marzo de 2016.

Dra. Amparo Querol Simón Roberto Pérez Torrado

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It was 1st September 2011 when we landed at the Aeroport de València, with no idea on Spanish culture, Spanish language or the amazing weather in Valencia. After almost five sunny years I am able to order a beer (although here in Spain one would prefer wine), I have finished my research and written this book. The beginnings were tough but there has always been someone who has given us a helping hand. Here comes the moment I'm going to express my thanks (hopefully not missing anyone out). During this PhD period, I have met a lot of people of various nationalities, so this is going to be a mix of Czech, Spanish and English words (without any correction and, obviously, with some errors).

V první řadě děkuji svým rodičům, bráchům, celé Řehůřkovic rodině a všem ostatním blízkým, protože bez vaší bezmezné podpory by tohle všechno bylo téměř nemožné. Jsem rád, že jste se všichni (někteří i vícekrát) dorazili ohřát tady k nám, a že jste nám vždy zpříjemnili náš zdejší pobyt.

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Gracias!

Thanks!

Don't stop me now!

Freddie Mercury
Queen

Preface

These first paragraphs will describe a broader context of the scope and objectives of this PhD thesis. This work was part of an international project called Cornucopia. This project was an international consortium of 10 academic and industrial partners funded by the European Commission FP7: Marie Curie Initial Network. Cornucopia was an acronym of the project of which main objective was to explore the potential of yeast biodiversity as a source of innovations in food and health. This objective was built up on a simple fact that although the yeast kingdom includes more than 1.500 species, only the species *Saccharomyces cerevisiae* has by far the predominant position in many fermentation-related industrial processes. However, such a huge divergent group represents a large untapped reservoir for development of new products in various industries including food, flavour, fragrance and health sectors.

Besides the screening and selection of yeasts with traits of interest, the Cornucopia research focused also on the detailed molecular understanding of the unique characteristics. The traits of interest involved also an unusual aroma and flavour production which became the objective of this PhD project. The attention was paid to *Saccharomyces kudriavzevii* and *Saccharomyces uvarum* which had been found to produce different amounts of several flavour compounds resulting in a distinct aroma profile, for instance, during wine fermentations. To fulfil the goals of the Cornucopia project, this work aimed to explore the metabolic pathways for the distinct flavour production, identify the responsible genes and characterise the corresponding enzymes.

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Introduction

Section I: Yeast as a little flavour maker

Flavours – from natural through synthetic back to natural

The story of the relationship between man and aromas and flavours began thousands of years ago when our earliest ancestors discovered pleasure in the aroma of a flower, and that addition of certain herbs improved taste of food. Later, union of nations and cultures during the era of Roman Empire, which connected Europe, the Arab world and the Orient, made plenty of spices available to flavour food. The fall of the Roman Empire then led to searching for new routes to the East. In fact, the demand for the oriental flavours and fragrances, as David Rowe describes (Rowe, 2009), was a major driving force in the European discovery of the Americas which, of course, had been discovered before by the people who actually lived there, but as they did not wear trousers, they did not count. Progresses in chemistry knowledge around the turn of the 20th century opened extensive development in flavour and fragrance detection and production. At that time, the first flavour and fragrance companies (such as Givaudan, one of the two largest flavour and fragrance companies) were founded and the flavour and fragrance industry originated. Over the years this industry has developed into a very profitable marked. This was mainly achieved after World War II. With an increase in the standard of living the food market became dominated by processed food and ready-meals which required flavourings to restore taste properties and to satisfy consumer preferences.

Until the middle of the 20th century most of the flavouring agents consisted of natural extracts and essential oils. In the 1960s first synthetic components started being used. The increasing number of food additives (both natural and synthetic) forced the appropriate authorities to establish a regulatory system. Worldwide, the two major regulators of food additives are the European Food Safety Authority (EFSA) and the Food and Drug Administration (FDA) of the United States. Even if they are to a certain extent

regulated, the use of components produced via chemical synthesis (even the so called "nature-identical", i.e. products that occur in nature but are produced via a chemical process) is strongly negative from the marketing point of view (Carocho *et al.*, 2014). Since health aspects and environmentally friendly production processes play an increasing role for the consumers, naturally obtained products (re)gain the flavour and food industry interest. In this context, an expansion of biotechnological processes with an important role of microorganisms, particularly yeasts, as cell factories is being observed (Krings and Berger, 1998; Vandamme and Soetaert, 2002).

Yeast

Like the relationship between man and aromas, the relationship between man and yeast began many years ago when consumption of fruits and grains left in covered containers for several days caused pleasure psychotropic effect. Until 17th century, however, it was not man-yeast relationship from human's perspective. Then Antoni van Leeuwenhoek, using his handcrafted microscopes, introduced to the world "*animalcules*", Leeuwenhoek's Latin naming for a "little animal". However, it took other two centuries till Louis Pasteur proved the hard work of the microbes during the fermentation process (Barnett, 2000; Pasteur *et al.*, 1879). Those were Dutch people who called the foam formed during the fermentation of beer with the Dutch word "*gist*", later transformed to the term yeast. Other well-known word referring to yeast originated no more than a few hundreds of kilometres to the south where French people used the word "*levure*" to refer to the role of yeast in causing bread dough to rise.

Hundreds of years lasting contribution of yeast in the production of wine, beer and bread has led to it being one of the most extensively studied organisms. The knowledge in turn allowed yeast to become an important workhorse in other industrial sectors, such as pharmaceutics, biofuel, wastewater treatment etc.

Saccharomyces cerevisiae

Because of their abundance and dominance in many spontaneous fermentations, *Saccharomyces cerevisiae* strains were selected for the majority of controlled fermentation processes, including the traditional uses for production of beverages and baking, but also for production of recombinant antibodies, vitamins, and drugs, such as insulin etc.(Bayne *et al.*, 1988; Gerngross, 2004; Kazemi Seresht *et al.*, 2013).

Besides its employment in industrial applications, *S. cerevisiae* plays an important role in scientific research. Many physiological processes which occur in plant and animal cells are highly similar in yeast cell. Knowledge gained in the studies of *S. cerevisiae* can therefore be applied to higher eukaryotic organisms. This has made *S. cerevisiae* one of the popular model organisms used for exploration of molecular aspects of cellular processes in other eukaryotic cells. Compared to plant or animal cell cultures, the use of *S. cerevisiae* as a model organism brings many benefits. Being unicellular with a short generation time, it is easy to cultivate and manipulate which simplifies an experimental work in terms of time and finances. Another advantage is a possibility to transform cells with artificially constructed recombinant DNA in order to perform heterologous expression of particular genes and study the corresponding proteins. Additionally, a use of an appropriate promoter enables to regulate the gene expression. These tools make also possible to express and study genes from bacteria, plants, and animals. The potential of genetic manipulations further increased with revealing of the entire *S. cerevisiae* genome by Goffeau *et al.* (1996). That new knowledge opened up a reservoir of new research questions and contributed to a progress of other biological fields, such as genomics, transcriptomics, proteomics, metabolomics etc. (Delneri *et al.*, 2001; Kitano, 2002; Oliver *et al.*, 2002; Oliver, 2002).

Taxonomy and its troubles

Additionally, together with the development of molecular methods of yeast characterisation the use of DNA sequencing enabled an improvement of yeast classification. Previously, the classification was based mainly on morphological, reproductive and physiological characteristics, such as fermentation capacity or carbon and nitrogen source assimilation. The current advance of sequencing technologies, such as high-throughput sequencing and next-generation sequencing tools, led and still leads to a re-arrangement of the taxonomic classification.

As its name gives a clue, *Saccharomyces cerevisiae* is a member of the genus *Saccharomyces* (previously called *Saccharomyces sensu stricto*). The genus *Saccharomyces* belongs to the kingdom Fungi, the phylum *Ascomycota* (as the sexual reproduction is based on the formation of ascospores), the subphylum *Saccharomycotina*, the class *Saccharomycetes*, the order *Saccharomycetales* and the family *Saccharomycetaceae*. The taxonomy of the *Saccharomyces* genus currently involves seven species (Figure I.1). Besides *S. cerevisiae*, there are *S. kudriavzevii*, *S. uvarum*, *S. paradoxus*, *S. mikatae*, *S. arboricolus*, and *S. eubayanus* (Boynton and Greig, 2014; Hittinger, 2013).

One of the most interesting mechanisms observed within the *Saccharomyces* genus (as well as in other yeast genera) is the formation of interspecific hybrids. As one can see, the genomic variation arising from the hybridisation makes a taxonomist's life even more entertaining.

The best described interspecific hybrid is *Saccharomyces pastorianus* (Walther *et al.*, 2014). For a relatively long time it was assumed to be a hybrid between *S. cerevisiae* and *Saccharomyces bayanus*. Today it is described as a hybrid between *S. cerevisiae* and the recently discovered species *S. eubayanus* (Hebly *et al.*, 2015; Libkind *et al.*, 2011). Formerly, the *S. bayanus* taxon involved two varieties, *S. bayanus var. bayanus* and *S. bayanus var. uvarum*. However, the recent isolation and characterisation of the new species

S. eubayanus prompted taxonomists to separate these two groups and establish the variety *uvarum* as the species *S. uvarum* and the variety *bayanus* as hybrids between *S. eubayanus* and *S. uvarum* (Pérez-Través *et al.*, 2014).

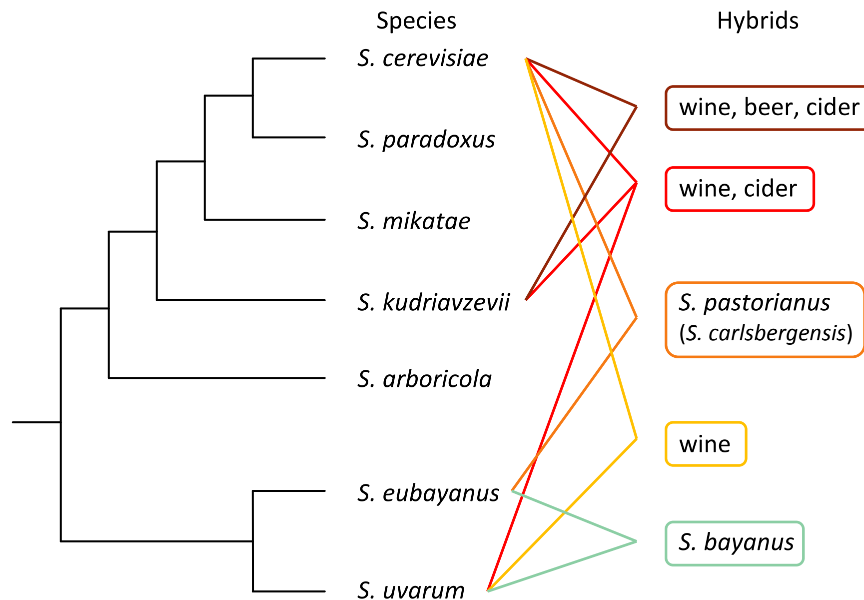

Figure I.1. Schematic illustration of *Saccharomyces* genus phylogeny. Lines indicate hybridisation among the species (adapted from Boynton and Greig (2014)).

Biotechnologist's fortune – a hybrid

Although the hybridisation of yeast species might make a taxonomist's life more complicated, a biotechnologist's life might be happier. It is because the hybrids generally combine optimal characteristics of both parents in order to adapt to specific environmental conditions, including both natural and biotechnological environments. As an example is the aforementioned *S. pastorianus,* also known as *S. carlsbergensis* indicating its employment in lager beer production typically brewed at 8-15°C. This low temperature selective pressure may have resulted in the hybridisation between *S. cerevisiae* providing robust fermentation characteristics, and the more

cryotolerant *S. eubayanus* providing the ability to grow at low temperature (Libkind *et al.*, 2011; Peris *et al.*, 2012). Similar tactics of the inheritance of good characteristics from both parents described in other *Saccharomyces* hybrids, such as *S. cerevisiae* x *S. kudriavzevii*, and *S. cerevisiae* x *S. uvarum*, that have mainly been isolated from wine, beer, and cider-related environments (Gonzalez *et al.*, 2006; Gonzalez *et al.*, 2008; Lopandic *et al.*, 2007; Masneuf *et al.*, 1998; Pérez-Torrado *et al.*, 2015). In addition to "pure" strains, the hybrids represent means for improving many attributes of industrial interest, including new flavours in food and fragrance industry, biofuels, and pharmaceutics. In this context, several research laboratories have been making an effort to develop methods to generate hybrid strains with desirable features (Alexander *et al.*, 2015; Pérez-Través *et al.*, 2012). This may be one of the reasons (amongst others) for understanding well the characteristics of the "pure" *Saccharomyces* species; in case of this dissertation *S. kudriavzevii* and *S. uvarum*. The entire genome of both species have been recently released which further helps elucidating the molecular aspects behind various interesting traits of these species (Scannell *et al.*, 2011).

Saccharomyces kudriavzevii

S. kudriavzevii was first isolated only from decaying leaves in Japan and has not so far been found in industrial fermentation environments. So researchers made queries how this species formed hybrids that were found in wine (Gonzalez *et al.*, 2006) and brewing (Gonzalez *et al.*, 2008) environments located in Europe (Naumov *et al.*, 2000a; Naumov *et al.*, 2000b). The answers came recently with an isolation of several *S. kudriavzevii* strains from oak trees in Portugal and Spain (Lopes *et al.*, 2010; Sampaio and Goncalves, 2008). These isolation events were made possible due to the decrease in isolation temperatures, which confirmed cryotolerant character of *S. kudriavzevii*. Other interesting advantages of *S. kudriavzevii*, as compared to *S. cerevisiae*,

were found in oenological properties. Although *S. kudriavzevii* does not appear to be present in wine environments, it is able to conduct fermentation of a sterile wine must (Gonzalez *et al.*, 2007). When compared to *S. cerevisiae*, *S. kudriavzevii* was found to produce higher amounts of glycerol and lower amounts of ethanol, which fulfil the new demands of the wine industry (Gonzalez *et al.*, 2007; Oliveira *et al.*, 2014).

Saccharomyces uvarum

Although several strains have been sporadically isolated from insects, tree fluxes or a mushroom (Naumov *et al.*, 2003; Naumov *et al.*, 2006), the natural habitats of *S. uvarum* have not been identified so far. It has been found mainly in industrial environments, such as wine and cider fermentation processes performed at low temperatures (Demuyter *et al.*, 2004; Naumov *et al.*, 2000b; Naumov *et al.*, 2001). Like *S. kudriavzevii*, due to the good fermentation capacity at low temperature, *S. uvarum* is considered cryotolerant. From the oenological point of view, as compared to *S. cerevisiae, S. uvarum* has shown higher production of glycerol and lower production of ethanol and acetic acid (Bertolini *et al.*, 1996; Giudici *et al.*, 1995). Additionally, *S. uvarum* has been characterised by its higher capability to release desirable flavour components, such as 2-phenylethanol and 2-phenylethyl acetate (Gamero *et al.*, 2013; Masneuf-Pomarede *et al.*, 2010). The wines produced by *S. uvarum* strains have been described to have a stronger aromatic intensity than those produced by *S. cerevisiae*.

Section II: Production of aroma-active compounds and beyond

From sugar to ethanol

As it has been shown in the case of the term yeast (derived from "*gist*"), the understanding of its origin usually helps getting to the core and meaning of the term. So it is with the term *Saccharomyces* which derives from Old Greek and means "sugar mould or fungus". This nicely indicates that the most important carbon and energy sources of *S. cerevisiae* are sugars, primarily hexose monosaccharides, such as glucose and fructose.

Glucose is in *S. cerevisiae* converted via metabolic pathway called glycolysis to pyruvate and thence, to pleasure for the majority of human society, to ethanol (Figure I.2). From energetic point of view, glycolysis is important because of ATP formation. Additionally, during the glycolytic pathway, namely during the conversion of glyceraldehyde-3-phosphate to 1,3-biphosphoglycerate, the redox cofactor NAD^+ is reduced to NADH. Since there are limited amounts of NADH/NAD^+ couple in yeast cells, in continuous glycolysis all of the NAD^+ would be depleted and glycolysis interrupted (Bakker *et al.*, 2001). Therefore, reduction of NAD^+ needs to be coupled with a continuous reoxidation of NADH. Under anaerobic conditions, when oxygen is not available, *S. cerevisiae* accomplishes the NAD^+ regeneration via the two-step conversion of pyruvate to ethanol and CO_2, referred to as alcoholic fermentation. The first step involves thyamine pyrophosphate (TPP)-dependent decarboxylation of pyruvate to acetaldehyde catalysed by pyruvate decarboxylase (TPP-dependent PDC). The second step, catalysed by alcohol dehydrogenase, is the reduction of acetaldehyde to ethanol, whereas NADH becomes NAD^+.

In the presence of oxygen, the NADH reoxidation occurs via respiration. In the respirative pathway, the pyruvate generated in glycolysis undergoes an oxidative decarboxylation in mitochondria and results in acetyl CoA formation which then enters citric acid cycle.

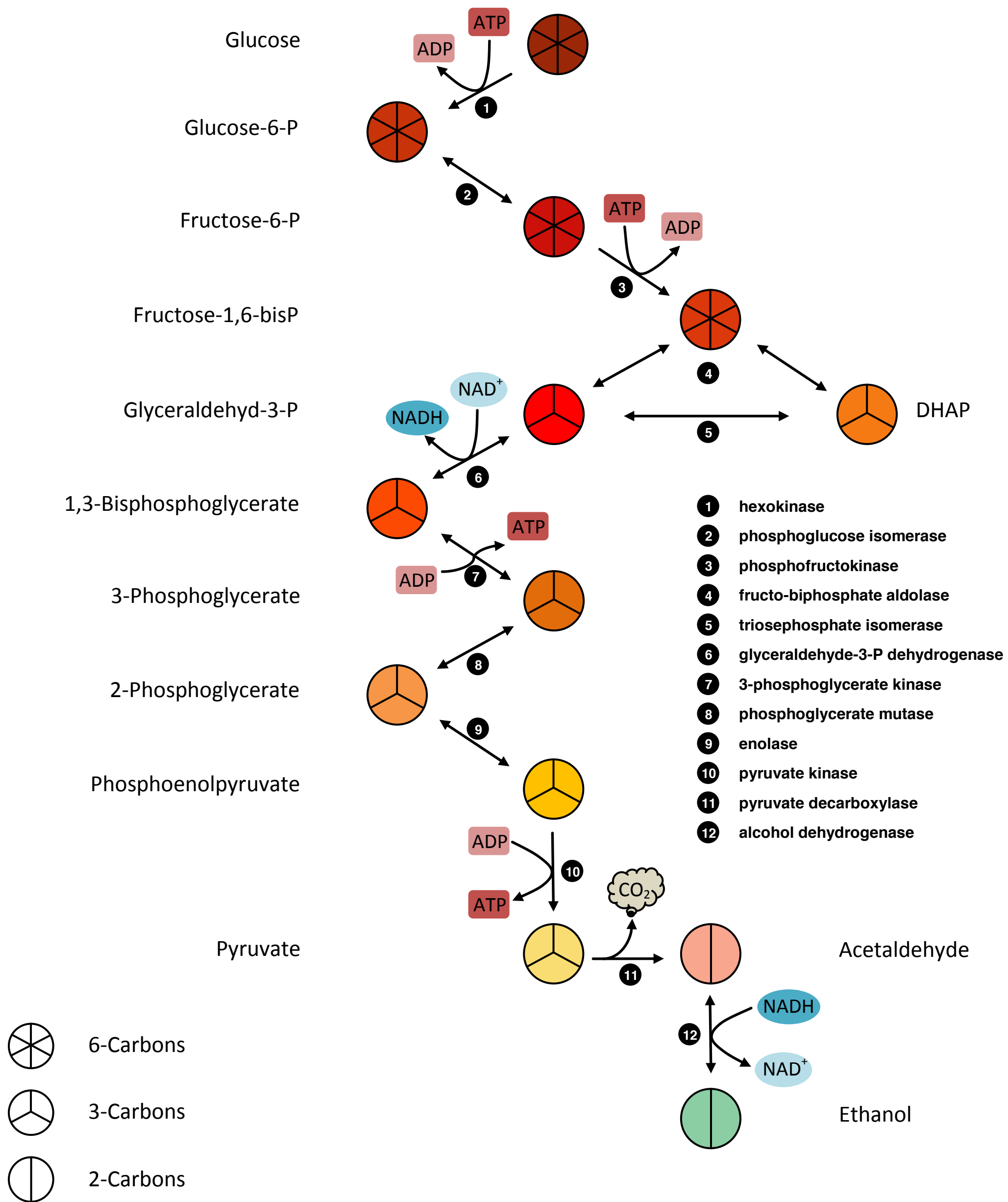

Figure I.2. Glycolysis followed by alcoholic fermentation.

Despite the fact that much more ATP can be achieved aerobically by means of citric acid cycle and electron transport chain, respiration in *S. cerevisiae* can take place only during sugar-limited cultivation and in the presence of oxygen. This predominant production of ethanol even under aerobic conditions and high glucose concentrations is commonly known as the Crabtree effect (De Deken, 1966). Due to the antiseptic nature of ethanol, one hypothesis interestingly explains this energetically inefficient mechanism as a tool of the yeast to defend nutrient resources in the fruit against rival microorganisms (Thomson *et al.*, 2005).

Secondary metabolism

From the biotechnological point of view, in addition to ethanol *S. cerevisiae* generates many other valuable secondary metabolites, such as glycerol, organic acids, sulphur-containing molecules, fatty acids, higher alcohols, and esters. An interaction between these numerous chemical compounds contributes to a certain extent to the quality, primarily to the aromatic and organoleptic quality, of the fermented foods and beverages, such as wine, beer, cider, sake, cheese or even chocolate (Fleet, 2003; Lambrechts and Pretorius, 2000; Meersman *et al.*, 2015a; Meersman *et al.*, 2015b). Among these aroma-active substances, volatile esters and higher alcohols represent the largest and most important group (Saerens *et al.*, 2010). Although most of these volatile flavour compounds are present at low concentrations in fermented beverages, they typically have very low sensory threshold, and therefore only small amounts of these molecules can have dramatic effect on the overall aroma profile. For instance, in terms of aroma quality of wine or beer, ethanol and CO_2, the major volatile products of yeast metabolism, make relatively small contribution respect to other volatile compounds. Conversely, the higher alcohols and esters formed during alcoholic fermentation strongly influence the sensory properties of the resulting product (Nykanen, 1986; Pérez-Torrado *et al.*, 2015; Romano *et al.*, 2003).

Higher alcohols

Since ethanol has two carbon atoms, the classification "higher" refers to alcohols which contain more than two carbons and thus have a higher molecular weight and boiling point than ethanol. Higher alcohols are also known under the term fusel alcohols, which derives its name from the German word "*fusel*" with simple meaning "bad liquor". This not very positive naming probably refers to the fact that higher alcohols, when present at high concentrations (in wine usually reported as higher than 400 mg/L), impart a harsh, unpleasant, solvent-like aroma. On the other hand, lower amounts of these compounds make a desirable contribution to the flavours and aromas of fermented foods and beverages (Lambrechts and Pretorius, 2000).

Table I.1. Overview of the most significant aroma-active higher alcohols produced by yeast during fermentation, their amino acidic precursors and odour (adapted from Lambrechts and Pretorius (2000)).

Compound	Amino acidic precursor	Odour
Propanol	Threonine	stupefying
Isobutanol	Valine	alcoholic
Isoamyl alcohol	Leucine	marzipan
Active amyl alcohol	Isoleucine	marzipan
2-phenylethanol	Phenylalanine	floral, rose
Tyrosol	Tyrosine	bees wax, honey

Higher alcohols are composed of aliphatic and aromatic alcohols (Swiegers and Pretorius, 2005). The aroma-active aliphatic alcohols mainly include branched-chain isoamyl alcohol, isobutanol, active amyl alcohol, and propanol. Among these isoamyl alcohol is quantitatively and qualitatively the most significant in many fermented beverages. Isobutanol and active amyl alcohol have been reported to be the most abundant minor components of brandy and other distillates. Its pleasant rose-like aroma makes

2-phenylethanol one of the most important aromatic alcohols required in fermented products as well as in the cosmetic and perfume industry (Table I.1) (Fabre *et al.*, 1998; Lambrechts and Pretorius, 2000).

Biosynthesis of higher alcohols

Higher alcohols are synthesised trough the catabolism of branched-chain aliphatic amino acids, valine, leucine and isoleucine, aromatic amino acids, phenylalanine, tyrosine and tryptophan, and sulphur-containing amino acid methionine. This metabolic pathway, also known as the Ehrlich pathway, involves three enzyme-catalysed reactions (Figure I.3).

The first reaction is amino acid transamination yielding the corresponding 2-keto acid, followed by its decarboxylation to the aldehyde, which is subsequently reduced by the alcohol dehydrogenase to the appropriate higher alcohol (Ehrlich, 1907; Hazelwood *et al.*, 2008). The transamination reaction is catalysed by four amino acid transaminases encoded by *BAT1, BAT2, ARO8* and *ARO9* genes. The *BAT1* and *BAT2* derived enzymes are responsible for the transamination of branched-chain amino acids (Kispal *et al.*, 1996). The individual *BAT* genes differ in their expression and cellular localisation (Eden *et al.*, 1996). While *BAT1* is localised in the mitochondria and is highly expressed in the exponential phase of growth, *BAT2* is cytosolic and is preferably expressed during the stationary phase.

The transaminases encoded by *ARO8* and *ARO9* catalyse the transamination of the aromatic amino acids. Enzymatic characterisation showed that both enzymes have broad-substrate specificity. Further mutation experiments revealed that despite differences in their kinetic properties, they complement each other (Urrestarazu *et al.*, 1998). The expression of *ARO8* and *ARO9* has been described to be different. *ARO8* is expressed constitutively whereas *ARO9* expression is induced by aromatic amino acids, indicating an important function of *ARO9* in aromatic amino acid catabolism (Iraqui *et al.*, 1998).

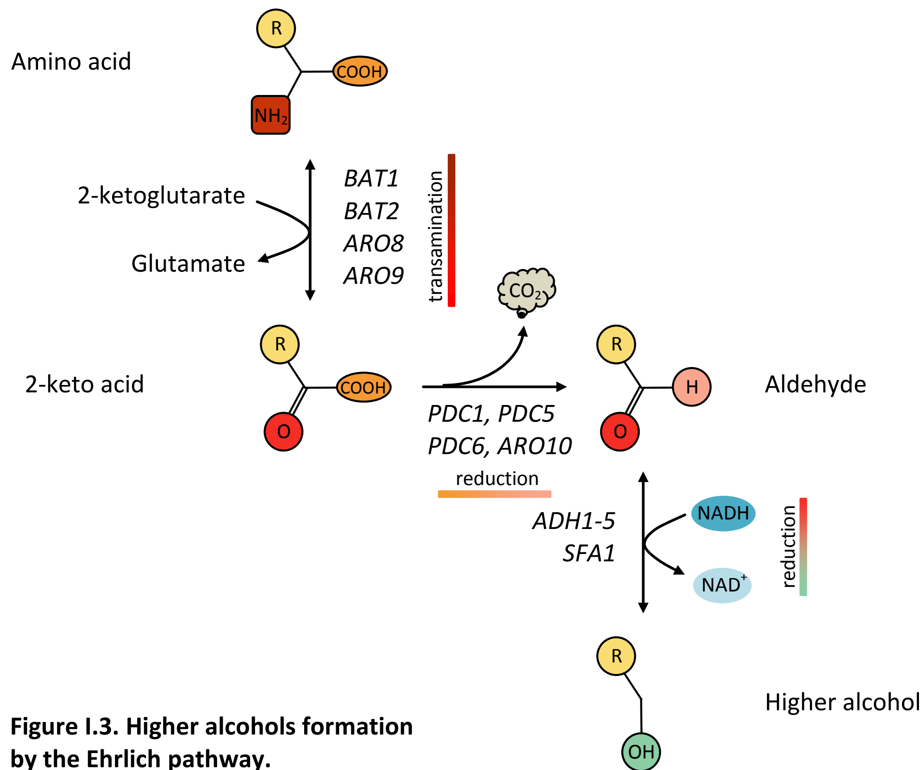

Figure I.3. Higher alcohols formation by the Ehrlich pathway.

The second reaction in the Ehrlich pathway is the decarboxylation of the 2-keto acid and the formation of the corresponding aldehyde. Like the conversion of pyruvate to acetaldehyde (described in the alcoholic fermentation), this step of the Ehrlich pathway is catalysed by thyamine pyrophospate (TPP)-dependent decarboxylase. The *S. cerevisiae* harbours five genes that show sequence similarity with TPP-dependent decarboxylase, namely *PDC1*, *PDC5*, *PDC6*, *ARO10*, and *THI3* (Hazelwood *et al.*, 2008). Since the decarboxylation reaction, and *ARO10* in particular, became an important part of this PhD work, this will be described in more detail later on in this introduction.

The final step in the higher alcohol formation via the Ehrlich pathway is

the reduction of the respective aldehyde, which is catalysed by an alcohol dehydrogenase. *S. cerevisiae* genome has been shown to harbour 16 alcohol dehydrogenases. Studies with deletion mutants reported by Dickinson et al. (2003) showed that the aldehyde reduction in the Ehrlich pathway can be catalysed by any of the ethanol dehydrogenases (Adh1p, Adh2p, Adh3p, Adh4p, and Adh5p) or by the formaldehyde dehydrogenase Sfa1p.

It should be noted that the final step of the Ehrlich pathway, i.e. the aldehyde conversion, can be the aforementioned reduction to the corresponding higher alcohol or oxidation to the corresponding carboxylic acid. These reduction/oxidation reactions involve the redox cofactors NADH/NAD^+ and therefore which of these two reactions occurs is thought to depend on the redox status of the cells (Styger *et al.*, 2011a; van Dijken and Scheffers, 1986; Vuralhan *et al.*, 2003).

In addition to the amino acid catabolism, another pathway has been suggested to be involved in the higher alcohols formation. The 2-keto acids can be derived from transamination of amino acids as described above, or they can be also derived from glucose via pyruvate during *de novo* synthesis of branched-chain amino acids (Ayrapaa, 1971; Mendes-Ferreira *et al.*, 2011; Nykanen, 1986).

Decarboxylation step in the Ehrlich pathway

Decarboxylation of the 2-keto acids derived from amino acid transamination is the only irreversible step in the Ehrlich pathway, and as already mentioned, is mediated by TPP-dependent decarboxylases (Hazelwood *et al.*, 2008). There are five genes (*PDC1*, *PDC5*, *PDC6*, *ARO10*, and *THI3*) in the *S. cerevisiae* genome, which were detected as TPP-dependent 2-keto acid decarboxylases (Figure I.4).

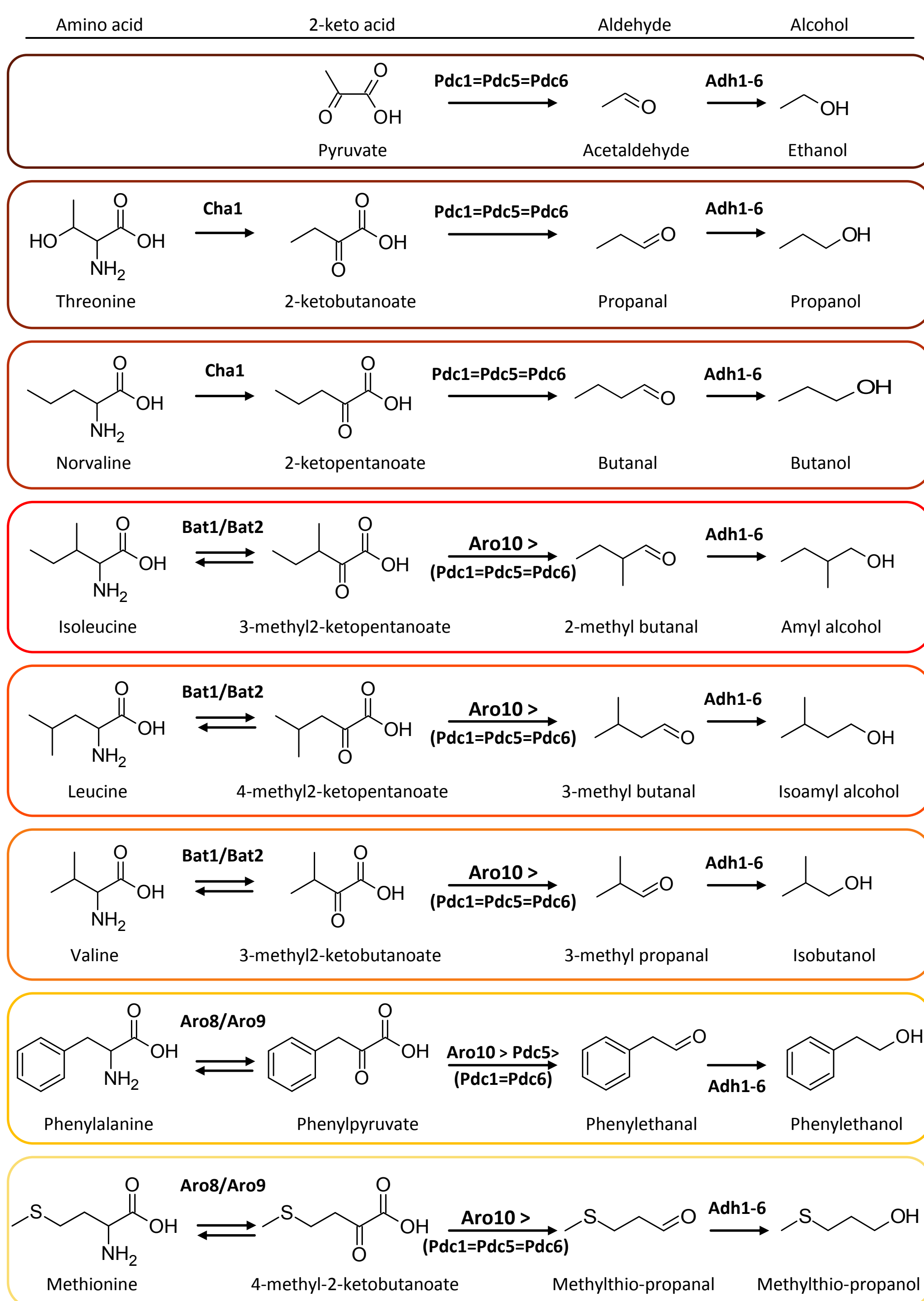

Figure I.4. The roles of individual 2-keto acid decarboxylases in higher alcohols formation (adapted from Romagnoli *et al.* (2012)).

The *PDC* genes encode differentially expressed pyruvate decarboxylase isoenzymes. *PDC1* encodes the major isoenzyme which exhibits high expression levels under most cultivation conditions. *PDC5*, on the other hand, was shown to be highly expressed in thiamine- and nitrogen-limited cultures, and in the cells lacking *PDC1* gene (Boer *et al.*, 2007; Seeboth *et al.*, 1990). The amino acid sequence analysis of the Pdc6 isoenzyme revealed a low content of sulphur-containing amino acids, and the expression analysis of *PDC6* revealed its induction mainly under low-sulphur conditions (Boer *et al.*, 2003; Tai *et al.*, 2005; Vuralhan *et al.*, 2005). *In vitro* enzyme assays performed by Romagnoli *et al.* (2012) revealed that the three isoenzymes have broad-substrate specificities with no significant differences in the enzyme activities, with the exception of a higher level activity of Pdc5 with phenylpyruvate. Additionally, Pdc isoenzymes are the sole decarboxylases that catalyses the decarboxylation of the linear 2-keto acids, pyruvate, 2-keto butanoate, and 2-keto pentanoate.

The *ARO10* derived enzyme has been characterised as a broad-substrate specificity 2-keto acid decarboxylase (Vuralhan *et al.*, 2005; Vuralhan *et al.*, 2003). Like the Pdc1, Pdc5 and Pdc6 isoenzymes, Aro10p is able to decarboxylate branched-chain and sulphur-containing 2-keto acids. Additionally, together with Pdc5p, Aro10p is the only decarboxylase catalysing the decarboxylation of aromatic substrates, such as phenylpyruvate. Aro10p, however, has a superior affinity for these substrates when compared with the Pdc isoenzymes (Romagnoli *et al.*, 2012). Gene expression analysis revealed that *ARO10* was expressed differentially for the different nitrogen sources. During growth on branched-chain or aromatic amino acids as the nitrogen source, the *ARO10* expression levels were much higher than in the presence of ammonium (Vuralhan *et al.*, 2005). Due to the catalytic activities and expression levels, Aro10p has been proposed to be the major decarboxylase involved in the production of branched-chain, aromatic, and sulphur-containing higher alcohols.

The fifth gene sharing sequence similarity with TPP-dependent decarboxylase is *THI3*. The Thi3 enzyme was initially described as the major decarboxylase involved in leucine catabolism (Dickinson *et al.*, 1997). However, further enzyme assays revealed no decarboxylation activity for Thi3p, indicating that *THI3* does not encode a functional 2-keto acid decarboxylase (Romagnoli *et al.*, 2012). Another function of Thi3p was observed in the regulation of thiamine biosynthesis (Nosaka *et al.*, 2005). In this context, the role of Thi3p in Ehrlich pathway has been assumed to be regulatory rather than catalytic. This hypothesis further supports the fact that while Pdc1p, Pdc5p, Pdc6p and Aro10p are cytosolic, Thi3p is localised in the cytosol as well as in the nucleus (Nosaka *et al.*, 2005).

Volatile esters

It has been mentioned in the previous paragraphs that volatile esters together with higher alcohols are important contributors to the sensorial quality of fermented foodstuffs. In addition, mostly fruity, candy, and floral aroma makes the volatile esters important fragrant components for cosmetic and perfume industry.

The most significant aroma-active esters produced by *S. cerevisiae* during the fermentation can be divided in two groups based on the components which they are derived from. In the first group, there are ethyl esters of which the acid component is medium-chain fatty acid, and the alcohol component is ethanol. The second group comprises the acetate esters formed from acetate as the acid component, and from ethanol or higher alcohol as the alcoholic component (Saerens *et al.*, 2010). The major aroma-active ethyl esters involve ethyl hexanoate, ethyl octanoate, and ethyl decanoate (Table I.2). The major acetate esters are ethyl acetate, isobutyl acetate, isoamyl acetate, and 2-phenylethyl acetate (Fujii *et al.*, 1994; Lambrechts and Pretorius, 2000; Verstrepen *et al.*, 2003a).

Table I.2. Overview of the most significant aroma-active esters produced by yeast during fermentation, and their odour (adapted from Lambrechts and Pretorius (2000)).

Ethyl esters	Odour	Acetate esters	Odour
Ethyl butanoate	floral, fruity	Ethyl acetate	nail polish, fruity
Ethyl hexanoate	apple, violets	Isobutyl acetate	fruity
Ethyl octanoate	pineapple, pear	Isoamyl acetate	banana
Ethyl decanoate	floral	2-phenylethyl acetate	rose, fruity, flowery

Chemically the esterification is a condensation reaction between an alcohol and an acid. In yeast cells, the ester formation is an enzyme-catalysed reaction where the alcohol component (ethanol or higher alcohol) interacts with the coenzyme A (CoA)-activated acid component (medium-chain fatty

acid as acyl-CoA, or acetate as acetyl-CoA). The thioester linkage between CoA and the acid component provides energy for the reaction (Maicas and Mateo, 2005; Mendes-Ferreira *et al.*, 2011). Thus, ethyl esters formation results from the reaction of acyl-CoA compounds with ethanol, and acetate esters formation from the reaction of acetyl-CoA with ethanol or higher alcohols (Figure I.5). In *S. cerevisiae*, the ethyl ester formation has been attributed to two acyl-CoA:ethanol *O*-acyltransferases encoded by the *EEB1* and *EHT1* genes, whereas the formation of acetate esters has been shown to be catalysed by alcohol *O*-acetyltransferases (AATases) (Saerens *et al.*, 2006). For the purposes of this thesis, further attention will be paid only to the formation of acetate esters and the respective genes *ATF1* and *ATF2*.

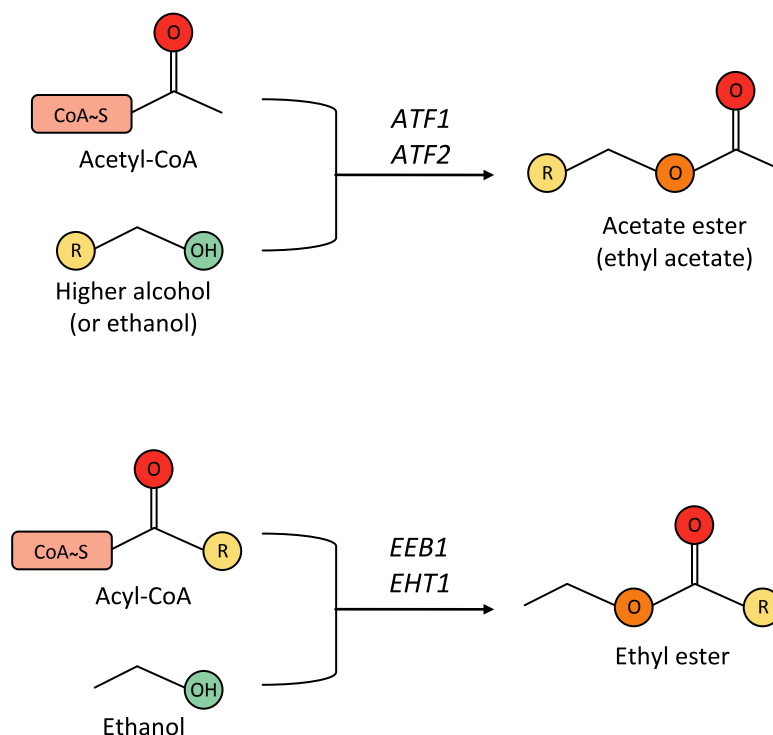

Figure I.5. Ester synthesis in *S. cerevisiae*.

Biosynthesis of acetate esters

Higher alcohols, the first co-substrate of alcohol acetyltransferase, are (as described) formed from the amino acids degradation. The other co-substrate, acetyl-CoA, can be formed either by oxidative decarboxylation of pyruvate or by direct activation of acetate with ATP (Mason and Dufour, 2000).

In *S. cerevisiae*, two genes which encode AATases, namely *ATF1* and *ATF2*, have so far been identified. The *ATF1* gene was first cloned and characterised by Fuji *et al.* (1994). The protein encoded by *ATF1* consists of 525 amino acids with a molecular weight of about 61 kDa. A few years later, Nagasawa *et al.* (1998) reported on cloning and characterisation of the second AATase encoded by *ATF2*. Similarly, the protein consists of 535 amino acids with a molecular weight of about 61 kDa. Sequence comparison between *ATF1* and *ATF2* showed 35% identity (Nagasawa *et al.*, 1998; van Laere *et al.*, 2008). Additionally, the comparison revealed a highly conserved heptapeptid region WRLICLP, which appears to be found only in the Atf proteins. Therefore, this conserved region has been hypothesised to be a part of the active site (Nagasawa *et al.*, 1998). Another conserved region is HXXXD, which was described as a characteristic motif of the yeast AATases as well as of plant AATase. In plant AATase, this motif has been shown to be the active site involved in CoA-dependent acyltransfer (D'Auria, 2006).

The AATases were first considered to be integral plasma membrane proteins. However, the data based on hydrophobicity analysis did not confirm any membrane-spanning regions in the Atf1p and Atf2p sequences. Further study performed by Verstrepen *et al.* (2004) revealed that the *ATF1*-derived enzyme is located in lipid particles. The localisation of Atf2p has so far not been conclusively determined. Data from subcellular fractionation suggest that Atf2p is mainly a soluble enzyme (Mason and Dufour, 2000).

Deletion and overexpression analysis has shown that in addition to the availability of the two co-substrates, the expression levels of *ATF1* and *ATF2*

genes also play very important role in the formation of aroma-active acetate esters. Overexpression of *ATF1* and *ATF2* results in an increase of acetate esters levels, particularly of isoamyl acetate, and ethyl acetate. Deletion of these genes, in contrast, leads to a significant decrease of the acetate esters concentrations (Lilly *et al.*, 2006; Verstrepen *et al.*, 2003b). Further characterisation has revealed that Atf1p and Atf2p in *S. cerevisiae* are able to transfer an activated acetate group to a wide variety of substrates with alcohol group and thus have broad substrate specificity for the alcohol cosubstrates. Mainly isoamyl alcohol but also other alcohols, such as ethanol, propanol, isobutanol, hexanol and 2-phenylethanol are esterified by Atf1p and Atf2p (Lilly *et al.*, 2006; Verstrepen *et al.*, 2003b). More precisely, the deletion of both, *ATF1* and *ATF2*, genes has been shown to result in totally abolished production of isoamyl acetate, pentyl acetate, hexyl acetate, or 2-phenylethyl acetate. On the other hand, the double deletion strain still produces significant amounts of ethyl acetate, propyl acetate, and isobutyl acetate. These data has led to hypothesis. First, Atf1p and Atf2p are the only enzymes involved in the synthesis of acetate esters from C_5- or longer-chain alcohols. Second, there is at least one other enzyme with AATase activity (Malcorps *et al.*, 1991; Verstrepen *et al.*, 2003b).

The amounts of acetate esters are also influenced by the activity of esterases, enzymes catalysing ester breakdown. In *S. cerevisiae*, the most studied ester-hydrolysing enzyme is Iah1p. The enzyme codified by *IAH1* gene was described as isoamyl acetate hydrolase (Fukuda *et al.*, 2000). However, Lilly *et al.* (2006) showed that Iah1p enzyme hydrolyses not only isoamyl acetate but also other acetate esters, such as 2-phenylethyl acetate and hexyl acetate. As described previously, the balance between ester-synthesizing and ester-degrading enzymes is important for the net rate of ester accumulation (Fukuda *et al.*, 1998).

Physiological role of aroma-active higher alcohols and acetate esters

Even if they are considered to be the oldest domesticated organisms, yeasts surely do not produce aroma-active higher alcohols and acetate esters (and other compounds) to make mankind happier. Although, one could raise an objection that as no research has disproved it, this hypothesis should not be rule out.

There are several (more serious) hypotheses which try to explain the physiological role of the higher alcohols production. Since the final step of a higher alcohol formation is the reduction of its respective aldehyde via NADH-dependent reaction, one hypothesis suggests that higher alcohols are formed to help tune the $NADH/NAD^+$ ratio and so the redox balance of the cell (Hazelwood *et al.*, 2008; van Dijken and Scheffers, 1986). Some authors, however, believe that the cell sufficiently maintain the redox balance via the aforementioned ethanol formation together with glycerol, acetate, acetaldehyde, and succinate metabolic pathways. Another hypothesis postulates that the physiological role of the higher alcohols is the removal of toxic aldehyde compounds or an alternative way how the cells obtain nitrogen (Styger *et al.*, 2011a).

There have been hypothesised various theories for the physiological relevance of acetate ester formation. The most interesting and relevant hypotheses are described in an extensive review published by Saerens *et al.* (2010). One hypothesis suggests the ester formation as a detoxification mechanism. Based on the relative contributions of Atf1p and Atf2p to total AATase activity, *ATF1*-encoded enzyme seems to play the major role in acetate ester formation (Lilly *et al.*, 2006; Verstrepen *et al.*, 2003b). It has been hypothesised that Atf2p might be important for different metabolic processes than Atf1p. For instance, Cauet *et al.* (1999) showed that Atf2p is highly important for acetylation, and thus detoxification, of 3β-hydroxysteroids, such

as pregnenolone.

Another hypothesis is that Atf1p has a specific role in fatty acid metabolism esters or that esterification catalysed by Atf1p is necessary to generate some specific lipid compounds needed under anaerobiosis. This is supported by the fact that the *ATF1* gene is repressed by trace amounts of oxygen and unsaturated fatty acids. Furthermore, since the synthesis of unsaturated fatty acids under anaerobic conditions is impaired, some esters could serve as analogues of unsaturated fatty acids and therefore might help maintain plasma membrane fluidity in low-oxygen conditions.

Last, but definitely not least, there is very interesting hypothesis that suggests ester formation as a mean for the dispersion of yeast in nature. It has been previously demonstrated that yeasts depend on insect vectors for their dispersal. To prove this hypothesis, Christiaens *et al.* (2014) have recently performed experiments to investigate whether acetate esters produced by *S. cerevisiae* help attract *Drosophila melanogaster*. The results have shown that *ATF1* gene expression and the production of acetate esters lead to an increased attraction of the fly to yeast (Figure 6). The authors, however, conclude that it is difficult to show that the yeast *ATF1* gene specifically evolved to stimulate the production of aroma compounds with the aim of attracting insects. On the other hand, several data support this theory. After some time, when nutrient levels are running low, the yeasts need to reach new environments. Under the nutrient-limiting conditions, the centre of yeast colonies is expected to be a hypoxic environment (Cap *et al.*, 2012) which may increase *ATF1* expression (Fujii *et al.*, 1997) and thus acetate ester formation (Lilly *et al.*, 2006; Malcorps and Dufour, 1992; Verstrepen *et al.*, 2003b). The production of aroma compounds then could help attract flies. Whereas some of the yeast cells are consumed by the insects, a fraction of cells will stick to the fly body and get dispersed to a different environment (Christiaens *et al.*, 2014).

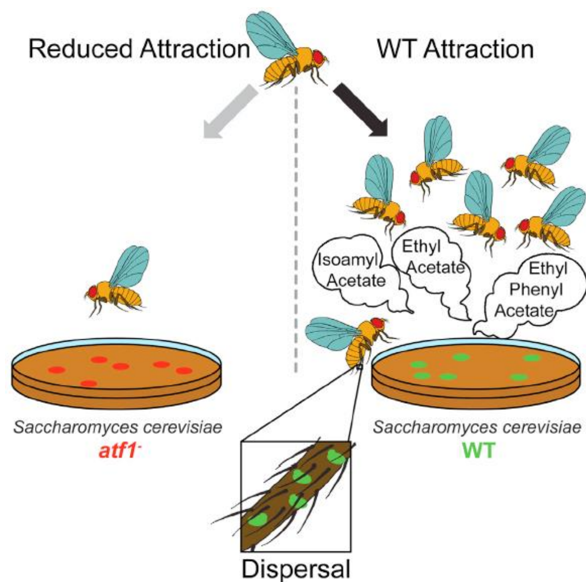

Figure 6. Graphical description of *S. cerevisiae* dispersion by the fruit flies *Drosophila melanogaster*. Christiaens *et al*. (2014) show that two acetate esters, ethyl acetate and isoamyl acetate, promote attraction of the flies. Deletion of the yeast *ATF1* gene drastically reduces *Drosophila* attraction and therefore limits yeast dispersal.

This hypothesis was further supported by the observation that the most commonly used *S. cerevisiae* laboratory strains show significantly lower production of aroma compounds compared to their wild and industrial relatives (Verstrepen *et al*., 2003b) and therefore the synthesis of these compounds has not been selected for under laboratory culture conditions and might rather be related to survival in complex natural environments (Christiaens *et al*., 2014).

In summary, as the number of stated hypothesis indicates, the production of the higher alcohols and esters might play several different roles. Nevertheless, the last hypothesis leaves open a possibility that yeast actually produce higher alcohols and esters just for their aromatic properties (even though with no intention to improve the taste of our beer or wine).

Background & Scope

It has been already mentioned in Preface that the work described in this thesis was part of the international project Cornucopia. More detailed background regarding the scope of the work arises from the fact that this was performed at the Institute of Agrochemistry and Food Technology (IATA-CSIC) in Valencia. More precisely, the work was performed within the research group headed by Prof. Amparo Querol. The general topic of this group aims yeasts with industrially relevant (especially wine-industry-relevant) properties. As evident from previous paragraphs, one of the group's interests is focused on a biotechnological potential of *S. kudriavzevii*, *S. uvarum* and their hybrids with *S. cerevisiae*. In the last years, extensive investigation has been carried out in order to characterise the fermentation capacities of these species under different conditions, such as different fermentation temperatures or different wine must (including natural and synthetic musts) (Arroyo-Lopez *et al.*, 2009; Arroyo-Lopez *et al.*, 2010; Arroyo-Lopez *et al.*, 2011; Gamero *et al.*, 2011; Gamero *et al.*, 2010; Gonzalez *et al.*, 2006; Gonzalez *et al.*, 2007; Pérez-Torrado *et al.*, 2015; Tronchoni *et al.*, 2009). Subsequent studies aimed to understand the molecular aspects behind several traits of interest (Combina *et al.*, 2012; Lopez-Malo *et al.*, 2013; Oliveira *et al.*, 2014; Peris *et al.*, 2012; Tronchoni *et al.*, 2014; Tronchoni *et al.*, 2012).

Broad analyses have been also performed concerning aroma and flavour production by these *Saccharomyces* species and their hybrids. Studies published by Gamero *et al.* (2013) and Pérez-Torrado *et al.* (2015) revealed remarkable differences in the production of higher alcohols and esters, the key components of overall flavour and aroma in the fermented products, when comparing the three species, *S. cerevisiae*, *S. kudriavzevii*, and *S. uvarum*. Furthermore, expression analysis performed by Gamero *et al.* (2014), showed significantly different expression levels of the genes involved in flavour compounds production by the three *Saccharomyces* species.

In order to shed more light on these differences, the main objective of this thesis was to explore the molecular bases underneath. As discussed above, the prime aroma-active acetate esters derive from higher alcohols and these derive from the corresponding branched-chain amino acids. In this context, in Chapter 1 several amino acids were used as the sole nitrogen source and the production of the appropriate higher alcohols and esters was analysed and compared among the three species.

Based on the results from Chapter 1, which indicated differences in the catabolism of aroma-related amino acids, Chapter 2 describes *in silico* comparative analysis of the enzymes involved in the branched-chain amino acids catabolism in *S. cerevesiae*, *S. kudriavzevii* and *S. uvarum*. To evaluate the severity of amino acid replacement in the orthologues enzymes, we applied Grantham scoring which quantitatively evaluates biochemical dissimilarity between amino acid side chain properties. The best hits released in the bioinformatic analysis were Aro10p, Atf1p and Atf2p.

The impact on higher alcohols and esters production, and the enzymatic properties of the enzymes encoded by *ARO10*, *ATF1* and *ATF2* alleles from *S. kudriavzevii* and *S. uvarum* were then tested in comparative analyses described in Chapter 3 and Chapter 4.

Materials & Methods

Culture media

Stock cultures were grown on standard complex or SC-Ura medium. Solid media were prepared by adding of 2% agar. Cultivations to study the production of the higher alcohols and acetate esters that derived from the corresponding amino acids were carried out in Synthetic medium supplemented by the appropriate amino acid or ammonium sulphate as the nitrogen source according to Bolat *et al.,* (2013) (Table M.1).

Table M.1. Culture media and their composition

Medium	Composition	
Standard complex medium	0.5% peptone	
	2% glucose	
	0.5% yeast extract	
SC-Ura medium	6.7% YNB	
	2% glucose	
	1.92 g/L Drop-out –Ura (Formedium, Norfolk, UK	
Synthetic medium	0.17% YNB w/o AAs & $(NH_4)_2SO_4$ (BD DIFCO™, Madrid, Spain)	
	2% glucose	
Nitrogen source*	$(NH_4)_2SO_4$	5 g/L
	leucine	10 g/L
	isoleucine	10 g/L
	phenylalanine	12.5 g/L
	valine	8.9 g/L

* Synthetic medium was supplemented with stated nitrogen sources either individually or as a mixture depending on the assay. Detailed description is provided in Cultivation to study the production of the higher alcohols and acetate esters that derived from the corresponding amino acids (page 57).

Micro-vinifications were performed in Synthetic wine must, which reproduces standard natural must composition and is very useful for reproducibility. The synthetic wine must was prepared according to Riou *et al.*, (1997) with small modifications according to Beltran *et al.*, (2004) as follows:

Synthetic wine must	Composition per 1L		
Sugars		Minerals	
Glucose	100 g	KH_2PO_4	0.75 g
Fructose	100 g	K_2SO_4	0.5 g
Organic acids		$MgSO_4.7H_2O$	0.25 g
Malic	5 g	$CaCl_2.7H_2O$	0.155 g
Citric	0.5 g	NaCl	0.2 g
Tartaric	3 g	NH_4Cl	0.46 g

The solution was sterilised at 121º C for 20 minutes. Subsequently, previously prepared stock solutions were added:

Amino acids	13.09 mL
Oligoelements	1 mL
Vitamins	10 mL

The final pH was adjusted to 3.3 with NaOH and the final synthetic wine must was filtered using an antimicrobial filter.

The composition of stock solutions was as follows:

Oligoelements (1 L)		Vitamins (1 L)	
$MnSO_4.H_2O$	4 g	Myo-inositol	2 g
$ZnSO_4.7H_2O$	4 g	Calcium pantothenate	15 g
$CuSO_4.5H_2O$	1 g	Thiamine HCl	0.025 g
KI	1 g	Nicotinic acid	0.2 g
$CoCl_2.6H_2O$	0.4 g	Pyridoxine	0.025 g
H_3BO_3	1 g	Biotin (100 mg/L)	3 mL
$(NH_4)_6Mo_7O_{24}$	1 g		

Amino acids (1 L):

Tyrosine	1.5g	Alanine	11.2g
Tryptophan	13.4g	Valine	3.4g
Isoleucine	2.5g	Methionine	2.4g
Aspartic acid	3.4g	Phenylalanine	2.9g
Glutamic acid	9.2g	Serine	6 g
Arginine	28.3g	Histidine	2.6g
Leucine	3.7g	Lysine	1.3g
Threonine	5.8g	Cysteine	1.5g
Glycine	1.4g	Proline	46.1g
Glutamine	38.4g		

Yeast strains

The yeast strains used in this dissertation are listed in Table M.2. *S.cerevisiae* Ta, a haploid strain that derives from commercial wine strain T73, was used as a host for cloning of *ARO10*, *ATF1* and *ATF2* alleles from *S. kudriavzevii* and *S. uvarum*. The JET01- and CEN.PK-indicated strains were used in the *ARO10*-related study which is discussed in Chapter 3. The strains indicated as JET02 and JET03, together with CLp-indicated strains, were used in the *ATF1*- and *ATF2*-related work which is described in Chapter 4.

The strategy of replacing endogenous *ARO10*, *ATF1* and *ATF2* in the Ta genome with the corresponding *ARO10* and *ATF* alleles from *S. kudriavzevii* or *S. uvarum* involved two steps i) deletion of either *ARO10, ATF1* or *ATF2* gene and ii) integration of *S. kudriavzevii* or *S. uvarum* orthologues into the locus. The genes deletions in the Ta genome were performed by integrating a nourseothricin resistance cassette by homologous recombination. Deletion cassettes were amplified using pAG25 (Goldstein and McCusker, 1999) as a template and specific primers (Table M.4). The resulting strains were named JET01 (*aro10Δ*), JET02 (*atf1Δ*) and JET03 (*atf2Δ*).

The integration of *SkARO10* into JET01 is described to illustrate the second step. The integration cassette was amplified from plasmid pG-SkARO10-kX with primers pGSkARO10f and pG-ARO10-R. The resulting PCR fragment included the *SkARO10* allele, followed by a kanamycin resistance marker, which was used in the subsequent transformation of the JET01 strain. The final Ta mutant that held the *SkARO10* allele was named JET01Sk. The same procedure was carried out with *ScARO10*, which resulted in the restoration of the endogenous allele by the undergone process. This strain, named JET01Sc, was used as a reference in the assays.

Table M.2. Yeast strains

Strain	Description	Reference
T73	*S. cerevisiae*, Wine strain, Alicante, Spain	Querol *et al*. (1992)
IFO1802	*S. kudriavzevii*, Type strain, NCBI	Kaneko and Banno (1991)
CECT12600	*S. uvarum*, Wine strain, Alicante, Spain	Spanish Culture Collection (CECT)
Ta	T73*hoΔ::loxP*	A. Querol
JET01	Ta *aro10Δ::NAT1*	This study
JET01Sk	Ta *aro10Δ::SkARO10-kX*	This study
JET01Su	Ta *aro10Δ::SuARO10-kX*	This study
JET01Sc	Ta *aro10Δ::ScARO10-kX*	This study
JET02	Ta *atf1Δ::NAT1*	This study
JET02Sk	Ta *atf1Δ::SkATF1-kX*	This study
JET02Su	Ta *atf1Δ::SuATF1-kX*	This study
JET02Sc	Ta *atf1Δ::ScATF1-kX*	This study
JET03	Ta *atf2Δ::NAT1*	This study
JET03Sk	Ta *atf2Δ::SkATF2-kX*	This study
JET03Su	Ta *atf2Δ::SuATF2-kX*	This study
JET03Sc	Ta *atf2Δ::SuATF2-kX*	This study
CEN.PK 711-7C	*MATa ura3-52 pdc1Δ pdc5Δ pdc6Δ aro10Δ thi3Δ*	Vuralhan *et al*., (2005)
CEN.PKpSkARO10	CEN.PK 711-7C pG-SkARO10-kX	This study
CEN.PKpSuARO10	CEN.PK 711-7C pG-SuARO10-kX	This study
CEN.PKpScARO10	CEN.PK 711-7C pG-ScARO10-kX	This study
BY4741atf1atf2iah1	*MAT*a *leu2-Δ0 his3-Δ*1 *met15-Δ0 ura3-Δ0 atf1Δ1*::loxP *atf2Δ1*::loxP *iah1Δ1*::loxP	Uber-García (2005)
CLpSkATF1	BY4741atf1atf2iah1 pG-SkATF1-TDH3p	This study
CLpSuATF1	BY4741atf1atf2iah1 pG-SuATF1-TDH3p	This study
CLpScATF1	BY4741atf1atf2iah1 pG-ScATF1-TDH3p	This study

Strain	Description	Reference
CLpSkATF2	BY4741atf1atf2iah1 pG-SkATF2-TDH3p	This study
CLpSuATF2	BY4741atf1atf2iah1 pG-SuATF2-TDH3p	This study
CLpScATF2	BY4741atf1atf2iah1 pG-ScATF2-TDH3p	This study

Plasmids

The *S. kudriavzevii ARO10*, *ATF1* and *ATF2* alleles (*SkARO10, SkATF1* and *SkATF2*) were amplified from the genomic DNA of *S. kudriavzevii* IFO1802. The *S. uvarum ARO10, ATF1* and *ATF2* alleles (*SuARO10, SuATF1* and *SuATF2*) were amplified from the genomic DNA of *S. uvarum* CECT12600. The *S. cerevisiae ARO10, ATF1* and *ATF2* alleles (*ScARO10, ScATF1* and *ScATF2*) were amplified by the PCR from the genomic DNA of *S. cerevisiae* T73. The primers that we used are listed in Table M.4. PCR fragments were then independently cloned into the pGREG526 vector (Jansen *et al.*, 2005), previously cut with NotI/SalI. The constructed plasmids that held the individual *ARO10* alleles were then introduced into the CEN.PK711-7C strain (Table M.2) used in Aro10 enzyme assays.

In order to increase expression levels of the *ATF* genes, before the start codon of the individual *ATF* genes within the pGREG526 vectors, the *TDH3* promoter (amplified by the PCR from the genomic DNA from the S288C strain) was inserted by homologous recombination. The resulting plasmids are listed in Table M.3. The constructed plasmids that contained *ATF* alleles behind the *TDH3* promoter were then introduced into the BY4741atf1atf2iah1 strain (Table M.2) used in Atf1 and Atf2 enzyme assays.

Table M.3. Plasmids used in this thesis

Plasmid name	Description	Reference
pGREG526	*URA3*, *kanMX*, *AmpR*	Jansen *et al.* (2005)
pGREG526 containing *ARO10* and *ATF* orthologues		
pG-SkARO10-kX	containing *S. kudriavzevii ARO10*	This study
pG-SuARO10-kX	containing *S. uvarum ARO10*	This study
pG-ScARO10-kX	containing *S. cerevisiae ARO10*	This study
pG-SkATF1-kX	containing *S. kudriavzevii ATF1*	This study
pG-SuATF1-kX	containing *S. uvarum ATF1*	This study
pG-ScATF1-kX	containing *S. cerevisiae ATF1*	This study
pG-SkATF2-kX	containing *S. kudriavzevii ATF2*	This study
pG-SuATF2-kX	containing *S. uvarum ATF2*	This study
pG-ScATF2-kX	containing *S. cerevisiae ATF2*	This study
pGREG526 harbouring *ATF* orthologues with *TDH3* promoter		
pG-SkATF1-TDH3p	containing *S. kudriavzevii ATF1*	This study
pG-SuATF1-TDH3p	containing *S. uvarum ATF1*	This study
pG-ScATF1-TDH3p	containing *S. cerevisiae ATF1*	This study
pG-SkATF2-TDH3p	containing *S. kudriavzevii ATF2*	This study
pG-SuATF2-TDH3p	containing *S. uvarum ATF2*	This study
pG-ScATF2-TDH3p	containing *S. cerevisiae ATF2*	This study

Primers and PCR

Primers listed in Table M.4 were used in standard PCR which were carried out in thermocycler Mastercycler (Eppendorf, Madrid, Spain). Depending on the result accuracy, two different polymerases were used in the reactions. During the construction of deletion cassettes Taq DNA polymerase (ThermoFischer, Madrid, Spain) was used. Integration cassettes for gene cloning were constructed with Phusion DNA polymerase (ThermoFischer,

Madrid, Spain) which has low error rates. The PCR conditions were set up according to provider's recommendations and according to primers' T_m values.

Table M.4. Primers

Primer	Sequence 5' – 3´
Cloning into pGREG526	
SkARO10-aF	CCTAGTACGGATTAGAAGCCGCCGAGCGGGTGACAACTTTTGATTT GTTCCCCGC
SkARO10-aR	GCGTGACATAACTAATTACATGACTCGAGGTCGACAAAGACAAAAT CGGCGGC
SuARO10-F	CCTAGTACGGATTAGAAGCCGCCGAGCGGGTGACATTCGGTTGCCT CGTATAGC
SuARO10-R	GCGTGACATAACTAATTACATGACTCGAGGTCGACCCATAGCTGAG ACGTTTTGC
ScARO10-F	CCTAGTACGGATTAGAAGCCGCCGAGCGGGTGACAATCTCTTAGGC ATGCTCTTGG
ScARO10-R	GCGTGACATAACTAATTACATGACTCGAGGTCGACTATAATTGCGCC CACAAGTTTC
SkATF1-F	CCTAGTACGGATTAGAAGCCGCCGAGCGGGTGACATCTTCAAAAGC CTCCTCATAC
SkATF1-R	GCGTGACATAACTAATTACATGACTCGAGGTCGACGCCTAAGGAAT GACGATACC
SuATF1-F	CCTAGTACGGATTAGAAGCCGCCGAGCGGGTGACAACAAAACCATA ACCGAATACG
SuATF1-R	GCGTGACATAACTAATTACATGACTCGAGGTCGACCGGCTAAAAGA ACGATACAA
ScATF1-F	CCTAGTACGGATTAGAAGCCGCCGAGCGGGTGACAGGTACTCATCG TAAAAGATTGC
ScATF1-R	GCGTGACATAACTAATTACATGACTCGAGGTCGACTAACCAACCAA AGCCGAG
SkATF2-F	CCTAGTACGGATTAGAAGCCGCCGAGCGGGTGACATTATCACCAGA CGGCTCAC
SkATF2-R	GCGTGACATAACTAATTACATGACTCGAGGTCGACGCTCTGTCCGAT ACACCG
SuATF2-F	CCTAGTACGGATTAGAAGCCGCCGAGCGGGTGACAATCACCAAAGT AACCACCAT

Primer	Sequence 5′ – 3´
SuATF2-R	GCGTGACATAACTAATTACATGACTCGAGGTCGACATACCGCTTCCT TGCTGT
ScATF2-F	CCTAGTACGGATTAGAAGCCGCCGAGCGGGTGACAAGGAAGCACG TCAGAAAAAG
ScATF2-R	GCGTGACATAACTAATTACATGACTCGAGGTCGACGCTCTGTCCGAT ACACTGC
ARO10 deletion cassette	
TaARO10-NAT1-F	ATGGCACCTGTTACAATTGAAAAGTTCGTAAATCAAGAAGGGTGTTT AGGTCGATGCCATC
TaARO10-NAT1-R	CTATTTTTTATTTCTTTTAAGTGCCGCTGCTTCAACCATGGGATGGCG GCGTTAGTATCG
ATF1/ATF2 deletion cassettes	
TaATF1-NAT1-F	ATGAATGAAATCGATGAGAAAAATCAGGCGCCCGTGCAACGGTGTT TAGGTCGATGCCATC
TaATF1-NAT1-R	CTAAGGGCCTAAAAGGAGAGCTTTATAAATGGAGCAAAGCGGATG GCGGCGTTAGTATCG
TaATF2-NAT1-F	ATGGAAGATATAGAAGGATACGAACCACATATCACTCAAGGGTGTT TAGGTCGATGCCATC
TaATF2-NAT1-R	TTAAAGCGACGCAAATTCGCCGATGGTTTGGTAGAAGAGCGGATG GCGGCGTTAGTATCG
Integration fragments	
pGSkARO10f	TAAAGTTTATTTACAAGATAACAAAGAAACTCCCTTAAGCATGACGC CTGTTACAATTAA
pGSuARO10f	TAAAGTTTATTTACAAGATAACAAAGAAACTCCCTTAAGCATGGCAC CTGTTACGATTGA
pGScARO10f	TAAAGTTTATTTACAAGATAAC
pG-ARO10-R	ACAATTGGTAGCAGTGTTTTATAATTGCGCCCACAAGTTTCTCACTAT AGGGCGAATTGG
pGSkATF1f	CTTCATCAGTATCACAAATACCATCAATTTATCAGCTCTCATGACTAA AATCAGCGAAGAG
pGSuATF1f	CTTCATCAGTATCACAAATACCATCAATTTATCAGCTCTCATGAATAC CTATAGTGAAAA
pGScATF1f	CTTCATCAGTATCACAAATAC
pG-ATF1-R	TCATATTGTCGAATAATATCAGTCAAGCATCATGTGAGATCTCACTA TAGGGCGAATTGG
pGSkATF2f	CGAATAATAACTTCAGCAATAAAAATTGTCCAGGTTAATTATGGATG ATATAGAGGAATAC

Primer	Sequence 5′ – 3′
pGSuATF2f	CGAATAATAACTTCAGCAATAAAAATTGTCCAGGTTAATTATGGATA GTTTAGAGGAATAC
pGScATF2f	CGAATAATAACTTCAGCAAT
pG-ATF2-R	TCGGCCGAGCTATACGAAGGCCCGCTACGGCAGTATCGCACTCACT ATAGGGCGAATTGG
Integration of *TDH3* promoter	
pG526-TDH3p-Fa	GAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCAGTTC GAGTTTATCATTATC
pG-TDH3p-CF1-R	CTGATAAATTGATGGTATTTGTGATACTGATGAAGCGAAACTAAGTT CTTGGTGTT
pG-TDH3p-KF1-R	GGTAGATTGATGATATTTGTTATACTGATGAGTGGCGAAACTAAGTT CTTGGTGTT
pG-TDH3p-UF1-R	GTTGGATTGATGGTTTTGTTAAACTGCTAAATTGGCGAAACTAAGTT CTTGGTGTT
pG-TDH3p-CF2-R	CCTGGACAATTTTTATTGCTGAAGTTATTATTCGTCGAAACTAAGTTC TTGGTGTT
pG-TDH3p-KF2-R	CGCAATTTTTTGTTCGTTTGAAGATCTAGTAGAGCCGAAACTAAGTT CTTGGTGTT
pG-TDH3p-UF2-R	GCAGTTTTTTGTTCTGAAACCTATTGTTCGTTTGTCGAAACTAAGTTC TTGGTGTT
Diagnostic	
T73AR10-UF	ATCTCTTAGGCATGCTCTTGG
T73ATF1-UF	GGTACTCATCGTAAAAGATTGC
T73ATF2-UF	AGGAAGCACGTCAGAAAAAG
K2	GGGACAATTCAACGCGTCTG
K3	CCTCGACATCATCTGCCC
SkARO10-R1	CATTGGAAACAAGGTGCGG
SuARO10-R1	CATGCGGTTCGTCTTGGTC
ScARO10-R1	GAAGTCACCAGGAACACCG
SkATF1-R1	GCCTAAGGAATGACGATACC
SuATF1-R1	CGGCTAAAAGAACGATACAA
ScATF1-R1	TTTGTGATACTGATGAAGTGCCG
SkATF2-R1	GCTCTGTCCGATACACCG
SuATF2-R1	ATACCGCTTCCTTGCTGT
ScATF2-R1	GCTCTGTCCGATACACTGC

Molecular techniques

DNA extraction

The extraction of yeast DNA was carried out from 3 mL overnight culture in standard complex medium at 30°C, using the method described by Querol *et al.* (1992). 1.5 mL of the cell culture was centrifuged at 10,000 rpm for 2 min and the medium was removed. The cell pellet was washed with 1 mL of distilled water and centrifuged at 10,000 rpm for 2 min to remove the water. 0.5 mL of Buffer 1 (sorbitol 0.9 M, EDTA 0.1 M pH 7.5) and 30 µL of Zymolyase (Seikagaku Corporation, Japan) were added. The samples were vortexed and the tubes were incubated at 37°C for 20 min. Samples were centrifuged at 10,000 rpm for 2 min to remove the supernatant. Protoplasts were re-suspended in 0.5 mL of Buffer 2 (Tris 50 mM pH 7.4, EDTA 20 mM). Afterwards, 13 µL of SDS 10% was added and the tubes were incubated at 65°C for 5 min. After the incubation, 0.2 mL of potassium acetate was added, the samples were mixed and incubated in ice for 5 min. Samples were centrifuged at 12,000 rpm at 4°C for 15 min to ensure the elimination of SDS. Supernatant was added to 0.7 mL of isopropanol (v/v) and incubated at room temperature for 5 min. Samples were centrifuged at 12,000 rpm at 4°C for 10 min. Supernatant was removed and 0.5 mL of ethanol 70% was added. Samples were centrifuged at 12,000 rpm at 4°C for 5 min. Supernatant was removed and samples were dried with a vacuum pump. Finally, DNA was resuspended in 40 µL of TE (Tris 10 mM ph 7.4, EDTA 1 mM pH 8.0).

For the yeast plasmid extraction a modified protocol described by Robzyk and Kassir (1992) was used. Cells were grown in 5 mL overnight culture in a selective SC-Ura medium in order to maintain the plasmid. 1.5 mL of the culture was transferred to new tube and centrifuged at 6,000 rpm for 5 min. Supernatant was removed and cells were resuspended in 100 µL of STET (8% sucrose, 5% Triton X-100, 50 mM Tris-HCl pH 8, 50 mM EDTA). 0.2 g glass

beads (0.45 mm) were added, and then samples were mixed with a mechanic shaker Mini Beadbeater-8 (BioSpec Products, USA) during 5 min, 30 sec shaking /30 sec incubating in ice cycles. Another 100 µL of STET were added, samples were mixed briefly and were incubated in boiling water for 3 min. The samples were cooled in ice and then were centrifuged at 12,000 rpm at 4°C for 10 min. 100 µL of the supernatant were transferred to new tube and 50 µL of ammonium acetate 7.5 M was added. Samples were centrifuged at 12,000 rpm at 4°C for 10 min. 100 µL of supernatant was added to 200 µL of absolute ethanol, and then it was precipitated at -20°C for 1h. Samples were centrifuged at 12,000 rpm at 4°C for 15 min. Pellets were washed with ethanol 70% and samples were centrifuged at 12,000 rpm at 4°C for 5 min. Supernatant was removed and samples were dried with a vacuum pump. Finally, DNA plasmid was resuspended with 20 µL distilled water.

S. cerevisiae transformation

Transformation was performed using a lithium acetate protocol described by Gietz and Woods (2002). Yeast cells were inoculated in 50 mL of standard complex medium and cultivated overnight at 30°C and 200 rpm. This culture was used to inoculate an OD_{600} of 0.2 in 50 mL standard complex medium and was incubated at 30°C and 200 rpm until the cells were completed at least 3 divisions (OD_{600} approximately 0.8). Then 5 mL culture was transferred to a new tube and centrifuged at 5,000 rpm for 5 min. The media was poured off and cells were resuspended in 2.5 mL of sterile water and centrifuged again. The water was removed and cells were resuspended in 100 µL of 0.1 M LiAc. Cells were centrifuged at 12,000 rpm for 15 sec and LiAc was removed with a micropipette. Cells were resuspended in 40 µL of 0.1 M LiAc, centrifuged and LiAc was removed again with a micropipette. Then transformation mix was added in this order: 240 µL of PEG (50% w/v), 36 µL of 1 M LiAc, 50 µL of ss-DNA 2 mg/mL (previously boiled for 5 min and quickly chilled in ice), and 34 µL of DNA fragment. Each tube was vigorously vortexed until the cell pellet

had been completely mixed. Tubes were incubated at 42°C for 40 min. Tubes were centrifuged at 3,000 rpm for 15 sec and the transformation mix was removed with a micropipette. Into each tube 1 mL of water was added and pellet was resuspended by pipetting it up and down gently. Then tubes were centrifuged at 3,000 rpm for 15 sec and water was removed. Pellet was resuspended in 500 µL of standard complex medium and incubated at 30°C for 3 h with a gentle shaking at 125 rpm. 200 µL (twice for each transformation) were plated onto selection plates (standard complex medium with Geneticin G418, 0.2 g/L, or SC-Ura medium) and incubated at 30 ºC until colonies appeared (2-3 days).

Analytical methods

Cultivation to study the production of the higher alcohols and acetate esters that derived from the corresponding amino acids

Cultivations were performed in Synthetic medium (described in Table M.1). Media were supplemented by different nitrogen sources. The concentration was 5 g/L when $(NH_4)_2SO_4$ was used as the nitrogen source. When individual amino acids were used as the nitrogen source, the concentrations were proportional to $(NH_4)_2SO_4$ (to obtain the same nitrogen content), as follows: 10 g/L leucine, 10 g/L isoleucine, 12.5 g/L phenylalanine, 8.9 g/L valine (Bolat *et al.*, 2013). The mix of these amino acids was also used as the nitrogen source. In this case the total amino acids concentration was 10 g/L and the proportional concentrations were 2.5 g/L leucine, 2.5 g/L isoleucine, 3 g/L phenylalanine and 2 g/L valine.

Starter cultures were prepared by pregrowing yeast in 15-mL tubes that contained 4 mL of the standard complex media. Before inoculating the experimental culture, the grown precultures were washed with water and resuspended in the same synthetic medium (with a certain nitrogen source), as used in the assay. Cells were resuspended in a volume that allowed an OD_{600}

of 1.7 to be achieved. These precultures (100 µL) were used to inoculate 1.6 mL of the synthetic media. At this stage the initial OD_{600} was 0.1. Cultivation was performed in 96-well plates with 2mL-deep wells. Wells were covered by transparent microplate sealer (Greiner bio-one, Germany) to avoid evaporation and loss of volatile flavour compounds. Cultures were incubated for 5 days at 25°C. The individual 1.7-mL cultures were later transferred to 2-mL tubes and were stored at -20°C for the analysis.

Yeast growth analysis

Yeast cells growth was followed using a 96-well plate. Synthetic media were supplemented with the amino acids as described above. Then 100 µL of media were inoculated in a well with 2 µL of cell suspension with $OD_{600} = 1$. Growth was monitored in a Spectrostar Nano absorbance reader (BMG Labtech, Ortenbert, Germany).

Synthetic wine must fermentation

Fermentations were performed in 250-mL glass bottles that contained 200 mL of synthetic wine must (the composition is described in Culture media section). Fermentations were done in triplicate at 25°C with continuous orbital shaking (150 rpm). Flasks were closed with Müller valves and monitored by decreasing weight caused by CO_2 release until a constant weight was obtained. Immediately after fermentation ended, yeast cells were removed by centrifugation and the contents of higher alcohols and esters in the supernatants were analysed by gas chromatography.

Determination of higher alcohols and esters

The samples stored in the 2-mL tubes were centrifuged (13,000 rpm, 2 min) and 1.5 mL of the supernatant was transferred to the 15-mL vials with 0.35 g of NaCl. The 20-µl volume of 2-heptanone (0.005%) was added as an internal standard. Higher alcohols and esters were analysed by the headspace

solid phase microextraction (HS-SPME) technique with a 100-µm poly-dimethylsiloxane (PDMS) fibre (Supelco, Sigma-Aldrich, Madrid, Spain). Solutions were maintained for 2 h at 25°C to establish the headspace-liquid equilibrium. The fibre was inserted into the headspace through a vial septum and was held for 7 min. The fibre was then inserted into the gas chromatograph inlet port for 4 min at 220°C with helium flow (1 mL/min) to desorb analytes. A Thermo Science TRACE GC Ultra gas chromatograph with a flame ionization detector (FID) was used, equipped with an HP INNOWax 30 m x 0.25 m capillary column coated with a 0.25-m layer of cross-linked polyethylene glycol (Agilent Technologies, Valencia, Spain). The oven temperature programme was: 5 min at 35°C, 2°C/min to 150°C, 20°C/min to 250°C and 2 min at 250°C. The detector temperature was kept constant at 300°C. A chromatographic signal was recorded by the ChromQuest programme. Volatile compounds were identified by the retention time for reference compounds. Quantification of the volatile compounds was determined using the calibration graphs of the corresponding standard volatile compounds.

Statistical analysis

The presented values are averages of biological triplicates with standard errors. The differences between the measured volatile compounds were determined by a one-way ANOVA, followed by Tukey's HSD test (statistical level of significance was set at $P \leq 0.05$). The analysis was performed using the STATISTICA 7.0 software (StatSoft, Inc., Tulsa, OK, USA).

Enzyme assays

2-keto acid decarboxylase assays (Aro10p)

The chemostat cultivation, preparation of cell extracts and 2-keto acid decarboxylase enzyme assays were performed as previously described by (Romagnoli *et al.*, 2012).

Chemostat culture cultivations

S. cerevisiae strains were grown in aerobic ethanol-limited chemostat cultures on a synthetic medium containing, per litre of demineralised water: 10 g phenylalanine, 3 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 5 g K_2SO_4, 5.7 g of ethanol, 1 mL of trace element solution, 1 ml of vitamin solution and 8% of antifoam-C emulsion (Sigma-Aldrich, Zwijndrecht, The Netherlands). Trace element and vitamin solutions were prepared as described previously (Verduyn *et al.*, 1992). Chemostat cultivation was performed in 2L bioreactors (Applikon, Schiedam, The Netherlands), with a working volume of 1L and a dilution rate of 0.05 h^{-1}, as described previously by Vuralhan *et al.*, (2005). Chemostat cultures were assumed to be in steady state after at least 5 volume changes and when culture dry weight and off gas CO_2 analyses differed by less than 2 % over two consecutive volume changes.

Cell extract preparation

For the preparation of cell extracts of *S. cerevisiae*, culture samples were harvested by centrifugation, washed twice with 10 mM potassium phosphate buffer (pH 7.5) containing 2 mM EDTA, and stored at -20°C. Before cell breakage, the samples were thawed at room temperature, washed, and resuspended in 100 mM potassium phosphate buffer (pH 7.5) containing 2 mM MgCl2 and 2 mM dithiothreitol. Extracts were prepared by sonication with 0.7-mm glass beads at 0°C for 2 min at 0.5 min intervals with an MSE sonicator (150 W output, 8 μm peak-to-peak amplitude). Unbroken cells and

debris were removed by centrifugation at 4°C (20 min at 36,000 x g). The purified cell extract was then used for enzyme assays. The protein concentrations in cell extracts were estimated by the Lowry method (Lowry *et al.*, 1951) where bovine serum albumin was used as a standard.

2-keto acid decarboxylase activity

Enzyme activity was measured at 30°C by coupling two reactions: i) decarboxylation of 2-keto acid to the corresponding aldehyde catalysed by the phenylpyruvate decarboxylase and ii) aldehyde oxidation coupled with the reduction of NAD^+ to NADH by an added excess of yeast aldehyde dehydrogenase. The increase in absorption was measured with spectrophotometer Tecan GENios Pro (Tecan, Giessen, The Netherlands) at 340 nm (ε_{NADH}=6.3 mM^{-1}). The assay mixture, in a total volume of 300 µL, contained 100 mM potassium phospahte buffer (pH 7.0), 2 mM NAD^+, 5 mM MgCl2, 15 mM pyrazole, 0.2 mM thiamine diphosphate, and 1.75 $U.mL^{-1}$ of aldehyde dehydrogenase from yeast (Sigma-Aldrich, Zwijndrecht, The Netherlands) dissolved in 1 mM dithiothreitol. The reaction was started by the addition of one of the following substrates: 5 mM phenylpyruvate, 10 mM ketoisocaproate, 10 mM ketoisovalerate, 10 mM ketomethylvalerate or 10 mM 4-methylthio-2-oxobutanoate. The kinetic parameters K_m and V_{max} were estimated for phenylpyruvate as the substrate at concentrations ranging from 0 to 5 mM. The data were fitted with GraphPad Prism 4.0 (GraphPad Software, Inc.) by using a nonlinear regression of the Michaelis-Menten equation.

Acetyl alcoholtransferase assays (Atf1p, Atf2p)

Preparation of cell extracts and AATase enzyme assays were performed as previously described by Rojas *et al.*,(2002) with minor modifications.

Preparation of yeast cell extracts

Yeasts were grown in SC-Ura at 25°C up to the late log phase and by taking optical density at 600 nm as a reference. Yeast cells were collected by centrifugation, washed twice with cold 0.85% NaCl solution and resuspended in ice-chilled disruption buffer (10 mM potassium phosphate, pH 7.5 that contained 10% (w/v) glycerol, 0.8 mM $MgCl_2$, 5 mM DTT). Protease inhibitor cocktail tablet Complete Mini, EDTA-free (Roche Diagnostics, Mannheim, Germany) was also added to the disruption buffer (one tablet per 10 mL). Disruption was performed with glass beads in a MillMix 20 Bead Beater cell disrupter (Domel Tehtnica, Zelezniki, Slovenia) at a frequency of 30 s^{-1} in 1-minute intervals over a 10-minute period. The resulting homogenate was centrifuged at 15,000 rpm for 30 min at 4°C and the resulting supernatant was used to assay alcohol acetyltransferase activity. The protein concentrations in the cell extracts were estimated by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard.

AATase activity

Enzyme assays were performed in a 25-ml glass syringe provided with a Luer lock cap. Reaction mixtures consisted in glycerol buffer (50 mM potassium phosphate, pH 7.5, 10% (w/v) glycerol) that contained higher alcohol as a cosubstrate, glycerol buffer that contained acetyl-CoA as the other cosubstrate and a cell extract. The higher alcohol, acetyl-CoA and cell extract showed the following volume ratio 1:0.1:0.4. The final volume was determined by the number of samples (1.5 mL). Isoamyl alcohol (at a final concentration of 0.01 mM – 100 mM), isobutanol (60 mM) or 2-phenylethanol (30 mM) was used as the substrate together with acetyl-CoA (0.8 mM). Substrate

concentrations were determined according to Rojas (2002). After adding all the components, entrapped air was removed by the plunger and the syringe was attached to an orbital shaker. After 30 min, the 1.5-mL samples were transferred to 15-mL vials with 0.35 g NaCl for ester quantification with gas chromatography. Enzyme activity was stopped by adding 60 µL of a saturated KSCN solution. The data were fitted with GraphPad Prism 4.0 (GraphPad Software, Inc.) by using a nonlinear regression of the Michaelis-Menten equation.

Chapter 1

S. kudriavzevii and *S. uvarum* differ from *S. cerevisiae* during the production of aroma-active higher alcohols and acetate esters using their amino acidic precursors

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Short introduction

It is widely accepted that the acetate esters and higher alcohols produced during fermentation by yeast are particularly important for the food industry. Given their desired fruity and floral aroma, acetate esters significantly contribute to the aroma character of alcoholic beverages (Lambrechts and Pretorius, 2000; Swiegers *et al.*, 2005). The most significant esters are ethyl acetate (solvent-like aroma), isobutyl acetate (fruity), isoamyl acetate (banana), and 2-phenylethyl acetate (flowery, rose-like) (Lambrechts and Pretorius, 2000; Styger *et al.*, 2011b). These esters are synthesised by alcohol acetyltransferases from acetyl-CoA and ethanol (ethyl acetate) or aliphatic or aromatic higher alcohols. Aliphatic (also branched-chain) higher alcohols, which include isobutyl alcohol, active amyl alcohol, and isoamyl alcohol, are formed as part of the degradation of branched-chain amino acids valine, isoleucine and leucine, respectively. The major component of aromatic higher alcohols is 2-phenylethanol, which is produced from aromatic amino acid phenylalanine (Dickinson *et al.*, 2003; Dickinson *et al.*, 2000; Dickinson *et al.*, 1998; Dickinson *et al.*, 1997; Nykanen, 1986).

Aroma compounds are synthesised mainly by the *Saccharomyces cerevisiae* species during food-related fermentations. However, other related species belonging to the *Saccharomyces* genus, such as *Saccharomyces kudriavzevii* and *Saccharomyces uvarum*, can potentially be of interest for aroma production. The phylogenetic similarities between these species and their closely related *S. cerevisiae* prompted the formation of natural interspecific hybrids, which are present in wine and brewing environments (Gonzalez *et al.*, 2008; Gonzalez *et al.*, 2007). In this context, the fermentative abilities of these two species, and their hybrids, have been recently investigated (Arroyo-Lopez *et al.*, 2010; Combina *et al.*, 2012; Gonzalez *et al.*, 2006; Lopez-Malo *et al.*, 2013; Naumov *et al.*, 2000b; Naumov *et al.*, 2001; Oliveira *et al.*, 2014; Sampaio and Goncalves, 2008; Tronchoni *et al.*, 2014).

The above-cited studies describe significant differences in the impact of these two species on the aromatic qualities of alcoholic beverages when compared to *S. cerevisiae*. Specifically, *S. kudriavzevii* and *S. uvarum* have interesting oenological properties which lead, for instance, to greater glycerol production or lower ethanol production compared to *S. cerevisiae* (Gamero *et al.*, 2013; Oliveira *et al.*, 2014). The wines produced by *S. uvarum* strains also have a stronger aromatic intensity than those produced by *S. cerevisiae* (Coloretti *et al.*, 2006; Eglinton *et al.*, 2000).

The study described in this chapter explores differences in the production of prime aroma-active acetate esters and higher alcohols by *S. kudriavzevii*, *S. uvarum* and *S. cerevisiae*. As valine, isoleucine, leucine and phenylalanine are the precursors of these higher alcohols, which subsequently lead to acetate esters, these four amino acids were used as the sole nitrogen source for the growth of these species. Next, the production of the corresponding higher alcohols and esters was analysed. Ammonium and the mixture of those four amino acids were also used as the nitrogen sources to better obtain a comparison of how the three *Saccharomyces* species deal with nitrogen in terms of major flavour-active volatile compounds formation.

Results

To determine differences during the production of the major aroma-active higher alcohols and esters from their corresponding precursors (branched-chain or aromatic amino acids) by *S. cerevisiae* T73 strain, *S. kudriavzevii* IFO 1802 and *S. uvarum* CECT 12600, yeasts were cultivated in a synthetic medium with particular nitrogen sources. Such a defined medium, using a specific amino acid or ammonium as the nitrogen source, allowed us to avoid the undesirable impact of other non-specific nitrogen sources. Under these conditions, growth was followed and the final aroma composition was determined.

Growth under different nitrogen sources

To test whether yeasts would grow under these specific conditions, the increment of populations over time was monitored (Figure 1.1). Although slight differences were observed among species at the beginning of the exponential phases, as seen when grown with leucine or valine as the nitrogen source, all the species presented a normal growth pattern, even when one amino acid was used as the only nitrogen source.

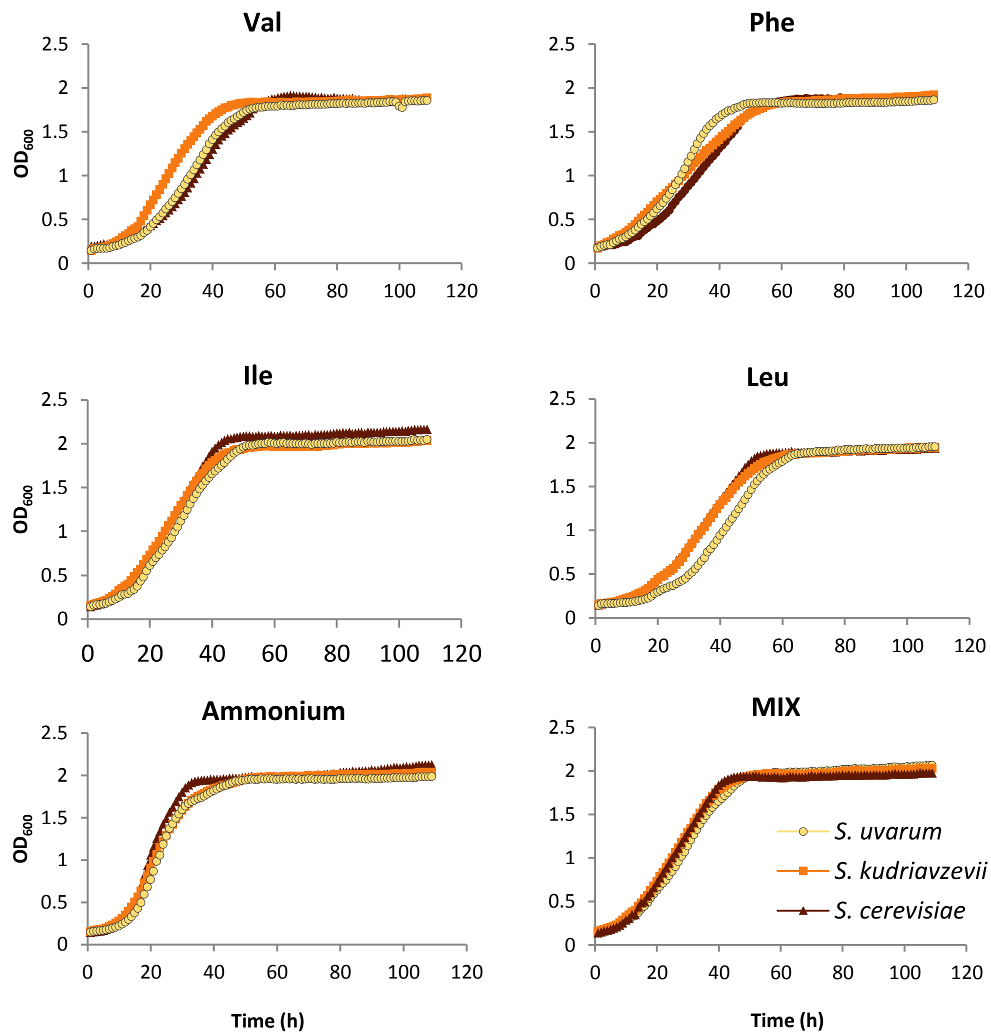

Figure 1.1. Growth of *S. cerevisiae* T73, *S. kudriavzevii* IFO 1802, and *S. uvarum* CECT 12600 with the indicated amino acids, with ammonium, or with an amino acid mixture as the nitrogen source (MIX).

Effect of different nitrogen sources on ethyl acetate production

We highlight ethyl acetate production in the analysis of the aroma compounds of the three species under the above-described conditions. Each species produced approximately the same amount of ethyl acetate regardless of the amino acid used as the nitrogen source (Figure 1.2). In this comparison the lowest concentrations were produced by *S. kudriavzevii*, while *S. uvarum* gave similar amounts to *S. cerevisiae*. When cultivated with either the mixture of amino acids or ammonium, *S. cerevisiae* did not change ethyl acetate production. However, *S. kudriavzevii* and *S. uvarum* produced significantly larger amounts of ethyl acetate when cultivated with the mixture of amino acids than with individual amino acids. When ammonium was employed as the nitrogen source, the ethyl acetate concentration produced by *S. kudriavzevii* was 3-fold higher, and 2.5-fold higher with *S. uvarum*, than *S. cerevisiae*.

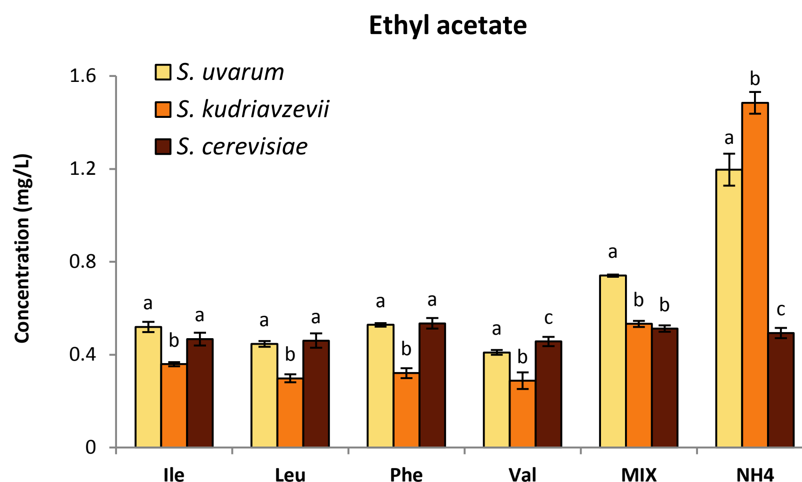

Figure 1.2. Ethyl acetate produced by *S. cerevisiae* T73, *S. kudriavzevii* IFO 1802, and *S. uvarum* CECT 12600 depending on the nitrogen source. The statistically significant differences among the species were determined independently for each nitrogen source and are indicated by labels above the columns.

Formation of the higher alcohols and esters derived from the corresponding amino acid

When valine was used as the sole nitrogen source, isobutanol production by *S. kudriavzevii* was similar to that of *S. cerevisiae*, whereas *S. uvarum* gave much smaller amount (approximately half) (Figure 1.3). A similar trend was observed when the mixture of amino acids was used as the nitrogen source. *S. uvarum* also produced the smallest amount of isobutanol when cultivated on ammonium. As expected, the highest isobutanol production values for the three species were observed when precursor valine was used. Strikingly, no major differences were observed during isobutyl acetate production for the different nitrogen sources. In all cases, the values ranged from about 0.025 mg/l to 0.070 mg/l.

The phenylalanine-grown cultures of *S. kudriavzevii* exhibited the highest 2-phenylethanol production, but the lowest 2-phenylethyl acetate production. On the contrary, *S. uvarum* formed the largest amounts of ester, but smaller (together with *S. cerevisiae*) amounts of the higher alcohol. A similar result was obtained when the nitrogen source used was the mixture of amino acids, which resulted in *S. kudriavzevii* with the highest 2-phenylethanol level and *S. uvarum* with the highest 2-phenylethyl acetate level. When ammonium was the nitrogen source, 2-phenylethyl acetate was not detected in any species. Negligible concentrations of 2-phenylethanol were found for *S. kudriavzevii* and *S. uvarum*, and none were seen for *S. cerevisiae*.

When isoleucine was the nitrogen source, *S. cerevisiae* was the highest producer of both corresponding compounds (active amyl alcohol and amyl acetate). The amyl acetate level was 3.7-fold and 2-fold higher than in *S. kudriavzevii* and *S. uvarum*, respectively.

When cultivated with leucine, the three species produced isoamyl alcohol similar concentrations, while they exhibited vast differences during isoamyl acetate production. *S. uvarum* was the highest producer of this compound.

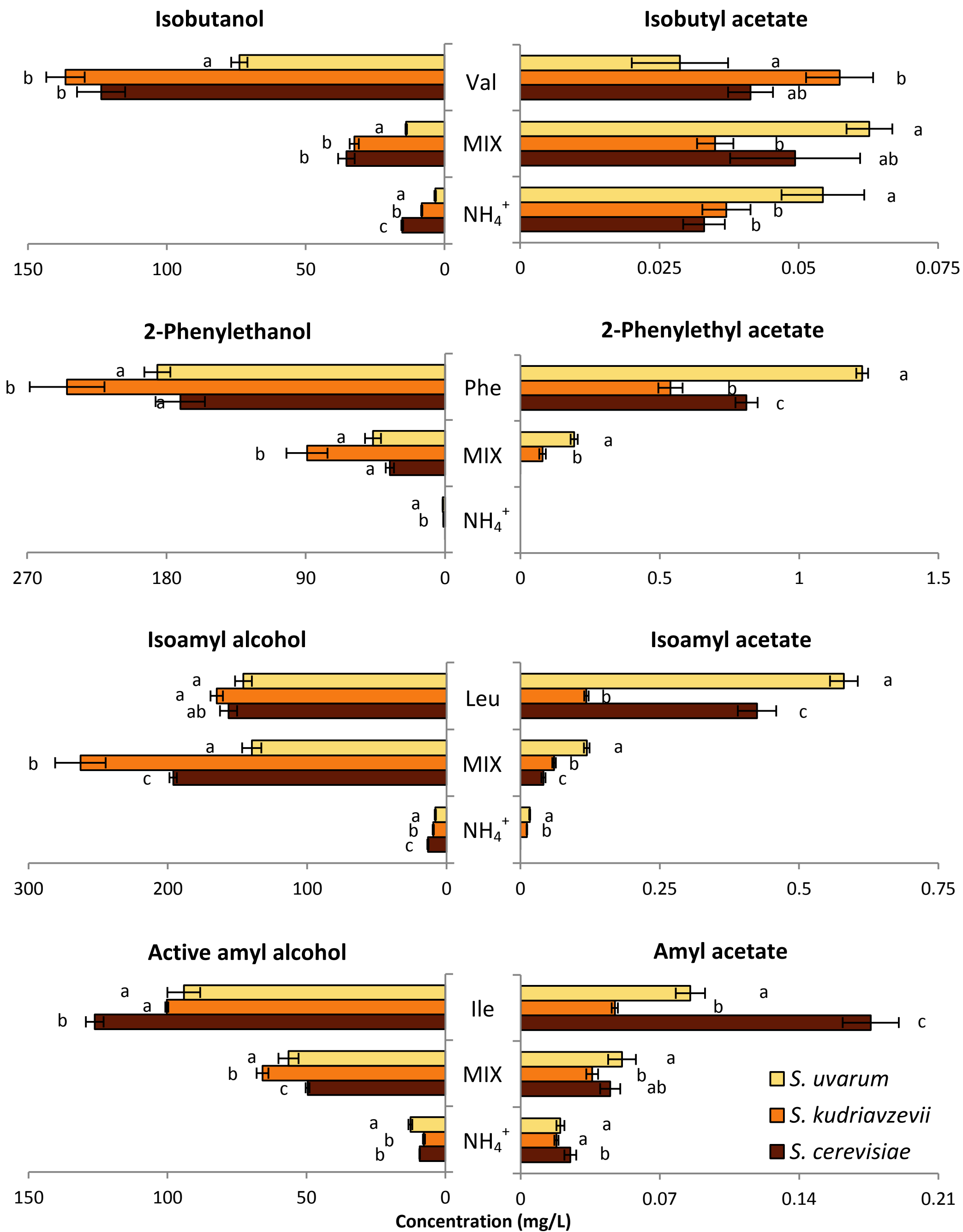

Figure 1.3. Production of the higher alcohols and esters derived from the stated amino acid and a comparison of these higher alcohols and esters when produced from the amino acids mixture (MIX) or ammonium sulphate used as the nitrogen source. The statistically significant differences among the species were determined independently for each nitrogen source and are indicated by labels beside the columns.

The detected concentration was 1.4-fold higher compared to *S. cerevisiae* and 5-fold higher vs. *S. kudriavzevii*. Similarly, *S. uvarum* exceeded the other two during isoamyl acetate production when grown with the amino acids mixture. *S. kudriavzevii* dominated during isoamyl alcohol production when the mixture of amino acids was used as the nitrogen source.

Comparison of the total higher alcohols and esters produced in response to different nitrogen sources

Apart from the major volatiles deriving from the particular amino acid, which was used as the nitrogen source, other higher alcohols and esters were detected. In this context, we also analysed the total amounts of the higher alcohols and esters produced by *S. kudriavzevii* and *S. uvarum* cultivated with different nitrogen sources. Then we compared the data to those of *S. cerevisiae*. As seen in Figure 1.4, the highest values of the total fusel alcohols were achieved by all the species when grown with the mixture of amino acids. This was not unexpected since each amino acid contributes to the formation of the corresponding higher alcohol. Nevertheless, the total amount of higher alcohols produced by *S. kudriavzevii* under these conditions was interesting as it resulted in a 2-fold larger amount than *S. uvarum*, and in a 1.7-fold larger one when compared to *S. cerevisiae*. In general, *S. kudriavzevii* exhibited the highest levels of the total fusel alcohol concentrations in four of the six nitrogen sources used (amino acid mixture, phenylalanine, leucine and valine), although the last two were comparable with those of *S. cerevisiae*.

Conversely, *S. kudriavzevii* did not excel in ester formation, while *S. uvarum* showed high levels of total esters, mainly when cultivated on phenylalanine and leucine. Likewise, *S. uvarum* produced a much larger quantity of esters than the other two *Saccharomyces* species when the nitrogen source was the amino acids mixture. When the amino acids mixture was used as the nitrogen source, the trend of the highest values was not observed for total esters, as it was in the case of the total higher alcohols.

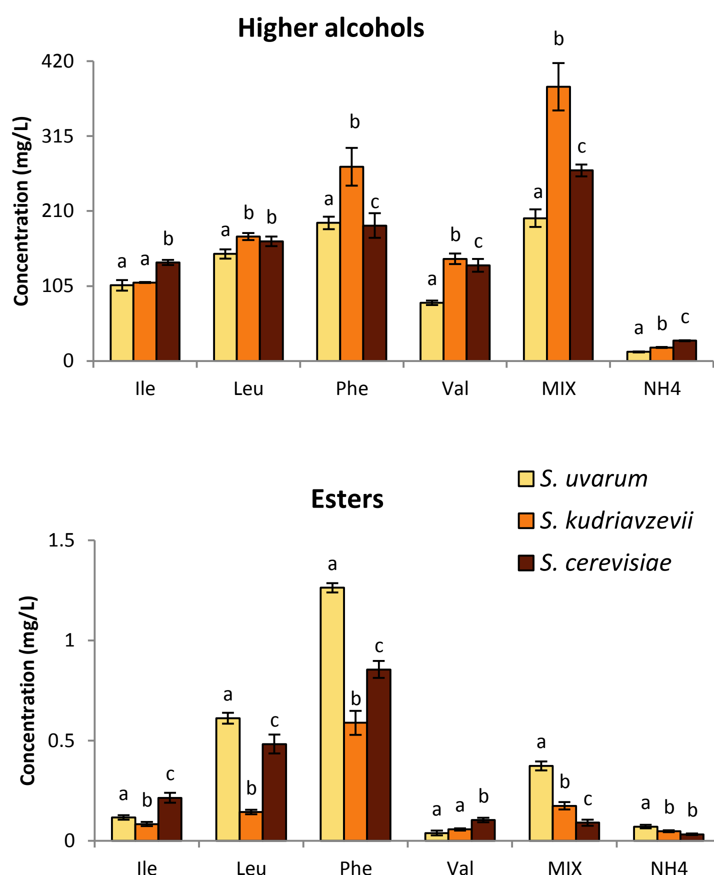

Figure 1.4. Total amount of the higher alcohols and esters produced by *S. cerevisiae* T73, *S. kudriavzevii* IFO 1802, and *S. uvarum* CECT 12600 depending on the nitrogen source used. The statistically significant differences among the species were determined independently for each nitrogen source and are indicated by labels above the columns.

Discussion

According to previous studies, *S. kudriavzevii* and *S. uvarum* show differences in several oenological traits, including the production of volatile aroma compounds during wine fermentation (Gamero *et al.*, 2014; Gamero *et al.*, 2013). To further examine these differences in detail, we explored the

responses to nitrogen sources compared to higher alcohols and acetate esters production. Indeed, the cultivation conditions used in our assays differed from typical fermentation processes. Yet, unlike complex media, the use of defined synthetic media allowed us to explore more precisely how the three species varied in the formation of the higher alcohols and acetate esters deriving from the corresponding amino acids and ammonium.

As the results show, the employment of individual amino acids as the nitrogen source led to a much higher production of the measured volatiles than with the other two nitrogen sources (amino acids mixture and ammonium) in all the species. Some exceptions were found during the production of isoamyl alcohol and isobutyl acetate. The largest amounts of isoamyl alcohol were obtained when produced from the amino acids mixture (by *S. kudriavzevii* and *S. cerevisiae*). Isobutyl acetate formation did not differ significantly when related to the nitrogen sources. This is surprising if we consider the significantly higher isobutanol (isobutyl acetate precursor) production with valine than with the other two nitrogen sources. This result indicates differences in the metabolism of isobutanol, particularly its subsequent esterification compared to the other higher alcohols.

The lowest concentrations of volatile compounds (with no particular differences found among species) were detected when ammonium was the nitrogen source. This result agrees with the observation published by Vuralhan *et al.* (2005; 2003), in which no activities of the 2-oxo-acid decarboxylase involved in the higher alcohol production pathway were detected in the cultures grown on ammonium used as the nitrogen source. Correspondingly, no or very low concentrations of higher alcohols were detected.

Relatively large differences were found among species in terms of concentrations of the higher alcohols and acetate esters deriving from their precursor. *S. uvarum*, for instance, surpassed the other two species for

2-phenylethyl acetate production. This result is consistent with previously reported conclusions, which indicated that good 2-phenylethyl acetate production was a typical trait of *S. uvarum* (Antonelli *et al.*, 1999; Gamero *et al.*, 2013; Masneuf-Pomarede *et al.*, 2010).

When we summarised and compared the amounts of the total higher alcohols and total esters produced by the three species, *S. uvarum* also showed reasonable ester formation, whereas *S. kudriavzevii* seemed to prefer the production of higher alcohols under these conditions. These differences could have been caused by distinct regulation mechanisms, different gene expression or diverse enzyme activities. For instance, remarkable differences among these species have been observed in the expression levels of those genes involved in the production of flavour compounds during winemaking (Gamero *et al.*, 2014). The genes that codify permeases, transaminases and other enzymes involved in amino acids metabolism were up-regulated in *S. uvarum* compared to *S. kudriavzevii*. *S. kudriavzevii* showed an up-regulation of *ATF2*. This gene, together with *ATF1*, encodes alcohol acetyl transferases, which catalyse the esterification of higher alcohols by acetyl coenzyme A. It has been previously shown in *S. cerevisiae* that Atf2p plays a minor role in esters formation compared to Atf1p (Verstrepen *et al.*, 2003b). In *S. kudriavzevii* and/or *S. uvarum*, these data suggest that the roles of Atf1p and Atf2p, and their substrate specificities, might be distinct from *S. cerevisiae*. Similar interspecific differences have also been observed between two subgenomes (*S. cerevisiae*-derived and *S. eubayanus*-derived) in lager-brewing hybrid *S. pastorianus* by Bolat and co-workers (2013). These authors described different roles for two 2-oxo-acid decarboxylase isoenzymes (involved in higher alcohols production), encoded by these two subgenomes. These different roles were based on distinct enzyme characteristics. The *S. cerevisiae* allele was preferentially involved in the production of the higher alcohols derived from the amino acids contained in

wort. In contrast, the allele from the *S. eubayanus* subgenome was involved in the formation of the higher alcohols derived from the amino acids synthesised *de novo* by the yeast.

Conclusion

It is obvious that the amino acid metabolism is controlled by a complex regulation system. The comparative analysis performed of three different species grown with distinct nitrogen sources revealed remarkable differences during the production of aroma-active higher alcohols and acetate esters. Our results indicate that despite *S. kudriavzevii* and *S. uvarum* being closely related to *S. cerevisiae*, the amino acid metabolism and subsequent production of flavour-active higher alcohols and acetate esters differed among these species. This knowledge can provide new possibilities for yeast-based applications in fragrance, flavour and food industries.

Chapter 2

In silico analysis of nucleotide divergences in the orthologous genes involved in aroma-active higher alcohols and acetate esters production by *S. kudriavzevii*, *S. uvarum* and *S. cerevisiae*

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Short introduction

Amino acid metabolism, which occurs during yeast fermentation processes, plays an important role in fragrance, flavour and food industries. Its importance arises from the fact that amino acid catabolism leads to the production of higher alcohols, which are precursors of acetate esters. The most significant acetate esters, i.e. isobutyl acetate (fruity-like aroma), isoamyl acetate (banana), and 2-phenylethyl acetate (flowery, rose-like), are products of a condensation reaction between the corresponding higher alcohol (isobutanol, isoamyl alcohol, 2-phenylethanol, respectively) and acetyl-CoA (Lambrechts and Pretorius, 2000; Styger *et al.*, 2011b). In *S. cerevisiae*, these higher alcohols derive directly from the catabolism of valine, leucine and phenylalanine (Dickinson *et al.*, 2003; Dickinson *et al.*, 2000; Dickinson *et al.*, 1998; Dickinson *et al.*, 1997) on a reaction pathway also known as the Ehrlich pathway (Ehrlich, 1907; Hazelwood *et al.*, 2008) (Figure 2.1). On this pathway, the amino acids, which are transported by amino acid permeases (codified by *GAP1*, *BAP2*, *BAP3*, *MUP3*) (Grauslund *et al.*, 1995; Isnard *et al.*, 1996; Jauniaux and Grenson, 1990; Regenberg *et al.*, 1999) are first transaminated to the corresponding 2-keto acids by transaminases (codified by *BAT1*, *BAT2*, *ARO8*, *ARO9*) (Eden *et al.*, 1996; Iraqui *et al.*, 1998; Kispal *et al.*, 1996). These 2-keto acids are then decarboxylated by decarboxylases (codified by *PDC1*, *PDC5*, *PDC6*, *ARO10*) (ter Schure *et al.*, 1998; Vuralhan *et al.*, 2003). The resulting aldehydes are reduced to their corresponding alcohols by dehydrogenases (codified by *ADH1-7*, *SFA1*) (Dickinson *et al.*, 2003). The subsequent acetate ester formation is mediated by the alcohol acetyltransferases codified by genes *ATF1* and *ATF2* (Fujii *et al.*, 1994; Mason and Dufour, 2000). Conversely, acetate ester breakdown is affected by the function of hydrolases, such as Iah1p (Fukuda *et al.*, 2000) which, together with Atf1p and Atf2p, maintain an optimal ester accumulation rate.

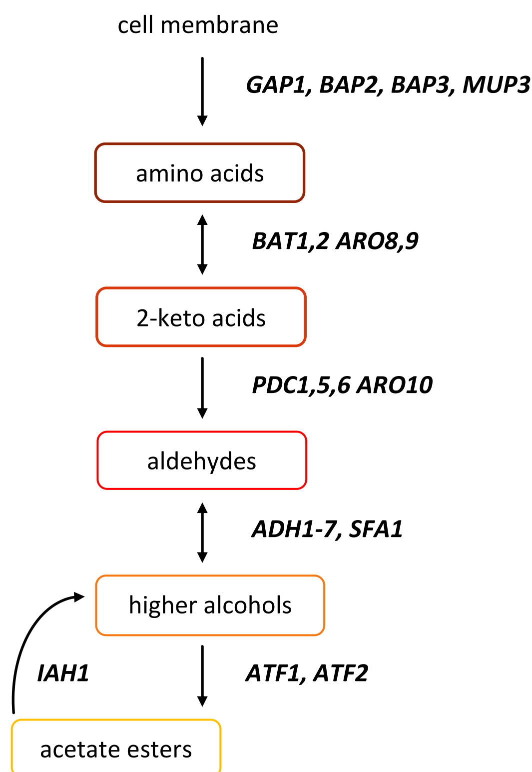


Figure 2.1. Amino acid catabolic pathway that leads to the production of higher alcohols and acetate esters.

Results from Chapter 1 show differences in the production of higher alcohols and acetate esters directly derived from amino acids by *S.kudriavzevii* and *S. uvarum* compared to *S. cerevisiae*. To gain a better insight into the molecular aspects of the aforementioned differences, the study described in this chapter aimed to explore nucleotide divergences in the genes (and, consequently, in the corresponding enzymes) involved in flavour compounds production. As aroma-active higher alcohols and esters derive from amino acid catabolism, computational tools were used to identify the most radical nonsynonymous nucleotide changes in the orthologue genes from *S. kudriavzevii/S. uvarum vs. S. cerevisiae* encoding 23 enzymes from amino acid catabolism pathway.

The bioinformatic analysis was based on the Grantham matrix (Grantham, 1974). This method classifies (dis)similarity in amino acids substitutions on the basis of physiochemical properties (composition, polarity, and molecular volume). The replacement of one amino acid with another that has similar biochemical properties (e.g. leucine *vs.* valine) is considered a conservative change, and a radical change if it has dissimilar properties. These (dis)similarities are then quantitatively evaluated according to the scoring designed by Grantham (Table 2.1). Using this method, we searched for *S. kudriavzevii/S. uvarum* genes encoding enzymes with the most radical changes in their amino acid sequences when compared to *S. cerevisiae*.

Arg	Leu	Pro	Thr	Ala	Val	Gly	Ile	Phe	Tyr	Cys	His	Gln	Asn	Lys	Asp	Glu	Met	Trp	
110	145	74	58	99	124	56	142	155	144	112	89	68	46	121	65	80	135	177	**Ser**
	102	103	71	112	96	125	97	97	77	180	29	43	86	26	96	54	91	101	**Arg**
		98	92	96	32	138	5	22	36	198	99	113	153	107	172	138	15	61	**Leu**
			38	27	68	42	95	114	110	169	77	76	91	103	108	93	87	147	**Pro**
				58	69	59	89	103	92	149	47	42	65	78	85	65	81	128	**Thr**
					64	60	94	113	112	195	86	91	111	106	126	107	84	148	**Ala**
						109	29	50	55	192	84	96	133	97	152	121	21	88	**Val**
							135	153	147	159	98	87	80	127	94	98	127	184	**Gly**
								21	33	198	94	109	149	102	168	134	10	61	**Ile**
									22	205	100	116	158	102	177	140	28	40	**Phe**
										194	83	99	143	85	160	122	36	37	**Tyr**
											174	154	139	202	154	170	196	215	**Cys**
												24	68	32	81	40	87	115	**His**
													46	53	61	29	101	130	**Gln**
														94	23	42	142	174	**Asn**
															101	56	95	110	**Lys**
																45	160	181	**Asp**
																	126	152	**Glu**
																		67	**Met**

Table 2.1.
The Grantham scoring which quantitatively evaluates (dis)similarities in amino acids substitutions on the basis of physio-chemical properties (composition, polarity, and molecular volume).

Results

To perform a comparative analysis, DNA sequences of the orthologue genes encoding 23 enzymes which are involved in amino acid catabolism leading to higher alcohols and acetate ester formation were obtained from 75 *S. cerevisiae* strains, two *S. kudriavzevii* strains and three *S. uvarum* strains, available in databases: SGD - *Saccharomyces* Genome Database (www.yeastgenome.org) (Cherry *et al.*, 2012), SGRP – *Saccharomyces* Genome Resequencing Project (www.moseslab.csb.utoronto.ca/sgrp) (Bergstrom *et al.*, 2014; Liti *et al.*, 2009), NCBI (www.ncbi.nlm.nih.gov), and *Saccharomyces sensu stricto* database (www.saccharomycessensustricto.org) (Scannell *et al.*, 2011). The strains used in the analysis are listed in Table 2.2. Amino acid translations of the DNA sequences were then aligned with MEGA 5.05 using the MUSCLE software (Tamura *et al.*, 2011). These alignments allowed us to search for amino acid substitutions between orthologues. The individual changes in *S. kudriavzevii* and *S. uvarum* sequences (with *S. cerevisiae* orthologues taken as references) were then quantified by Grantham matrix. According to increasing biochemical dissimilarity, Li et al. (Li *et al.*, 1984) proposed a Grantham scale classification which comprised four classes as follows: conservative (0-50), moderately conservative (51-100), moderately radical (101-150) and radical (≥151). In our analysis we decided to simplify the classification and we set up the scores of 120 and higher as a radical change.

Table 2.2. List of *S. cerevisisae*, *S. kudriavzevii* and *S. uvarum* strains involved in the bioinformatic analysis. The strains were obtained from corresponding databases as indicated.

S. cerevisiae strains				*S. kudriavzevii* strains	*S. uvarum* strains
SGRP database		SGD		*Sss* database	
273614N	S288c	AWRI1631	K11	IFO1802	CBS7001
322134S	SK1	AWRI796	Kyokai7	ZP591	
378604X	W303	BY4741	LalvinQA23		CECT12600*
BC187	Y9	BY4742	M22		BMV58*
DBVPG1106	Y10	CBS7960	PW5		
DBVPG1373	Y12	CEN.PK	SEY6210		
DBVPG1788	Y55	CLIB215	Sigma1278b		
DBVPG1853	YIIc17_E5	CLIB324	T7		
DBVPG6040	YJM789	CLIB382	T73		
DBVPG6044	YJM975	D273-10B	UC5		
DBVPG6765	YJM978	EC1118	VL3		
K11	YJM981	EC9-8	Vin13		
L_1374	YPS128	FL100	X2180-1A		
L_1528	YPS606	FY1679	YJM269		
NCYC110	YS2	FostersB	YJM339		
NCYC361	YS4	FostersO	YPH499		
RM11_1A	YS9	JAY291	YPS163		
UWOPS03_461.4		JK9-3d	ZTW1		
UWOPS05_217.3					
UWOPS05_227.2					
UWOPS83_787.3					
UWOPS87_2421					

SGRP – (www.moseslab.csb.utoronto.ca/sgrp) (Bergstrom *et al.*, 2014; Liti *et al.*, 2009)
SGD – (www.yeastgenome.org) (Cherry *et al.*, 2012)
Sss (*Saccharomyces sensu stricto*) – (www.saccharomycessensustricto.org) (Scannell *et al.*, 2011)
* *S. uvarum* strains that are being sequenced and annotated by the research group of A. Querol

Across the 23 assessed sequences, three were evaluated with significantly higher Grantham scores: 2-keto acid decarboxylase encoded by *ARO10*, and two alcohol acetyltransferases encoded by *ATF1* and *ATF2* (Table 2.3). These three sequences surpassed the other two highest sequences (Aro9p, Bap2p) by about 40%. Equally for *S. kudriavzevii* and *S. uvarum*, the highest total Grantham score and the largest amount of substitutions were found in Atf2p (SuAtf2p – 132 and SkAtf2p – 110). However, the largest amount of radical substitutions contained the Aro10p sequences. SuAro10p contained 15 substitutions with a score of 2313, and SkAro10p 11 substitutions with a score of 1629. The Atf1p and Atf2p sequences from *S. uvarum* contained four and five radical substitutions with a score of 650 and 755, respectively. Atf1p and Atf2p from *S. kudriavzevii* contained six and four with a score of 942 and 609, respectively.

Table 2.3. Amino acid substitutions between the orthologous enzymes from *S. cerevisiae* and *S. kudriavzevii* or *S. uvarum* evaluated by the Grantham score. The enzymes are involved in the production of aroma-active higher alcohols and acetate esters.

Name	AAs		Total substitutions		Radical substitutions (≥120)		$\frac{\sum \text{Gr. score of radicals}}{\sum \text{Gr. score total}} * 100$ (%)
			No.	∑ Grantham score	No.	∑ Grantham score	
Permeases							
Gap1	602	*Sk*	34	2050	2	275	13.4
		Su	68	3961	4	541	13.7
Bap2	609	*Sk*	58	3350	6	797	23.8
		Su	62	3641	4	535	14.7
Bap3	604	*Sk*	45	2276	2	301	13.2
		Su	55	2574	2	248	9.6
Mup3	546	*Sk*	49	2500	3	478	19.1
		Su	53	2613	2	388	14.8

Name	AAs		Total substitutions No.	Total substitutions ∑ Grantham score	Radical substitutions (≥120) No.	Radical substitutions (≥120) ∑ Grantham score	$\frac{\sum \text{Gr. score of radicals}}{\sum \text{Gr. score total}} * 100$ (%)
Transaminases							
Bat1	393	*Sk*	18	817	0	0	0
		Su	20	1222	0	0	0.0
Bat2	376	*Sk*	41	2640	4	649	24.6
		Su	45	2865	3	524	18.3
Aro8	500	*Sk*	34	2154	1	180	8.4
		Su	44	2725	2	335	12.3
Aro9	517	*Sk*	72	3560	3	421	11.8
		Su	87	4198	0	0	0.0
Decarboxylases							
Pdc1	563	*Sk*	10	567	1	125	22.0
		Su	13	786	0	0	0.0
Pdc5	563	*Sk*	30	1266	0	0	0
		Su	36	1642	0	0	0.0
Pdc6	563	*Sk*	NF	-	-	-	-
		Su	36	2031	2	263	12.9
Aro10	635	*Sk*	87	5764	11	1629	28.3
		Su	98	6443	15	2313	35.9
Dehydrogenases							
Adh1	348	*Sk*	15	727	0	0	0
		Su	17	1207	3	519	43.0
Adh2	348	*Sk*	22	812	0	0	0
		Su	21	1039	1	144	13.9
Adh3	375	*Sk*	15	821	1	149	18.1
		Su	12	843	1	149	17.7
Adh4	382	*Sk*	31	1602	0	0	0
		Su	46	2426	2	147	6.1
Adh5	351	*Sk*	25	1172	0	0	0
		Su	34	1881	0	0	0.0
Adh6	360	*Sk*	29	1428	0	0	0
		Su	30	1636	0	0	0.0
Adh7	361	*Sk*	40	2605	4	584	22.4
		Su	NF	-	-	-	-

Name	AAs		Total substitutions No.	Total substitutions ∑ Grantham score	Radical substitutions (≥120) No.	Radical substitutions (≥120) ∑ Grantham score	$\frac{\sum \text{Gr. score of radicals}}{\sum \text{Gr. score total}} * 100\ (\%)$
Sfa1	385	*Sk*	28	1640	5	700	42.7
		Su	25	1688	2	310	18.4
Acetyltransferases							
Atf1	524	*Sk*	89	5350	6	942	17.6
		Su	99	6172	4	650	10.5
Atf2	535	*Sk*	110	6187	4	609	9.8
		Su	132	7788	5	755	9.7
Esterase							
Iah1	238	*Sk*	49	2849	3	449	15.8
		Su	45	2811	3	375	13.3

NF – sequence not found in the databases; Radical substitutions involve each substitution with a Grantham score ≥120.

Discussion

As aroma-active higher alcohols and esters derive from the amino acid catabolism, we used computational tools to identify the most radical non-synonymous nucleotide changes in the orthologues genes from *S. kudriavzevii*/*S. uvarum vs. S. cerevisiae* that encode the enzymes from the amino acid catabolism pathway. To evaluate the severity of amino acid replacement, we applied Grantham scoring based on the assessment of biochemical dissimilarity between amino acid side chain properties (Grantham, 1974).

The largest number of radical substitutions was observed in SuAro10p and SkAro10p (Table 2.3). In SuAro10p, the analysis revealed 15 of the 98 substitutions as being radical which is *c.* 15%. Similarly, in SkAro10p 11 of the 87 substitutions were radical, which is *c.* 12.5%. When expressing these substitutions as a Grantham score, the ratio was *c.* 36% for SuAro10p and 28% for SkAro10p. However, the highest ratio of radical *vs.* total substitution

was observed in Sfa1p from *S. kudriavzevii*. Sfa1p is a bifunctional enzyme that displays the glutathione-dependent formaldehyde dehydrogenase activity required for formaldehyde detoxification, and the alcohol dehydrogenase activity involved in the formation of higher alcohols (Wehner *et al.*, 1993). In spite of the fact that the radical substitutions represented only 5 of 28, according to the Grantham scale, these five substitutions comprise *c.* 43% of the Grantham score for all the substitutions. In *S. uvarum* Sfa1p, however, the same calculation resulted in only 19%.

Moreover, since Aro10p was detected among the best hits, we further performed *in silico* analysis of Aro80p. This protein has been described to be a key transcriptional regulator of *ARO10* gene. Aro80p activates expression of the *ARO10* gene in response to aromatic amino acids (Iraqui *et al.*, 1999). Interestingly, the bioinformatic analysis evaluated the Aro80p from *S. kudriavzevii* and *S. uvarum* with approximately double Grantham scores than those of Aro10p, Atf1p, and Atf2p (Table 2.4). Similarly, the comparison of the Aro80p orthologues revealed relatively high number of amino acid substitutions (including the radical ones) when compared with the other gene comparisons. The differences in the Aro80p sequences from the three *Saccharomyces* species probably arise from the regulatory function of this protein.

Table 2.4. Amino acid substitutions in the orthologues Aro80p from *S. kudriavzevii* and *S. cerevisiae* evaluated by the Grantham score.

Name	AAs		Total substitutions		Radical substitutions (≥120)		$\frac{\sum \text{Gr. score of radicals}}{\sum \text{Gr. score total}} * 100$ (%)
			No.	∑ Grantham score	No.	∑ Grantham score	
Aro80	950	*Sk*	191	13232	26	3719	28.1
		Su	157	10712	18	2483	23.2

Conclusions

Notwithstanding the interesting results obtained for Sfa1p and Aro80p, further investigation of this thesis aimed to characterise catalytic activities of the enzymes encoded by the orthologous genes *ARO10*, *ATF1* and *ATF2*, and to examine their implication in higher alcohols and acetate esters production.

It is worth mentioning that, to date, there are publicly available genome sequences from two *S. kudriavzevii* strains (IFO1802 and ZP591) and from one *S. uvarum* strain (CBS7001) (Scannell *et al.*, 2011). Sequencing and annotation of other two *S. uvarum* genomes (CECT12600 and BMV58) is being in progress by the research group of A. Querol. Since the analysis revealed only minor differences between the *S. kudriavzevii* strains and between the *S. uvarum* strains (e.g. no differences were observed in the selected *ARO10*), the *S. kudriavzevii* type strain IFO1802 and *S. uvarum* CECT12600 were used for further experiments.

Chapter 3

Characterisation of the broad substrate specificity 2-keto acid decarboxylase Aro10p of *S. kudriavzevii* and *S. uvarum*, and their implication in aroma development

Partially published in Microbial Cell Factories 15: 51-62

Short introduction

In the work described in Chapter 2, we searched for the *S. kudriavzevii* and *S. uvarum* genes that encode amino acid catabolism-related enzymes of which amino acid sequences have the most radical changes compared to *S. cerevisiae*. The bioinformatic analysis revealed *ARO10* to be the candidate with the highest score for radical changes.

In *S. cerevisiae*, the product of the *ARO10* gene was described as a 2-keto acid decarboxylase which catalyses the decarboxylation of the 2-keto acids derived from the amino acid transamination on the Ehrlich pathway (Vuralhan *et al*., 2003). This decarboxylation step is the only irreversible reaction that takes place on the Ehrlich pathway. Besides Aro10p, other decarboxylases (Pdc1p, Pdc5p, Pdc6p) also catalyse the conversion of 2-keto acids into the corresponding aldehydes. Nevertheless, Aro10p showed superior kinetic parameters for branched-chain, aromatic, and sulphur-containing 2-keto acids than the other decarboxylases (Romagnoli *et al*., 2012). This broad-substrate specificity, together with the kinetic properties suggested that Aro10p was the major decarboxylase involved in the formation of the aroma-active higher alcohols that derived from the branched-chain, aromatic and sulphur-containing aroma acids (Romagnoli *et al*., 2012).

This chapter focused on cloning of the *S. kudriavzevii ARO10* (*SkARO10*) and *S. uvarum ARO10* (*SuARO10*) alleles into *S. cerevisiae* in order to examine its impact on the production of higher alcohols and acetate esters. The substrate specificities and kinetic properties of the encoded enzymes were also analysed and compared to *S. cerevisiae*.

It is necessary to notify that Bolat *et al*., (2013) recently performed detailed functional analysis and enzymatic characterisation of the decarboxylase encoded by *ARO10* gene from *S. eubayanus* subgenome of the lager-brewing yeast *S. pastorianus*. Considering the relatively low genomic divergence (7%; Libkind *et al.,* 2011) between *S. uvarum* and *S. eubayanus*,

together with the fact that the decarboxylase activity assessment is relatively complex method, we primarily aimed to assay the enzyme properties for *S. kudriavzevii* Aro10p.

Results

Effect of *SkARO10* and *SuARO10* on the formation of the higher alcohols and esters

In order to verify the effect of *SkARO10* and *SuARO10* during amino acid catabolism, which leads to the production of higher alcohols and/or acetate esters, the native *ARO10* (*ScARO10*) allele of a haploid strain of the wine *S. cerevisiae* T73 strain was swapped with *SkARO10* or *SuARO10* allele resulting in the mutant strain JET01Sk or JET01Su (Table M.2, page 49). To exclude any other mutations that may have occurred in the allele replacement step, the original *ScARO10* allele was returned to its native position which resulted in the strain JET01Sc. Subsequently, the formation of the major aroma-active higher alcohols and acetate esters was measured and compared between JET01Sc and JET01Sk/JET01Su.

Cells were cultivated with individual amino acids valine, leucine or phenylalanine as the sole nitrogen source, and the corresponding higher alcohols and their esters were analysed. Such a defined medium allowed us to observe the *in vivo* effect of the *SkARO10* and *SuARO10* alleles with no undesirable impact of other non-specific nitrogen sources. Under the tested conditions, all the strains exhibited a normal growth with no significant differences among them (Figure 3.1). This confirmed that the three decarboxylases showed activity with the formed 2-keto acids, enabling the strains to use these amino acids as the sole nitrogen source.

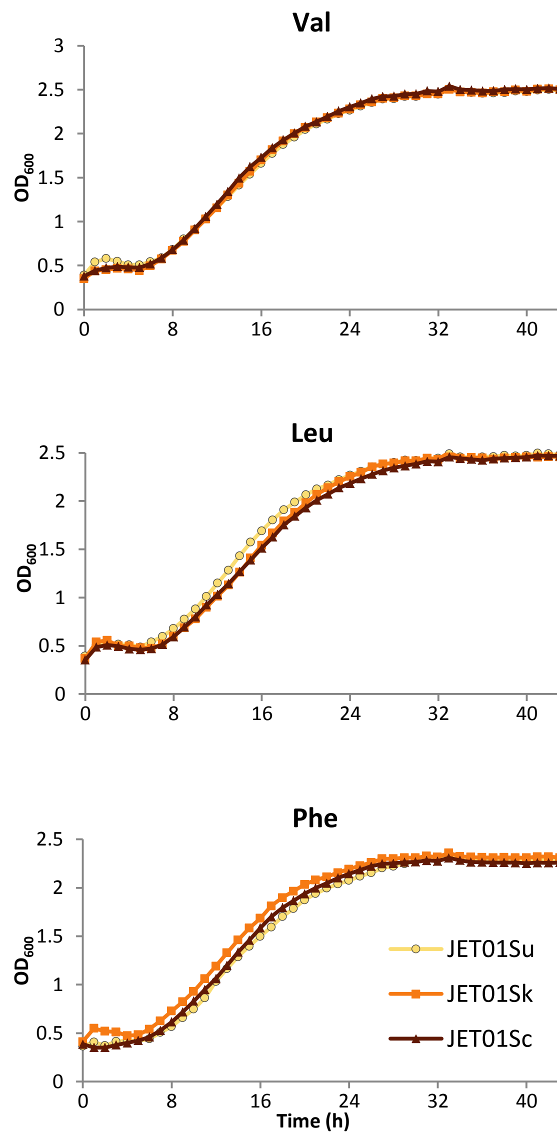


Figure 3.1. Growth of JET01Sk, JET01Su and JET01Sc with the indicated amino acids as the nitrogen source.

With valine as the nitrogen source, both corresponding derivatives (isobutanol and isobutyl acetate) were produced by the strains that carried the *SkARO10* or *SuARO10* allele in significantly larger amounts (Figure 3.2). Particularly, the isobutyl acetate concentration was *c.* 9-fold higher than that produced by JET01Sc. A similar upward trend in favour of JET01Sk and JET01Su was observed in the production of isoamyl alcohol and isoamyl acetate when their amino acidic precursor leucine was used as the nitrogen source. Both, JET01Sk and JET01Su showed an almost 2-fold increase in the isoamyl alcohol concentration. Both strains also showed an increase in the isoamyl acetate concentration (JET01Sk *c.* 3.6-fold and JET01Su *c.* 3-fold) compared to JET01Sc. The phenylalanine-grown cultures exhibited slight, but statistically insignificant, differences during the formation of the corresponding products, these being 2-phenylethanol and 2-phenylethyl acetate.

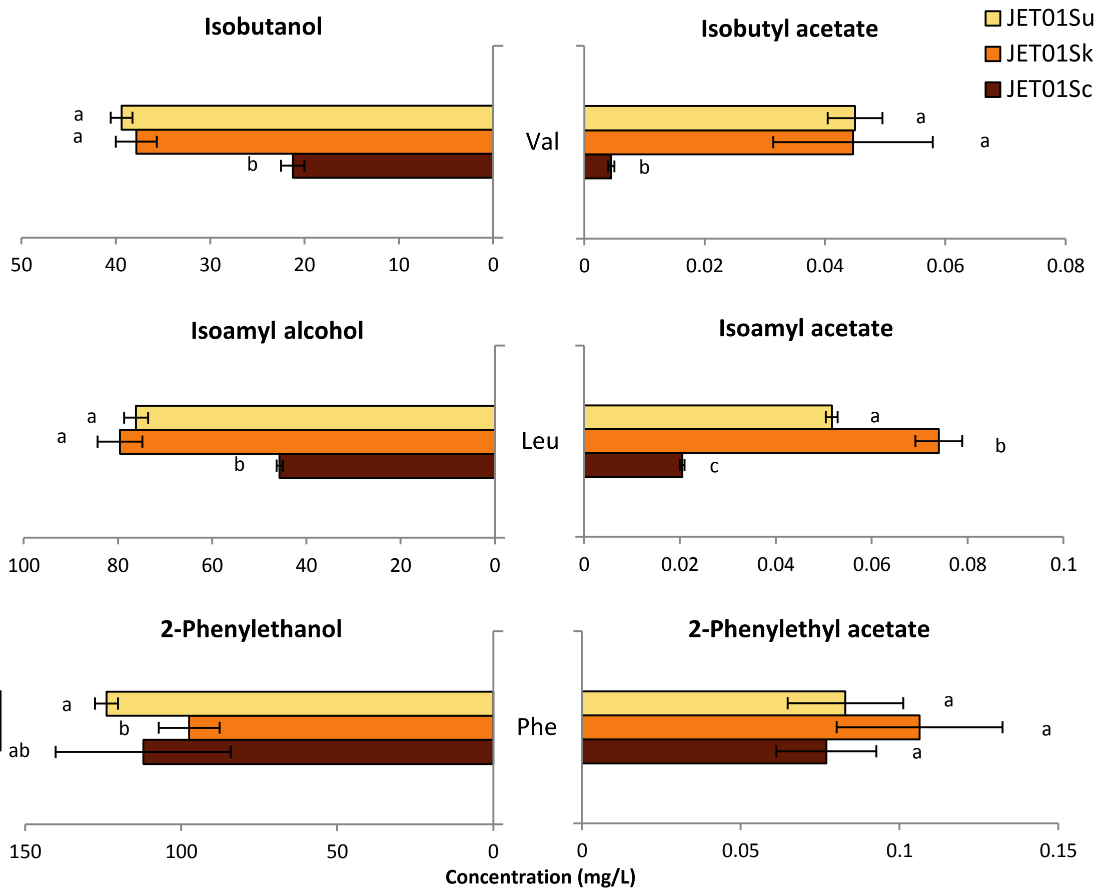


Figure 3.2. Production of the higher alcohols and esters derived from the corresponding amino acidic precursors which were used as the nitrogen source. The corresponding amino acids are indicated in the graphs between the columns. The statistically significant differences among the strains were determined independently for each nitrogen source and are indicated by labels beside the columns.

Additionally, in order to verify the effect of the *SkARO10* and *SuARO10* alleles on the formation of higher alcohols and esters in a more complex medium, synthetic wine must fermentations by the strains JET01Sk, JET01Su and JET01Sc were performed, and the production of higher alcohols and acetate esters was analysed. Weight loss monitoring revealed that all the strains had similar fermentation rates with no differences (Figure 3.3).

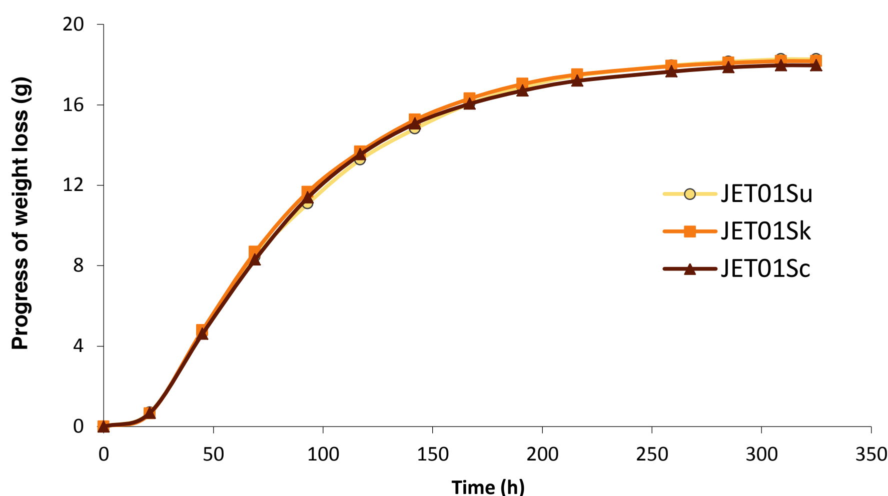


Figure 3.3. Progress of synthetic wine must fermentation. The fermentations were monitored as the progress of weight loss between two following measurements until the weight loss was negligible and the weight of the fermenting culture was constant.

Interestingly, while JET01Su showed similar data, JET01Sk showed the contrary to the data observed in the cultivations with the individual amino acids as the nitrogen source. Regarding higher alcohols, JET01Sk only exhibited an increased amount of 2-phenylethanol (Figure 3.4A) whereas the amounts of isobutanol and isoamyl alcohol did not differ significantly when compared to JET01Sc. Regarding acetate esters, JET01Sk showed larger amounts of isoamyl acetate and 2-phenylethyl acetate when compared to JET01Sc (Figure 3.4B).

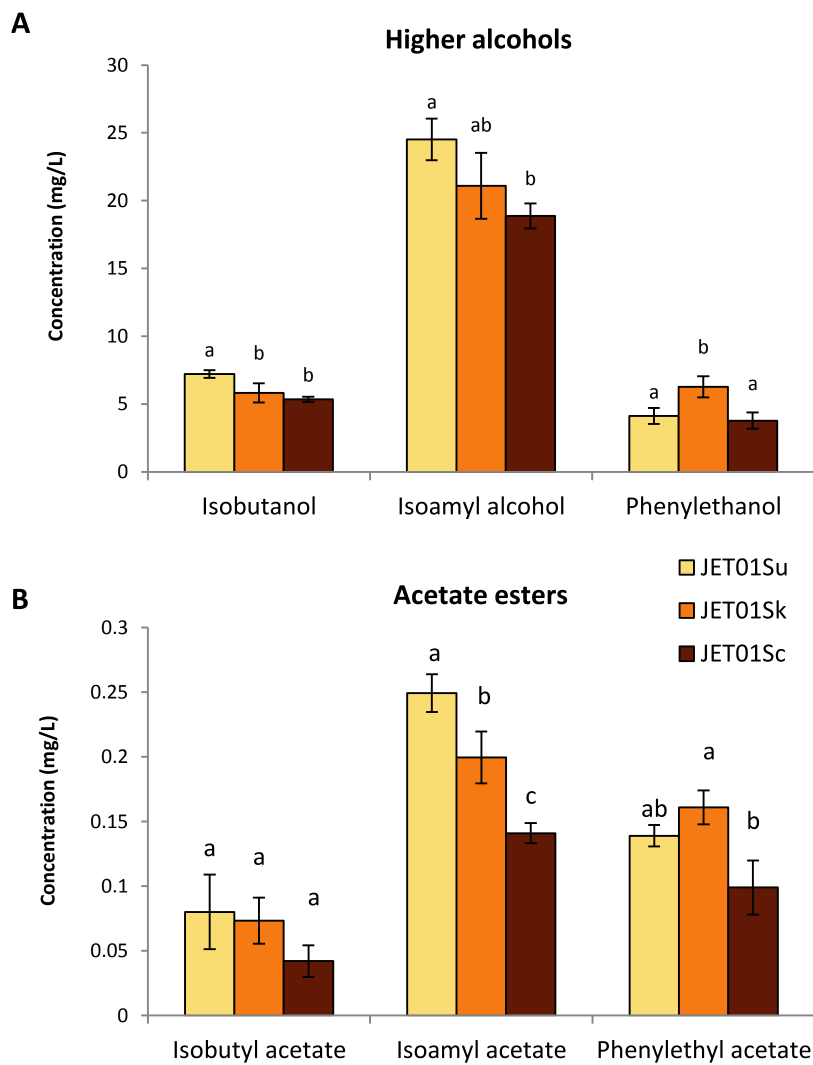


Figure 3.4. Production of the higher alcohols (A) and acetate esters (B) by JET01Sk and JET01Sc during the fermentation of the synthetic wine must. The statistically significant differences among the strains were determined independently for each measured compound and are indicated by labels above the columns.

Comparison of the substrate specificity of SkAro10p and ScAro10p

To relate these metabolites concentration differences to the presence of either *SkRO10* or *ScARO10* the substrate specificity of SkAro10p and ScAro10p were compared. To achieve this, the individual decarboxylase genes were expressed in a host *S. cerevisiae* strain that lacked all the 2-keto acid decarboxylase genes involved in the decarboxylation step of the Ehrlich pathway (CEN.PK711-7C *pdc1Δ pdc5Δ pdc6Δ aro10Δ thi3Δ*). Absence of the pyruvate decarboxylase genes (*PDC1, PDC5, PDC6*) has been previously shown to inhibit growth on glucose (Flikweert *et al.*, 1996). Therefore, ethanol was used as a carbon source in the chemostat cultivations. Eventually, to overcome the tight transcriptional control of *ARO10* gene (Boer *et al.*, 2003) and the regulation of the Aro10p activity by the nitrogen sources (Vuralhan *et al.*, 2005), phenylalanine was used as the nitrogen source rather than ammonium sulphate. Decarboxylase activity was measured in the cell extracts from the chemostat cultures and was compared for five different substrates phenylpyruvate, ketoisocaproate, ketoisovalerate, ketomethylvalerate, and 4-methylthio-2-oxobutanoate. Substrates were used at saturating concentrations of 10 mM, except for phenylpyruvate (5 mM). The cell extracts of both strains (CEN.PKpSkARO10 and CEN.PKpScARO10) exhibited activities for all five substrates (Figure 3.5). Nevertheless, when comparing the individual substrates, the strain that carried *ScARO10* displayed significantly greater activity for phenylpyruvate than for the other substrates. In contrast, the cell extracts of the strain that expressed *SkARO10* exhibited similar activities for all substrates.

Furthermore, the kinetic properties of the two decarboxylases were assessed for phenylpyruvate. The typical Michaelis-Menten saturation kinetics was observed for the enzymatic activities measured in the cell extracts of both strains. The K_m for this substrate was 2-fold lower for *S. kudriavzevii* Aro10p than the K_m found for *S. cerevisiae* Aro10p (0.07±0.005 mM *vs.*

0.15±0.01 mM, respectively) and SkAro10p showed 3-fold lower V_{max} than ScAro10p (6.95±0.15 *vs.* 21.7±0.5 nmol·min^{-1}·(mg protein)$^{-1}$).

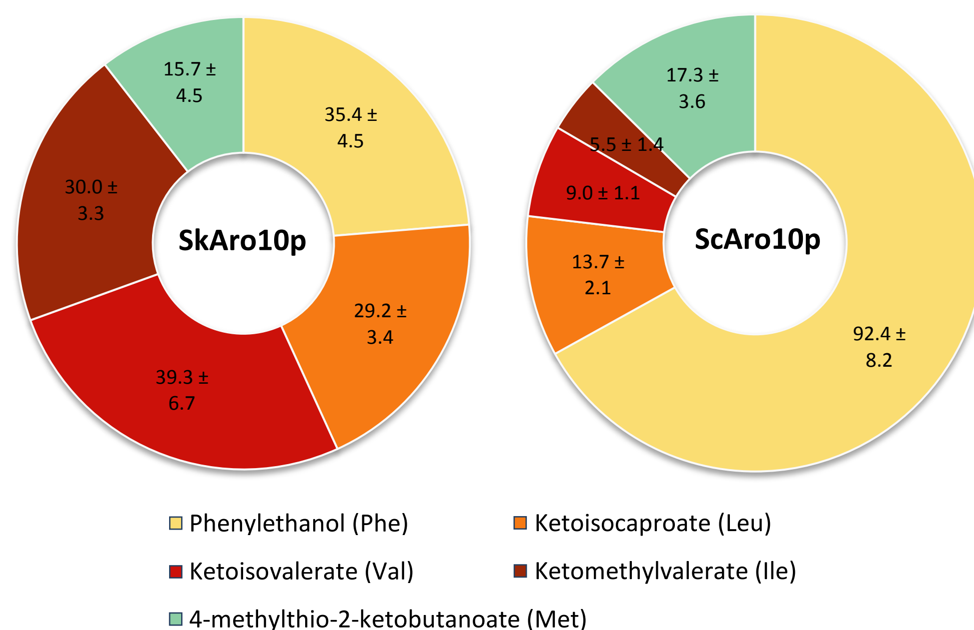


Figure 3.5. Activities (nmol·min^{-1}·(mg protein)$^{-1}$) of SkAro10p and ScAro10p measured in the cell extracts of *S. cerevisiae* strain with *pdc1 pdc5 pdc6 aro10 thi3* deletion. Activities were measured at a concentration of 5 mM for phenylpyruvate and at 10 mM for the other substrates. The amino acidic precursors of the corresponding substrates are offered in the parentheses following the substrates.

Discussion

The enzymatic activities of SkAro10p, assayed in a *pdc1 pdc5 pdc6 aro10 thi3* quintuple-null *S. cerevisiae* strain, were observed for all the tested substrates. This result indicates that the substrate specificity of SkAro10p is as broad as that of ScAro10p. Yet significant differences between SkAro10p and ScAro10p were observed for substrate preferences. When individual enzymatic activities were expressed as a percentage distributed among the

total enzymatic activity of the measured substrates, SkAro10p was found to be more or less evenly proportional of the enzymatic activities. In contrast, ScAro10p showed considerably greater activity towards phenylpyruvate than the other substrates. This suggests phenylpyruvate to be a preferred substrate. This phenylpyruvate-preference of Aro10p from the wine *S. cerevisiae* T73 strain was consistent with previously observed data for Aro10p from the laboratory *S. cerevisiae* CEN.PK113-7D strain (Romagnoli *et al.*, 2012), and for the Aro10 isoenzymes encoded by the two (*S. cerevisiae*-derived and *S. eubayanus*-derived) subgenomes of the lager-brewing *S. pastorianus* strain (Bolat *et al.*, 2013).

The impact of *SkARO10* and *SuARO10* on the production of higher alcohols and their esters was analysed by heterologous expression in a host *S.cerevisiae*. The results showed a remarkable increase in the detected amounts of valine- and leucine-derived higher alcohols (isobutanol and isoamyl alcohol, respectively) produced by the strains that carried *SkARO10* compared to the isogenic reference strain with active *ScARO10*. However, no differences were observed in the formation of 2-phenylethanol from phenylalanine. This phenomenon might be explained by the aforementioned differences in substrate specificities, particularly the phenylpyruvate-preference of ScAro10p. With its strong activity towards phenylpyruvate, ScAro10p produced more 2-phenylethanol and fewer other higher alcohols. Apparently SkAro10p also was sufficiently effective with ketoisovalerate and ketoisocaproate and, therefore, the production of isobutanol and isoamyl alcohol, respectively, was greater than in ScAro10p. Synthetic must fermentations revealed an opposite result with the differences in 2-phenylethanol formation and no significant differences in the production of isobutanol and isoamyl alcohol when compared the strain that carried *SkARO10* to the reference strain with *ScARO10*. This discrepancy might be due to the complexity of the synthetic must and a possible impact of other

compounds on the formation of the higher alcohols.

On the other hand, the comparison between the strain with *SuARO10* and the reference strain with active *ScARO10* revealed more or less equal results under both conditions, i.e. synthetic must fermentations as well as with individual amino acids as the nitrogen source. However, to suggest a hypothesis involving enzyme activity as for SkAro10p, it is necessary to assess the substrate specificity of SuAro10p.

The *S. cerevisiae* that harboured *SkARO10* or *SuARO10* yielded larger amounts of acetate esters. This improved acetate ester formation was probably the result of the larger production of higher alcohols as they are precursors. Yet in the previous study described in Chapter 1, despite the fact that *S. kudriavzevii* produced larger amounts of higher alcohols than *S. cerevisiae* and *S. uvarum*, *S. kudriavzevii* did not produce larger amounts of acetate esters. This indicates interspecific variations in acetate ester formation; for instance, it has been already mentioned during this thesis that in *S. cerevisiae* the two alcohol acetate transferases (Atf1p and Atf2p), the enzymes that catalyse the esterification of higher alcohols by acetyl coenzyme A, act differently during ester production. It has been shown that Atf2p plays a minor role in ester formation compared to Atf1p (Verstrepen *et al.*, 2003b). In *S. kudriavzevii* and/or *S. uvarum*, the roles of Atf1p and Atf2p, and their substrate specificities, might differ from *S. cerevisiae*. Hypothetically speaking, one possible explanation might be the amino acid variations in the orthologous Atf1p and Atf2p between *S. cerevisiae* and *S. kudriavzevii*/*S. uvarum* observed in our sequence analysis. The suggested hypotheses, together with the provided *in silico* sequence comparison, indicated that *ATF1* and *ATF2*, from *S. kudriavzevii* and *S. uvarum,* were good targets for further investigation, which would have clarified their potential to enhance biotechnological flavour production. This investigation is described in the following chapter.

Conclusions

In this study we detected 2-keto acid decarboxylase (Aro10p) from *S. kudriavzevii* and *S. uvarum* as the possible aspirant to modify the aroma production capacity of *S. cerevisiae*. The heterologous *SkARO10* or *SuARO10* expression in a host *S. cerevisiae* resulted in increased production of isobutanol and isoamyl alcohol, and their acetate esters, when their amino acidic precursors were used as the nitrogen source. Significant differences in the aroma profile were also found during the fermentations of synthetic must. The analysis of decarboxylase activities in cell extracts revealed remarkable differences between SkAro10p and ScAro10p. Although both enzymes indicated similarly broad substrate specificity, ScAro10p showed a marked preference for phenylpyruvate (the precursor of 2-phenylethanol that confers a rose-like flavour), while the activities of SkAro10p for all the tested substrates were more or less equal. Hence employment of SkAro10p could lead to an overall aroma with a new flavour composition and a more complex profile. To postulate any analogous conclusion for SuAro10p, the enzyme assays needs to be completed.

Chapter 4

Comparison of enzymatic properties of the *S. kudriavzevii* and *S. uvarum* alcohol acetyltransferases and their impact on aroma-active compounds production

Submitted for publication in Frontiers in Microbiology

Short introduction

The *in silico* analysis described in Chapter 2 detected the *ATF1*- and *ATF2*-encoded proteins among the hits with the highest Grantham scores. Equally for *S. kudriavzevii* and *S. uvarum*, both alcohol acetyltransferases Atf1p and Atf2p clearly yielded the largest number of amino acid substitutions (together with Aro10p).

Alcohol acetyltransferases I and II (AATase I and II), codified by the *ATF1* and *ATF2* genes, catalyse in *S. cerevisiae* the reaction of higher alcohols with acetyl-CoA, which results in acetyl esters (Fujii *et al.*, 1994; Malcorps *et al.*, 1991; Minetoki *et al.*, 1993; Nagasawa *et al.*, 1998; Yoshioka and Hashimoto, 1981). It has been reported that Atf1p and Atf2p are responsible for the acetylation of a broad range of higher alcohols. Mainly isoamyl alcohol, but also other alcohols, such as propanol, isobutanol, hexanol and 2-phenylethanol, are esterified by Atf1p and Atf2p (Verstrepen *et al.*, 2003b). Nevertheless, the contributions of each enzyme seem to differ. The deletion and overexpression of *ATF2* resulted only in minor changes in the formation of acetate esters, which indicated that the *ATF2*-encoded enzyme plays only a minor role compared with the *ATF1*-encoded enzyme (Lilly *et al.*, 2006; Verstrepen *et al.*, 2003b).

Similarly to the study described in Chapter 3, this work aimed to clone genes *ATF1* and *ATF2* from *S. kudriavzevii* and *S. uvarum* into *S. cerevisiae* in order to examine and compare their impact on the production of the prime aroma-active higher alcohols and acetate esters. Another goal was to assess the substrate specificities and kinetic properties of the encoded enzymes.

Results

Effect of the *ATF1* and *ATF2* homologous genes on the formation of higher alcohols and esters

In order to verify the impact of the *ATF1* and *ATF2* genes from *S. kudriavzevii* and *S. uvarum* on the final content of higher alcohols and/or acetate esters, individual alleles (*SkATF1, SuATF1, SkATF2, SuATF2*) were cloned into the locus of *ATF1* or *ATF2* in a haploid strain of the wine *S. cerevisiae* T73 strain. The constructed mutants were named JET02Sk, JET02Su, JET03Sk and JET03Su, respectively (Table M.2, page 49). The formation of the major aroma-active higher alcohols and acetate esters was compared to that of *S. cerevisiae*. To exclude any other mutations that may have occurred in the allele replacement step, the original *ATF1* (or *ATF2*) allele was returned to its native position which resulted in the strain JET02Sc (or JET03Sc) that was then used as a reference in the aroma comparison.

Synthetic wine must fermentations by the strains with the different *ATF* alleles were performed, and the production of higher alcohols and acetate esters was analysed. Weight loss monitoring revealed that all the strains had similar fermentation rates with no differences (Figure 4.1).

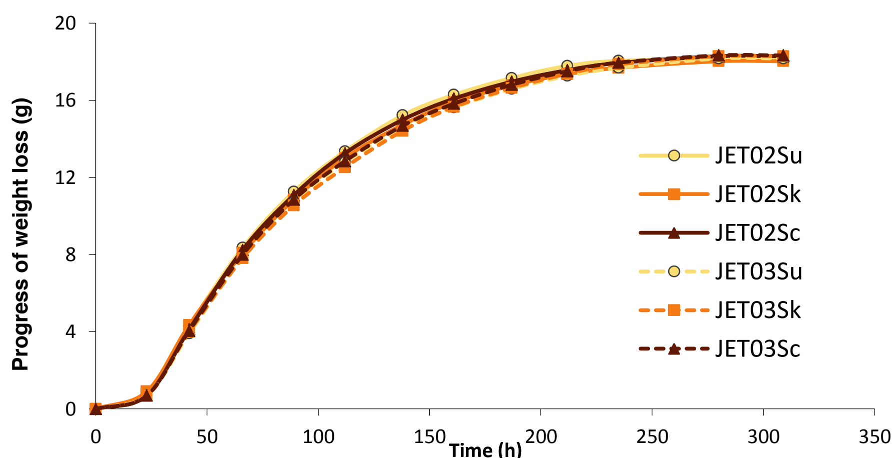


Figure 4.1. Progress of synthetic wine must fermentation. The fermentations were monitored as the progress of weight loss between two following measurements until the weight loss was negligible and the weight of the fermenting culture was constant.

Regarding aroma compounds production, the most significant differences were observed during 2-phenylethyl acetate production (Figure 4.2). The strains that harboured *ATF1* or *ATF2* from *S. kudriavzevii* and *S. uvarum* produced almost 2-fold larger amounts of 2-phenylethyl acetate compared to the corresponding reference strains with *ScATF1* or *ScATF2*. Strain JET02Sk also revealed *c.* 3-fold and JET02Su *c.* 2-fold larger amounts of isobutyl acetate than JET02Sc.

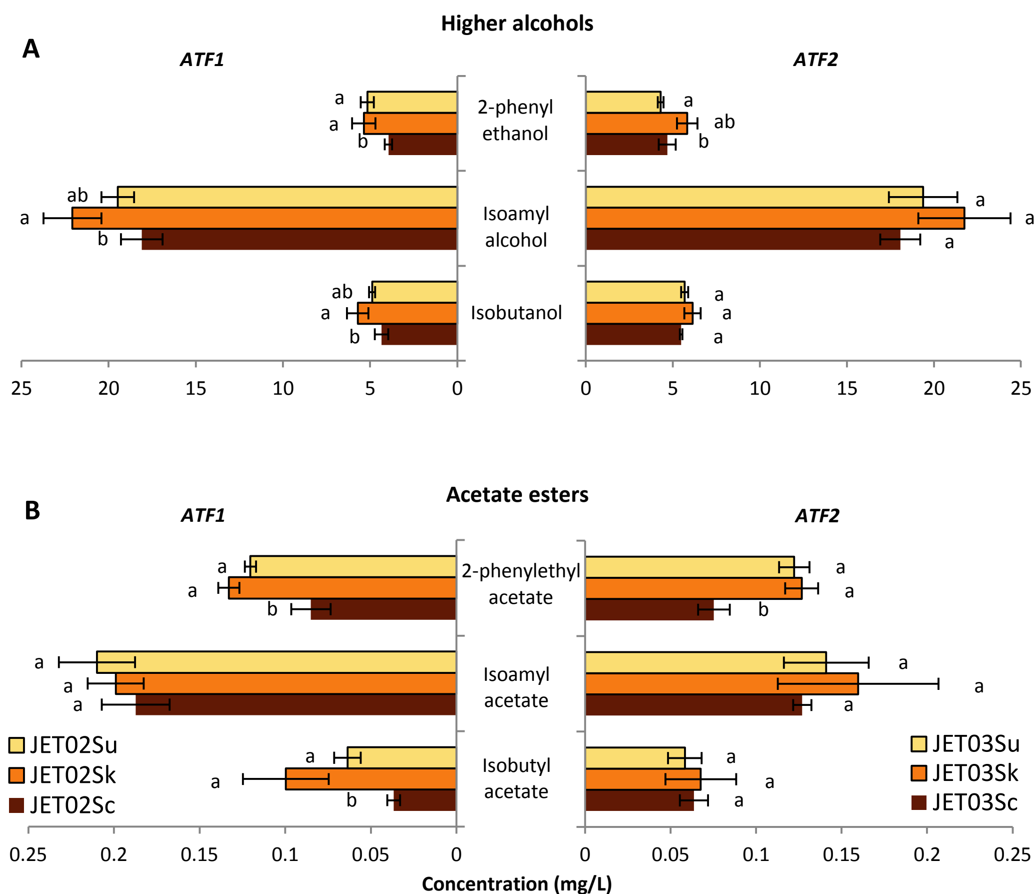


Figure 4.2. Production of higher alcohols (A) and acetate esters (B) by the JET02-derived strains that harboured the individual *ATF1* orthologues (left-oriented bars) and by the JET03-derived strains that harboured the individual *ATF2* orthologues (right-oriented bars) during synthetic wine must fermentation. The statistically significant differences among the strains were determined independently for each measured compound and are indicated by labels beside the columns.

During synthetic must fermentation several ethyl esters (ethyl hexanoate, ethyl decanoate, ethyl octanoate) were also detected. The comparison of the concentrations of the ethyl esters produced by the strains with the different *ATF* alleles did not reveal any significant differences (data not shown).

Since aroma active higher alcohols, and subsequently acetate esters, derive directly from their amino acidic precursors, another aim was to verify whether, and to what extent, the individual *ATF* alleles played a role in the production of higher alcohols and acetate esters from their corresponding amino acidic precursors. Cells were therefore cultivated with individual amino acids valine, leucine or phenylalanine as the sole nitrogen source and the corresponding higher alcohols and their esters were analysed. In the assay done of the increment in populations over time, all the strains presented a normal growth pattern under these conditions (Figure 4.3). This confirmed their ability to utilise these amino acids as the sole nitrogen source. The analysis of the higher alcohols and acetate esters produced by the JET02-derived strains (strains that harboured *ATF1* alleles from *S. kudriavzevii* or *S. uvarum*) showed significantly higher concentrations of 2-phenylethyl acetate in the phenylalanine-grown culture of the JET02Sk strain (Figure 4.4A). This strain, which harboured *SkATF1*, produced *c.* 1.9-fold higher concentrations of 2-phenylethyl acetate than the strain with *SuATF1* and *c.* 2.7-fold higher concentrations than reference strain JET02Sc. With the other analysed compounds only small and statistically insignificant differences were detected. In contrast, the JET03-derived strains (strains with introduced *SkATF2* or *SuATF2*) grown with the individual amino acids exhibited more differences during higher alcohols and acetate esters production. With valine as the nitrogen source, both corresponding derivatives, isobutanol and isobutyl acetate, were produced by the strain that carried *SkATF2* or *SuATF2* alleles in larger amounts (Figure 4.4B). Particularly, the isobutyl acetate concentrations were around 3-fold higher in

both strains than that produced by JET03Sc. A similar upward trend in favour of JET03Sk and JET03Su was observed during isoamyl acetate production when their amino acidic precursor leucine was used as the nitrogen source. JET03Sk showed a 2-fold and JET03Su a 2.5-fold increase in the isoamyl acetate concentration. The phenylalanine-grown cultures of JET03Sk and JET03Su exhibited 2.2-fold and 1.9-fold larger amounts of 2-phenylethyl acetate than the reference JET03Sc.

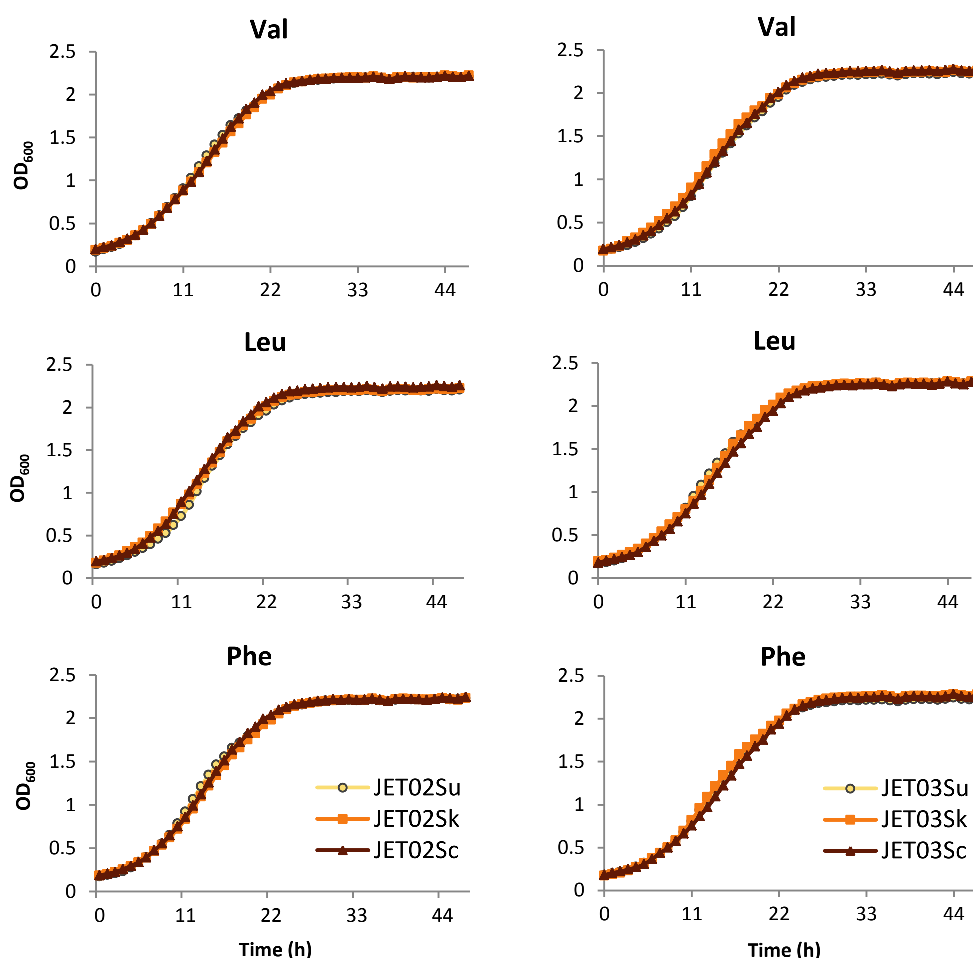
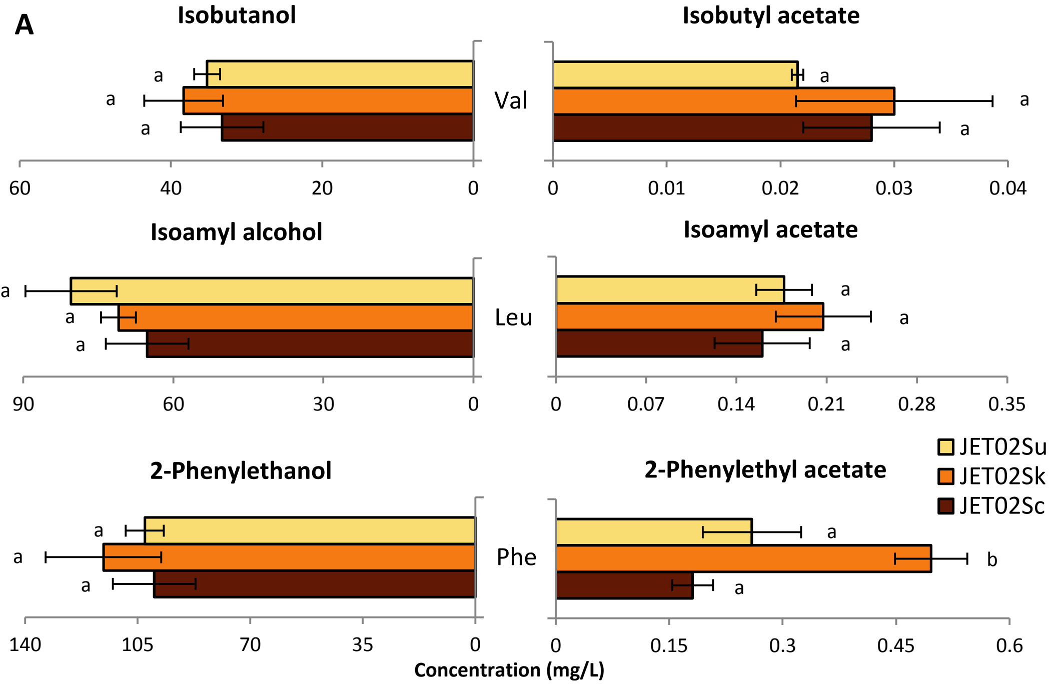


Figure 4.3. Growth of the JET02-derived strains that harboured the individual *ATF1* orthologues (A) and of the JET03-derived strains that harboured the individual *ATF2* orthologues (B) with the indicated amino acids as the nitrogen source.


A
Isobutanol
Isobutyl acetate
Val
Isoamyl alcohol
Isoamyl acetate
Leu
2-Phenylethanol
2-Phenylethyl acetate
Phe
JET02Su
JET02Sk
JET02Sc
Concentration (mg/L)

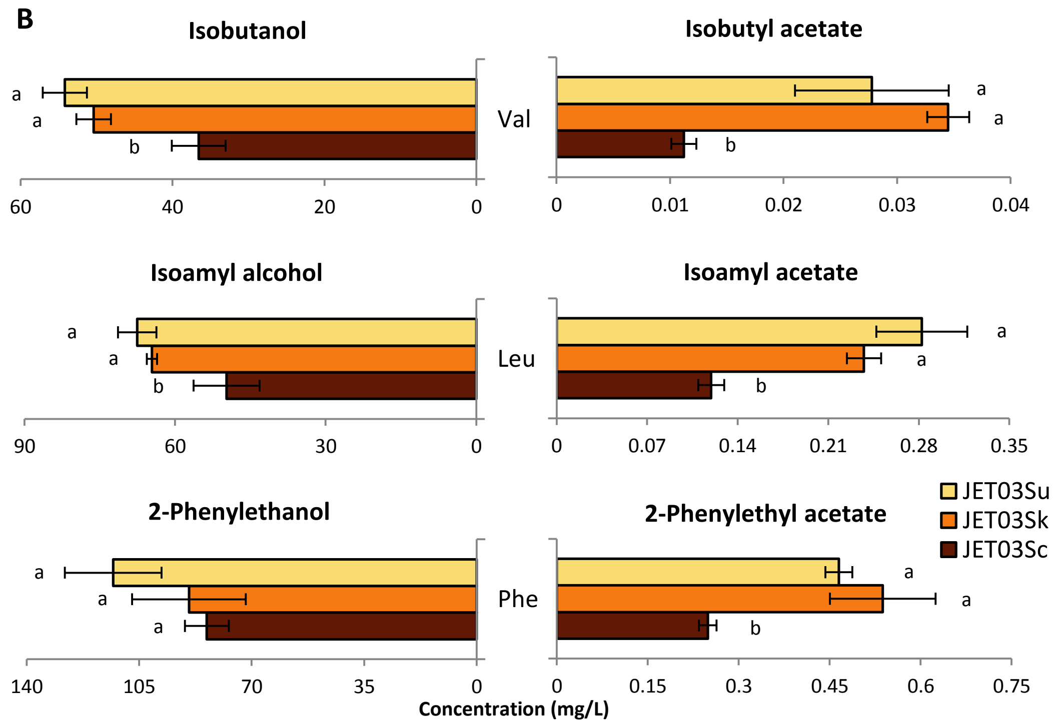

B
Isobutanol
Isobutyl acetate
Val
Isoamyl alcohol
Isoamyl acetate
Leu
2-Phenylethanol
2-Phenylethyl acetate
Phe
JET03Su
JET03Sk
JET03Sc
Concentration (mg/L)

Figure 4.4. Production of the higher alcohols and esters derived from the corresponding amino acidic precursors used as the nitrogen source (indicated in the graphs). A – Production of the higher alcohols and esters by the JET02-derived strains that harboured the individual *ATF1* orthologues. B - Production of the higher alcohols and esters by the JET03-derived strains that harboured the individual *ATF2* orthologues. The statistically significant differences among the strains were determined independently for each nitrogen source and are indicated by labels beside the columns.

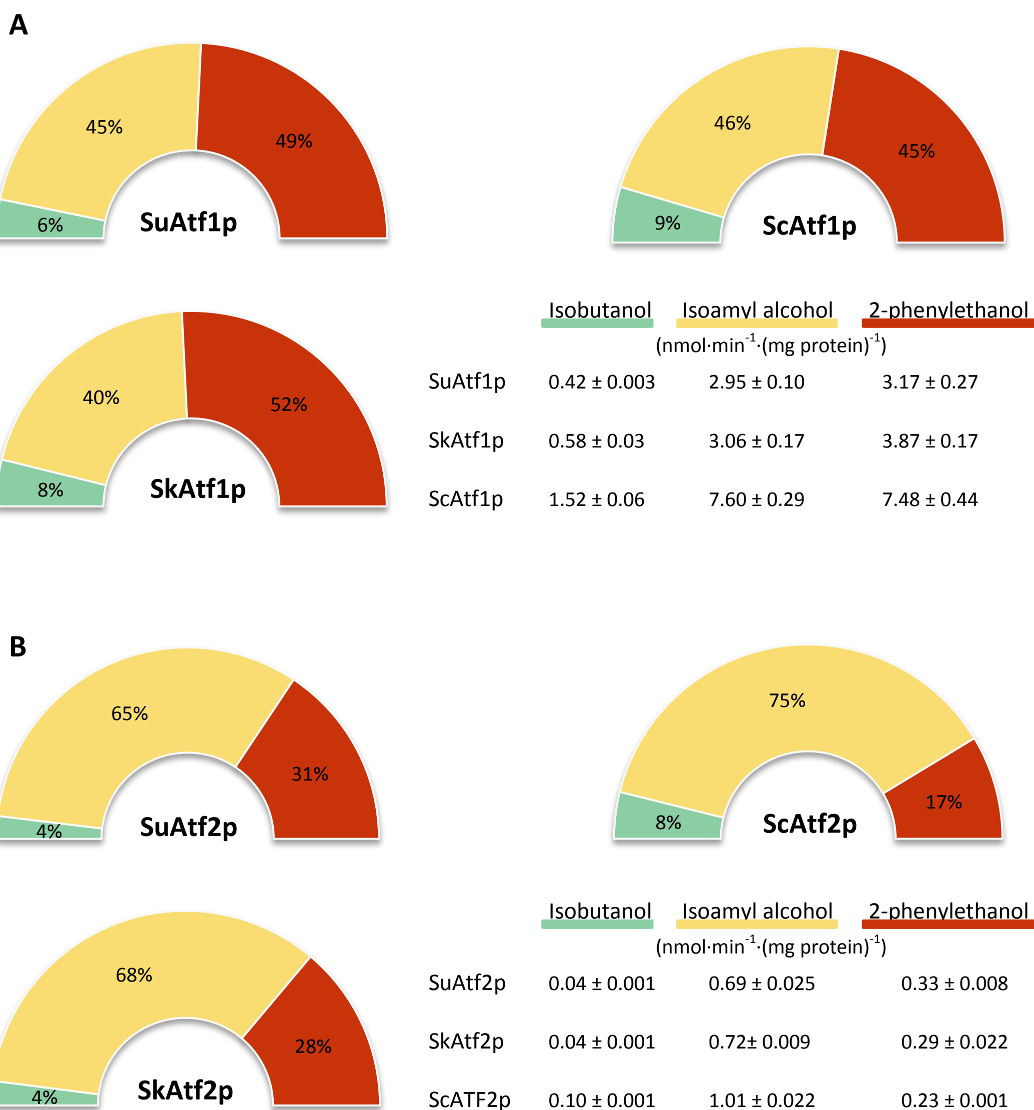
Comparison of the AATase substrate specificities

To determine and compare the AATase activities of Atf1p and Atf2p from the three different *Saccharomyces* species, the individual *ATF* genes were cloned into the plasmid pGREG526 and expressed in a host *S. cerevisiae* strain with the deleted *ATF1* and *ATF2* genes. In order to obtain sufficient AATase activity, the *ATF* genes were expressed under the control of the constitutive *TDH3* promoter. The kinetic parameters (K_m and V_{max}) were assessed for isoamyl alcohol as a substrate since isoamyl alcohol acetylation is known to be catalysed only by Atf1p and Atf2p. To avoid the isoamyl acetate breakdown, the measured catalytic reaction product, *IAH1* gene that codifies ester hydrolase, was also deleted in the host strain (BY4741atf1atf2iah1). AATase activity was determined in cell extracts over a wide range of isoamyl alcohol concentrations (0.01 mM – 100 mM) and was measured by isoamyl acetate formation which was analysed by head space gas chromatography. The activity measured in the cell extracts that expressed the *ATF1* orthologues showed typical Michaelis-Menten saturation kinetics. In contrast, the activity of all the AATases encoded by *ATF2* displayed an increasing tendency over the entire range of tested concentrations, without reaching saturation. Therefore, K_m and V_{max} were determined only for the enzymes encoded by the *ATF1* orthologues. The K_m and V_{max} values calculated for isoamyl alcohol displayed considerable differences among the individual Atf1 enzymes (Table 4.1). SkAtf1p showed almost 2-fold higher and SuAtf1p *c.* 3-fold higher K_m than ScAtf1p. Regarding V_{max}, both SkAtf1p and SuAtf1p exhibited around 2-fold lower values than ScAtf1p.

Table 4.1. The kinetic parameters of SkAtf1p, SuAtf1p and ScAtf1p measured in the cell extracts of the *S. cerevisiae* strain with *atf1 atf2 iah1* deletions

Cell extract	K_m (mM)	V_{max} (nmol.min^{-1}.(mg protein)$^{-1}$)
CLpSkATF1	57.4 ± 7.1	4.80 ± 0.26
CLpSuATF1	92.9 ± 10.9	5.69 ± 0.35
CLpScATF1	32.2 ± 2.2	9.99 ± 0.24

To further characterise enzymatic properties, substrate specificities were compared for three different amino-acid-derived higher alcohols as follows: isobutanol, isoamyl alcohol, and 2-phenylethanol. Specific activities were analysed at fixed substrate concentrations of 60 mM for isobutanol, 100 mM for isoamyl alcohol, and 30 mM for 2-phenylethanol. The cell extracts of all the strains that expressed the individual *ATF1* and *ATF2* orthologues exhibited activities for all the measured substrates. Interesting differences were observed between enzymes Atf1 and enzymes Atf2. When individual enzymatic activities were expressed as a percentage distributed among the total enzymatic activity of the measured substrates, enzymes Atf1 were found to be more or less evenly proportional of the enzymatic activities towards isoamyl alcohol and 2-phenylethanol (Figure 4.5A). In contrast, enzymes Atf2 showed considerably stronger activity towards isoamyl alcohol than the other substrates. Moreover, while all three Atf1 enzymes displayed an almost identical percentage distribution of individual enzymatic activities, the proportion of the enzyme activity of SkAtf2p and SuAtf2p to 2-phenylethanol was almost 2-fold greater, and 2-fold lower to isobutanol than for ScAtf2p (Figure 4.5B).



	Isobutanol	Isoamyl alcohol	2-phenylethanol
		(nmol·min^{-1}·(mg protein)$^{-1}$)	
SuAtf1p	0.42 ± 0.003	2.95 ± 0.10	3.17 ± 0.27
SkAtf1p	0.58 ± 0.03	3.06 ± 0.17	3.87 ± 0.17
ScAtf1p	1.52 ± 0.06	7.60 ± 0.29	7.48 ± 0.44

	Isobutanol	Isoamyl alcohol	2-phenylethanol
		(nmol·min^{-1}·(mg protein)$^{-1}$)	
SuAtf2p	0.04 ± 0.001	0.69 ± 0.025	0.33 ± 0.008
SkAtf2p	0.04 ± 0.001	0.72± 0.009	0.29 ± 0.022
ScATF2p	0.10 ± 0.001	1.01 ± 0.022	0.23 ± 0.001

Figure 4.5. The relative alcohol acetyltransferase activities of the Atf1 (A) and Atf2 (B) enzymes expressed as a percentage distributed among the total enzymatic activity of the measured substrates. The presented values are averages and mean deviations of 3 replicates. The activities of the different Atf1 and Atf2 enzymes were measured in the cell extracts of the *S. cerevisiae* strain with *atf1 atf2 iah1* deletion at a concentration of 60 mM for isobutanol, 100 mM for isoamyl alcohol and 30 mM for 2-phenylethanol.

Discussion

One of the goals aimed to investigate and compare the effects of *ATF1* and *ATF2* from *S. kudriavzevii* and *S. uvarum* on the production of the aroma-active higher alcohols and acetate esters that derived from the corresponding amino acids. The gas chromatography analysis revealed increasing amounts of acetate esters for the strains that expressed alleles *SkATF2* and *SuATF2*. One possible explanation is the amino acid substitutions observed during the aforementioned *in silico* analysis. Both sequences, SkAtf2p and SuAtf2p, revealed a large number of substitutions (20% and 25%, respectively) compared to ScAtf2p. Only four substitutions in SkAtf2p and five in SuAtf2p were classified as radical according to the Grantham scale. None of the substitutions were found in the two conserved regions, WRLICLP and HXXXD, hypothesised as parts of the active site (D'Auria, 2006; Nagasawa *et al.*, 1998). Despite the small number of radical substitutions, or none, at the putative active site, the large number of total substitutions, or combinations of some, might play role in the differences observed during acetate ester production. While the Atf1p sequences also showed a large number of amino acid changes (SkAtf1p *vs.* ScAtf1p 17%, SuAtf1p *vs.* ScAtf1p 18%), the comparison of the strains that expressed the individual *ATF1* orthologues gave no significant differences during the production of higher alcohols and acetate esters (except for 2-phenylethyl acetate). Further research by mutagenesis assays could clarify a possible correlation between amino acid substitutions and the different higher alcohol and acetate ester productions.

The JET03-derived strains with introduced *SkATF2* and *SuATF2* (JET03Sk and JET03Su, respectively) also exhibited larger amounts of higher alcohols than JET03Sc. One possible explanation could be the catalytic activity of esterases that catalysed ester breakdown, such as Iah1p (Fukuda *et al.*, 2000; Lilly *et al.*, 2006). In hypothetical terms, the superior concentrations of the higher alcohols produced by JET03Sk and JET03Su could result from Iah1p,

which degraded the large amounts of acetate esters produced as a result of SkAtf2p and SuAtf2p activity. As previously described (Fukuda *et al.*, 1998), the balance between ester-synthesising and ester-degrading enzymes leads to an optimal balance between higher alcohols and acetate esters.

It has been shown that Atf1p and Atf2p in *S. cerevisiae* are able to transfer an activated acetate group to a wide variety of substrates with an alcohol group, and thus display wide substrate specificity for alcohol cosubstrates (Verstrepen *et al.*, 2003b). Similarly, our findings showed wide substrate specificity for Atf1p and Atf2p from *S. kudriavzevii* and *S. uvarum*. The activities observed in the extracts of the cells that expressed the individual *ATF2* genes were an order of magnitude lower than the activities in the cell extracts that expressed *ATF1*genes. It has been hypothesised that Atf2p might be important for different metabolic processes other than Atf1p. For instance, Cauet *et al.* (1999) suggested that Atf2p plays a key role in the detoxification of 3β-hydroxysteroids. Our results also indicate that the Atf2 proteins from *S. kudriavzevii* and *S. uvarum* play only a minor role in acetate ester formation compared to Atf1p, similarly to that observed in *S. cerevisiae* (Lilly *et al.*, 2006; Verstrepen *et al.*, 2003b).

Interesting differences were observed in the kinetic properties of the alcohol acetyltransferases among the Atf1p from *S. cerevisiae*, *S. kudriavzevii* and *S. uvarum* towards isoamyl alcohol. Of the two cosubstrates, isoamyl alcohol was chosen for determining the kinetic properties because previous studies have shown alcohol to be a rate-limiting cosubstrate during alcohol acetyltransferases reactions (Minetoki *et al.*, 1993). However, we did not observe any correlation between the different K_m values and isoamyl acetate formation. The strains that expressed the individual *ATF2* orthologues revealed marked differences in isoamyl acetate concentrations. However, unlike the kinetic data of the Atf1 enzymes, the individual Atf2p showed a linear increase of isoamyl acetate synthesis with isoamyl alcohol

concentrations, and did not reach saturation, not even with the highest concentration (100 mM) used in the assay. Higher concentrations resulted in non-specific peaks, as detected by gas chromatography. As Malcorps *et al.* (1991) explained, one possible reason is that a high isoamyl alcohol concentration in the enzyme assay could modify the enzyme properties through conformational changes or denaturation, or could induce non-specific alcoholysis of the acetyl-enzyme intermediate. The higher isoamyl alcohol concentrations needed to determine the Atf2p kinetic properties indicated that the K_m values of the Atf2 enzymes were higher than those of Atf1p, which is in accordance with the observations made by Nagasawa et al. (1998). Regarding the Atf1 enzymes, the K_m (32.2 mM) for *S.cerevisiae* Atf1p calculated in our study was similar to that observed for *S. cerevisiae* sake strain Kyokai No. 7 (29.8 mM) (Minetoki *et al.*, 1993) and for *S. cerevisiae* beer strain NCYC 366 (25mM) (Malcorps and Dufour, 1992). These similar values indicate that the range of isoamyl alcohol concentrations used in our assays did not lead to the aforementioned modifications in the Atf1 enzymatic properties.

In the work discussed in Chapter 1 we observed differences in the production of higher alcohols and acetate esters by *S. kudriavzevii* and *S. uvarum* compared to *S. cerevisiae*. These differences could be explained by the different kinetic properties of the individual Atf1 enzymes and/or the activities of the Atf2 enzymes observed in this study. Another reason could be variations in the expression levels of the *ATF* genes as the expression levels have been demonstrated to be an important factor for ester synthesis (Malcorps *et al.*, 1991; Verstrepen *et al.*, 2003b). For instance, (Gamero *et al.*, 2013) observed considerable differences in aroma production during wine fermentation by these three *Saccharomyces* species. The subsequent expression analysis revealed, besides others, the up-regulation of *ATF1* in *S. uvarum* and the up-regulation of *ATF2* in both *S. uvarum* and *S. kudriavzevii*

compared to *S. cerevisiae* (Gamero *et al.*, 2014). The existence of another as yet unknown enzyme with AATase activity in the yeast proteome has also been proposed (Malcorps and Dufour, 1992; Verstrepen *et al.*, 2003b). Its putative effect on acetate ester production by the three different *Saccharomyces* species cannot be ruled out.

Conclusion

The amino acid variations noted in the orthologues Atf1p and Atf2p of *S. kudriavzevii, S. uvarum* and *S. cerevisiae* indicated a possible impact on the distinct properties of the enzymes characterised herein. Together with differences in gene expression levels, these distinct enzymatic properties appear to play an important role in the differences among these three *Saccharomyces* species during acetate ester formation.

General summary

Probably every researcher, who dedicated its scientific life to yeast, has heard, read or said that yeasts have been associated with human activities for millennia. This is because people like alcoholic beverages and yeasts are able to produce alcoholic beverages. Of course, this would be an underrated statement because yeasts are highly valued for other merits, not just for their ethanol production. Besides wine and beer they are essential during the production of bread and other fermented foodstuff, such as cheese or sake. During these "traditional" yeast-related applications, *S. cerevisiae* has become the main workhorse. *S. cerevisiae* also serves as a tool and model to understanding of eukaryotic cell biology. In this context, the knowledge accumulated on numerous molecular basis of a wide variety of industrially relevant properties has made *S. cerevisiae* an important microorganism in a large number of other production processes, such as drug and biofuel production.

During the last years several projects have been started to explore the academic and industrial potential of other, so-called non-conventional, yeast species. Part of one of these projects, namely Cornucopia project, was also the work described in this thesis. The objectives of the Cornucopia project were based on biodiversity of hundreds of yeast species, which may harbour sources for novel applications, or for innovation or improvement of already existing applications.

One of the Cornucopia aims focused on yeast biodiversity in terms of aroma and flavour compounds production. It is known that different yeast species and strains contribute to the sensory and organoleptic characteristics of the fermented products in a different way (Romano *et al.*, 2003). The main objects of the research presented in this dissertation were *S. kudriavzevii* and *S. uvarum*. In previous studies these two species, closely related to *S. cerevisiae*, showed remarkable differences during the production of higher alcohols and esters when comparing the three species (Gamero *et al.*, 2013;

Pérez-Torrado *et al.*, 2015).

Higher alcohols and esters formed by yeast belong to key components of overall flavour and aroma in the fermented products. In spite of having aroma themselves, the main importance of higher alcohols in terms of overall aroma lies in the fact that they are precursors of acetate esters. Given their floral and fruity character, acetate esters are regarded as very desirable aroma contributors. Acetate esters are formed from acetyl-CoA and higher alcohol in a condensation reaction mediated by alcohol acetyltransferases I and II (encoded by *ATF1* and *ATF2*). Higher alcohols are synthesised from 2-keto acids which derive either from glycolysis or from transamination of amino acids during amino acid catabolic pathway, also known as Ehrlich pathway. This pathway consists of amino acid transamination that yields the 2-keto acid, which is subsequently decarboxylated to the corresponding aldehyde. The higher alcohol then originates from a reduction of the aldehyde. Among the most significant aroma-active acetate esters belong isobutyl acetate, isoamyl acetate and 2-phenylethyl acetate, of which precursors are isobutanol, isoamyl alcohol and 2-phenylethanol. These higher alcohols derive from valine, leucine and phenylalanine degradation, respectively.

As already mentioned, *S. kudriavzevii* and *S. uvarum* revealed significant differences during the formation of these important flavour-active compounds as compared to *S. cerevisiae*. The main goal of this PhD thesis was to obtain a deeper fundamental understanding of the molecular aspects behind these differences.

Chapter 1 investigates how the three *Saccharomyces* species differ during the production of higher alcohols and acetate esters that derived directly from their amino acidic precursors. This objective was fulfilled by carrying out cultivations of *S. cerevisiae*, *S. uvarum* and *S. kudriavzevii* strains with individual amino acids as the sole nitrogen source, followed by analysis and comparison of the produced higher alcohols and acetate esters. The global

volatile compound analysis revealed that *S. kudriavzevii* produced large amounts of higher alcohols, whereas *S. uvarum* excelled in the production of acetate esters. Particularly from phenylalanine, *S. uvarum* produced the largest amounts of 2-phenylethyl acetate, while *S. kudriavzevii* obtained the greatest 2-phenylethanol formation from this precursor. Altogether, the data discussed in Chapter 1 indicated differences in the amino acid metabolism and subsequent production of flavour-active higher alcohols and acetate esters among these closely related *Saccharomyces* species.

In Chapter 2 we put a question whether these differences could be caused by certain genes from amino acid catabolic pathway. Of course, one can consider this inquiry as oversimplification because amino acid catabolism and the production of higher alcohols and esters are controlled by complex regulation mechanisms. Yet, we decided to explore the amino acid divergences in 23 orthologues enzymes from *S. kudriavzevii* or *S uvarum* vs. *S. cerevisiae*, involved in higher alcohols and acetate esters production. *In silico* screening was based on multiple sequence alignments and the Grantham scoring. The Grantham scoring provides a quantitative assessment of the biochemical distances between amino acids, on the basis of their composition, polarity and molecular volume, and according to increasing dissimilarity classifies the amino acid substitutions as conservative or radical (Grantham, 1974; Li *et al.*, 1984). The analysis released three sequences that were evaluated with significantly higher Grantham scores: 2-keto acid decarboxylase encoded by *ARO10*, and two alcohol acetyltransferases encoded by *ATF1* and *ATF2.*

The studies described in Chapter 3 and Chapter 4 were then designed to evaluate the effect of individual *ARO10*, *ATF1* and *ATF2* alleles from *S. kudriavzevii* and *S. uvarum* on the production of higher alcohols and the corresponding acetate esters. The studies also aimed to assess kinetic properties and substrate specificities of the respective enzymes.

Chapter 3 was focused on *ARO10*. The results showed that a replacement

of *ScARO10* in *S. cerevisiae* by *SkARO10* or *SuARO10* influenced differently production of higher alcohols and esters when the cells were cultivated in the presence of individual amino acids or in the synthetic wine must. The analysis of higher alcohols and esters revealed particularly enhanced formation of isobutanol, isoamyl alcohol and their esters when their amino acidic precursors were used as the nitrogen source. Although not completed for SuAro10p, also enzyme assays yielded interesting results. Significant differences were found when substrate specificities of SkAro10p were compared with those of ScAro10p by their expression in a 2-keto acid decarboxylase-null *S. cerevisiae* strain. The cell extracts with expressed ScAro10p showed greater activity for phenylpyruvate (the precursor of 2-phenylethanol that confers a rose-like flavour), which suggests this phenylalanine-derivative to be the preferred substrate, whereas the decarboxylation activities measured in the cell extracts with SkAro10p ranged with all the tested substrates at the same level.

Analogous to Chapter 3, the study from Chapter 4 examined the impact of the *ATF1* and *ATF2* alleles from *S. kudriavzevii* (*SkATF1*, *SkATF2*) and *S. uvarum* (*SuATF1*, *SuATF2*) on the production of aroma compounds and the kinetic properties of the enzymes. The heterologous expression of the individual *ATF1* and *ATF2* alleles in a host *S. cerevisiae* resulted in the enhanced production of several higher alcohols and acetate esters. Particularly, an increase of 2-phenylethyl acetate production by the strains that harboured *ATF1* and *ATF2* alleles from *S. kudriavzevii* and *S. uvarum* was observed. When grown with individual amino acids as the nitrogen source, the strain that harboured *SkATF1* showed particularly high 2-phenylethyl acetate production and the strains with introduced *SkATF2* or *SuATF2* revealed increased production of isobutyl acetate, isoamyl acetate, and 2-phenylethyl acetate compared to the reference strains with endogenous *ATF1/ATF2* alleles. The alcohol acetyltransferase activities of the individual *ATF1*-encoded

and *ATF2*-encoded enzymes measured in the cell extracts of the *S. cerevisiae atf1 atf2 iah1* triple-null strain were detected for all the measured substrates. This indicated that *S. kudriavzevii* and *S. uvarum* Atf enzymes had broad range substrate specificity like *S. cerevisiae* Atf enzymes. Individual Atf1 enzymes exhibited markedly different kinetic properties since SkAtf1p showed *c.* 2-fold higher and SuAtf1p *c.* 3-fold higher K_m for isoamyl alcohol than ScAtf1p.

All together the data described and discussed in this thesis indicate that the amino acid variations observed between the decarboxylases encoded by the orthologues *ARO10* genes and between the AATases encoded by the orthologues *ATF1* and *ATF2* genes could be the reason for the distinct enzyme properties, which possibly lead to the enhanced production of several flavour compounds.

In addition to Aro10p, Atf1p and Atf2p, the *in silico* analysis revealed an interesting result in Sfa1p, a bifunctional enzyme that displays the glutathione-dependent formaldehyde dehydrogenase activity required for formaldehyde detoxification, and the alcohol dehydrogenase activity involved in the formation of higher alcohols (Wehner *et al.*, 1993). When compared with *S. cerevisiae*, the Sfa1p sequence from *S. kudriavzevii* contained 28 amino acid substitutions, of which 5 were detected as radical. Interestingly, when the substitutions were expressed as a Grantham score these five radical substitutions comprised c. 43% of the Grantham score for all the substitutions. The same calculation performed in Sfa1p from *S. uvarum* resulted in only 19%. Furthermore, an additional *in silico* analysis, in which we examined amino acid divergences in the key transcriptional regulator of *ARO10* gene, i.e. Aro80p (Iraqui *et al.*, 1999), revealed approximately 2-fold higher Grantham scores than those of Aro10p, Atf1p, and Atf2p. These results make *SFA1* and *ARO80* interesting candidates for further investigation.

The increased yields of aroma components observed herein may interest several industry sectors. Evidently, the research in this dissertation was based

mainly on GMO strategy, which still remains unacceptable to food and beverage industry. Yet, the knowledge on the important enzymes involved in higher alcohols and acetate esters biosynthesis could be of scientific as well as of applied interest to other industries, such as fragrance and perfume industry, where a GMO strategy is not so controversial. This knowledge could also prove useful for hybridisation or directed evolution approaches allowing production of novel species with improved fermentative characteristics, which could contribute to modulating the aroma of fermentation products.

Conclusions

Conclussions

The species *S. kudriavzevii* and *S. uvarum* differed from their closely related species *S. cerevisiae* during the production of aroma-active higher alcohols and acetate esters using their amino acid precursors.

The global volatile compound analysis revealed that *S. kudriavzevii* produced large amounts of higher alcohols, whereas *S. uvarum* excelled in the production of acetate esters when grown with distinct nitrogen sources.

In silico analysis, that used the Grantham scoring to measure the biochemical distances of amino acid substitutions in the orthologues enzymes involved in higher alcohols and acetate esters formation, showed the most radical non-synonymous changes in Aro10p orthologues, Atf1p orthologues, and Atf2p orthologues from *S. kudriavzevii* and *S. uvarum* vs. *S. cerevisiae*.

The expression of the individual *ARO10* or *ATF1*/*ATF2* alleles from *S. kudriavzevii* or *S. uvarum* improved capacity of *S. cerevisiae* for the production of several higher alcohols and acetate esters. This observation could be of interest to aroma-related industries.

Comparative analysis of the enzymes encoded by the orthologous *ARO10*, *ATF1* and *ATF2* showed remarkable differences in their substrate specificities and kinetic properties. ScAro10p showed a marked preference for phenylpyruvate (the precursor of 2-phenylethanol that confers a rose-like flavour), while the activities of SkAro10p for all the tested substrates were more or less equal. Employment of SkAro10p could lead to an overall aroma with a new flavour composition and a more complex profile.

The alcohol acetyltransferase activities of the individual *ATF1*-encoded and *ATF2*-encoded enzymes indicate that *S. kudriavzevii* and *S. uvarum* Atf enzymes have broad range substrate specificity like *S. cerevisiae* Atf enzymes. Individual Atf1 enzymes exhibited markedly different kinetic properties since

SkAtf1p showed c. 2-fold higher and SuAtf1p c. 3-fold higher K_m for isoamyl alcohol than ScAtf1p.

The differences found among the three *Saccharomyces* species in the aroma-active higher alcohols and acetate ester formation may be due, to some extent, to the distinct properties of Aro10 and Atf enzymes, possibly caused by the amino acid variations.

Additional *in silico* analysis revealed significant differences with regard to radical substitutions in Sfa1p orthologues and Aro80p orthologues. Their kinetic properties and impact on the aroma production will likely to be a subject of future investigation.

Resumen general

Probablemente todos los investigadores que han dedicado su vida científica al conocimiento de las levaduras, ha escuchado, leído o mencionado, que las levaduras se han asociado con la actividad humana durante miles de años. Esto es porque las levaduras son los microrganismos responsables de la fermentación alcohólica y, por tanto, son capaces de producir bebidas alcohólicas. Por supuesto esto sería una definición incompleta, ya que las levaduras también tienen un papel relevante en otros procesos biotecnológicos. Además de ser esenciales para la elaboración del vino y la cerveza, también son imprescindibles en la producción de pan y otros alimentos fermentados, como el queso o sake. *Saccharomyces cerevisiae* también sirve como una herramienta y organismo modelo para la comprensión de la biología de células eucariotas. En este contexto, el conocimiento científico acumulado, y su amplia variedad de propiedades de interés industrial, ha hecho a *S. cerevisiae* un microorganismo importante en un gran número de procesos, tales como la producción de ingredientes, las fermentaciones alcohólicas o la obtención de biocombustible.

Durante los últimos años, en varios proyectos de investigación se ha comenzado a explorar el potencial académico e industrial de otras especies de levaduras, y que se conocen como levaduras no convencionales. Uno de estos proyectos es Cornucopia (Marie Curie Training), proyecto en el que se enmarca la presente tesis. El objetivo global del proyecto Cornucopia fue estudiar la biodiversidad de levaduras no convencionales, buscando nuevas aplicaciones, así como el uso de estas levaduras para mejora la innovación en procesos ya existentes. Sin embargo, uno de los subjetivos, y donde se enmarca la presente tesis, es el estudio de la biodiversidad de las levaduras, centrándose en la producción de compuestos relacionados con el aroma y el sabor. Se sabe que diferentes cepas y especies de levaduras contribuyen a las características sensoriales y organolépticas de los productos fermentados de manera diferente (Romano *et al.*, 2003). En la presente tesis nos hemos

centrado en el estudio del papel de las especies *S. uvarum* y *S. kudriavzevii* en la síntesis de aromas y como principal aplicación en la elaboración de vinos. En estudios anteriores, estas dos especies, estrechamente relacionadas con *S. cerevisiae*, mostraron diferencias notables durante la producción de alcoholes superiores y ésteres cuando se comparaban con *S. cerevisiae* (Gamero *et al.,* 2013; Pérez-Torrado *et al.*, 2015).

Los alcoholes superiores y ésteres formados por las levaduras, son componentes claves en el sabor y el aroma de los productos fermentados. En el caso de los alcoholes superiores, además del interés por si solos, también son relevantes por ser los precursores de los ésteres de acetato. Dado el carácter floral y afrutado de los ésteres de acetato, estos son considerados como compuestos muy deseables en el aroma de bebidas fermentadas. Los ésteres de acetato se forman a partir de acetil-CoA y alcoholes superiores en una reacción de condensación mediada por el alcohol acetiltransferasas I y II (codificado por *ATF1* y *ATF2*).

Tal y como hemos mencionado previamente, *S. kudriavzevii* y *S. uvarum* presentan diferencias muy significativas en la formación de estos compuestos aromáticos al comparar con *S. cerevisiae*. Por lo tanto, el principal objetivo de la presenten tesis fue profundizar en la comprensión de los aspectos moleculares básicos responsables de las estas diferencias.

En el capítulo 1 se estudia cómo las tres especies del género Saccharomyces difieren en la producción de aromas a partir de los aminoácidos precursores de dichos compuestos. Este objetivo se llevó a cabo mediante el crecimiento de cepas de *S. cerevisiae*, *S. uvarum* y *S. kudriavzevii* usando como única fuente de nitrógeno, los correspondientes aminoácidos de forma individual, y siguiendo la producción de alcoholes superiores y ésteres de acetato. El análisis global de los resultados obtenidos en este capítulo indicó que *S. kudriavzevii* produce una mayor cantidad de alcoholes superiores, mientras que *S. uvarum* se caracteriza por ser la especie que

produce mayor cantidad de ésteres de acetato. En concreto, *S. uvarum* es la especie que produce las mayores cantidades de acetato de 2-feniletilo y *S. kudriavzevii* del precursor de dicho acetato, el 2-feniletanol. Los resultados obtenidos en este capítulo nos indican diferencias importantes en el metabolismo de los aminoácidos y la producción de alcoholes superiores, así como de ésteres de acetato entre las tres especies del género *Saccharomyces*, estrechamente relacionadas.

En el capítulo 2, planteamos si estas diferencias podrían ser debidas a variaciones en algunos de los genes implicados en la ruta catabólica de los aminoácidos. Por supuesto, esta aproximación se puede considerar demasiado simplista ya que el catabolismo de aminoácidos y la producción de alcoholes superiores y ésteres son rutas muy complejas. Sin embargo, decidimos explorar las divergencias aminoacídicas en 23 enzimas ortólogos que participan en la producción de alcoholes superiores y ésteres de acetato en las especies *S. kudriavzevii* o *S. uvarum*, comparadas con las secuencias de *S. cerevisiae*. Para alcanzar este objetivo se realizó un estudio *in silico* de un alineamiento múltiple de secuencias y un posterior análisis de las distancias de Grantham, basadas en las diferencias de las propiedades físico-químicas de los reemplazamientos aminoácidos (Grantham, 1974; Li *et al.*, 1984). El análisis reveló que tres secuencias mostraron valores de Grantham significativamente más elevados: la 2-cetoácido descarboxilasa codificada por el gen *ARO10*, y dos alcohol acetil transferasas, codificadas por los genes *ATF1* y *ATF2*.

Los estudios llevados a cabo en los capítulos 3 y 4 se diseñaron para evaluar el efecto de forma individual de los alelos *ARO10*, *ATF1* y *ATF2* de las especies *S. kudriavzevii* y *S. uvarum*, en la producción de alcoholes superiores y ésteres de acetato respectivamente. En estos capítulos también nos centramos en evaluar las propiedades cinéticas y de especificidad por sustrato de las respectivas enzimas.

En el capítulo 3 nos centramos en *ARO10*. Los resultados mostraron que cuando reemplazamos el alelo *ARO10* en una cepa de *S. cerevisiae* donde hemos sustituido dicho gen por el alelo de *S. kudriavzevii* o de *S. uvarum*, se observan diferencias en la producción de alcoholes superiores y de ésteres de acetato al cultivar dichas cepas en un medio usando como única fuente de nitrógeno los diferentes aminoácidos de forma individual, o bien usando como medio de cultivo mosto sintético. Las diferencias más significativas se observan en la formación de isobutanol y alcohol isoamílico. Aunque no se ha completado para el alelo de *S. uvarum*, los ensayos enzimáticos para el enzima de *S. kudriavzevii* dieron resultados interesantes. Se encontraron diferencias significativas cuando se analizó la especificidad de sustrato del enzima SkAro10p, comparando con el enzima ScAro10p. Los extractos celulares cuando se expresaba ScAro10p mostraron una mayor actividad por el fenilpiruvato (el precursor de 2-feniletanol que confiere aroma a rosa), lo que sugiere que este derivado de la fenilalanina es el sustrato preferido para dicho enzima; mientras que las actividades de descarboxilación medidas en los extractos celulares con SkAro10p mostraron afinidad por todos los sustratos ensayados al mismo nivel.

Similar al capítulo 3, en el capítulo 4 estudiamos el impacto en la producción de aromas y de las propiedades enzimáticas de las proteínas codificadas por los alelos de *ATF1* y *ATF2* procedentes de *S. kudriavzevii* (*SkATF1, SkATF2*) y *S. uvarum* (*SuATF1, SuATF2*). La expresión individual de los alelos *ATF1* y *ATF2* procedentes de *S. kudriavzevii* y *S. uvarum* en una cepa de *S. cerevisiae* donde se habían disrumpido dichos genes, mostraron, al igual que con *ARO10*, diferencias en la producción de alcoholes superiores y ésteres de acetato. Se observó sobre todo un incremento en la producción del acetato de 2-feniletanol en las cepas con los alelos procedentes de *S. kudriavzevii* y *S. uvarum*. Cuando se cultivaron dichas cepas en un medio usando como única fuente de nitrógeno los diferentes aminoácidos de forma

individual, la cepa que tenía el alelo *SkATF1*, mostró una alta producción de acetato de 2-feniletilo y las cepas con los alelos *SkATF2* o *SuATF2* mostraron un aumento en la producción de acetato de isobutilo, acetato de isoamilo, y acetato de 2-feniletilo, en comparación con la cepas de referencia con los alelos *ATF1*/*ATF2* endógenos. Las actividades alcohol acetil transferasa de los enzimas codificados por *ATF1* y *ATF2* se midieron en extractos celulares en una cepa de *S. cerevisiae* triple mutante *atf1 atf2 iah1*. Los resultados indicaron que los enzimas Atf de *S. kudriavzevii* y *S. uvarum* tenían una amplia especificidad de sustrato comparado con los enzimas de *S. cerevisiae*. Las propiedades enzimáticas de SkAtf1p mostraron diferencias en la K_m para el alcohol isoamílico siendo estas dos unidades superiores a la mostrada por ScAtf1p y de 3 veces superior para SuAtf1p.

Todos los datos descritos y analizados en este trabajo indican que las variaciones de aminoácidos observadas entre las descarboxilasas codificadas por los genes ortólogos *ARO10* y entre las AATases codificadas por los genes ortólogos *ATF1* y *ATF2* podrían ser la razón de las diferencias enzimáticas observadas, y por tanto también podrían ser la razón de la mejora en el aroma de los vinos al fermentar con las especies *S. kudriavzevii* y *S. uvarum* respecto a *S. cerevisiae*.

Además de Aro10p, Atf1p y Atf2p, el análisis *in silico* reveló un resultado interesante en Sfa1p, una enzima bifuncional que muestra una actividad formaldehído deshidrogenasa dependiente de glutatión requerida para la desintoxicación de formaldehído, y la actividad alcohol deshidrogenasa, implicada en la formación de alcoholes superiores (Wehner *et al.,* 1993). Cuando se compara la secuencia Sfa1p de *S. kudriavzevii* con la del gen de *S. cerevisiae*, se observaron 28 sustituciones de aminoácidos, de los cuales 5 fueron detectados como radicales. Curiosamente, cuando estos cambios se analizaron usando las distancias de Grantham, estas cinco sustituciones fueron las responsables del 43% de la puntuación de Grantham para todas las

sustituciones. El mismo cálculo se realizó en Sfa1p de *S. uvarum* y en este caso dicho parámetro resultó ser sólo del 19%. Además, un análisis adicional *in silico*, en el que se examinaron las divergencias de aminoácidos del factor transcripcional del gen *ARO10*, es decir Aro80p (Iraqui *et al.,* 1999), reveló aproximadamente 2 veces superior el valor de Grantham que el observado para Aro10p, Atf1p, y Atf2p. Estos resultados hacen a *SFA1* y *ARO80* dos candidatos interesantes para futuras investigaciones.

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List of Publications related to this thesis

Stribny J., Pérez-Torrado R., Querol A. (2015) *Saccharomyces kudriavzevii* and *Saccharomyces uvarum* differ from *Saccharomyces cerevisiae* during the production of aroma-active higher alcohols and acetate esters using their amino acidic precursors. Int J Food Microbiol 205: 41-46

Stribny J., Romagnoli G., Pérez-Torrado R., Daran J.M., Querol A. (2016) Characterisation of the broad substrate specificity 2-keto acid decarboxylase Aro10p of *Saccharomyces kudriavzevii* and its implication in aroma development. Microbial Cell Factories 15: 51-62

Stribny J., Querol A., Pérez-Torrado R. (2016) Comparison of enzymatic properties of the *Saccharomyces kudriavzevii* and *Saccharomyces uvarum* alcohol acetyltransferases and their impact on aroma-active compounds production. Frontiers in Microbiology, *submitted for publication*