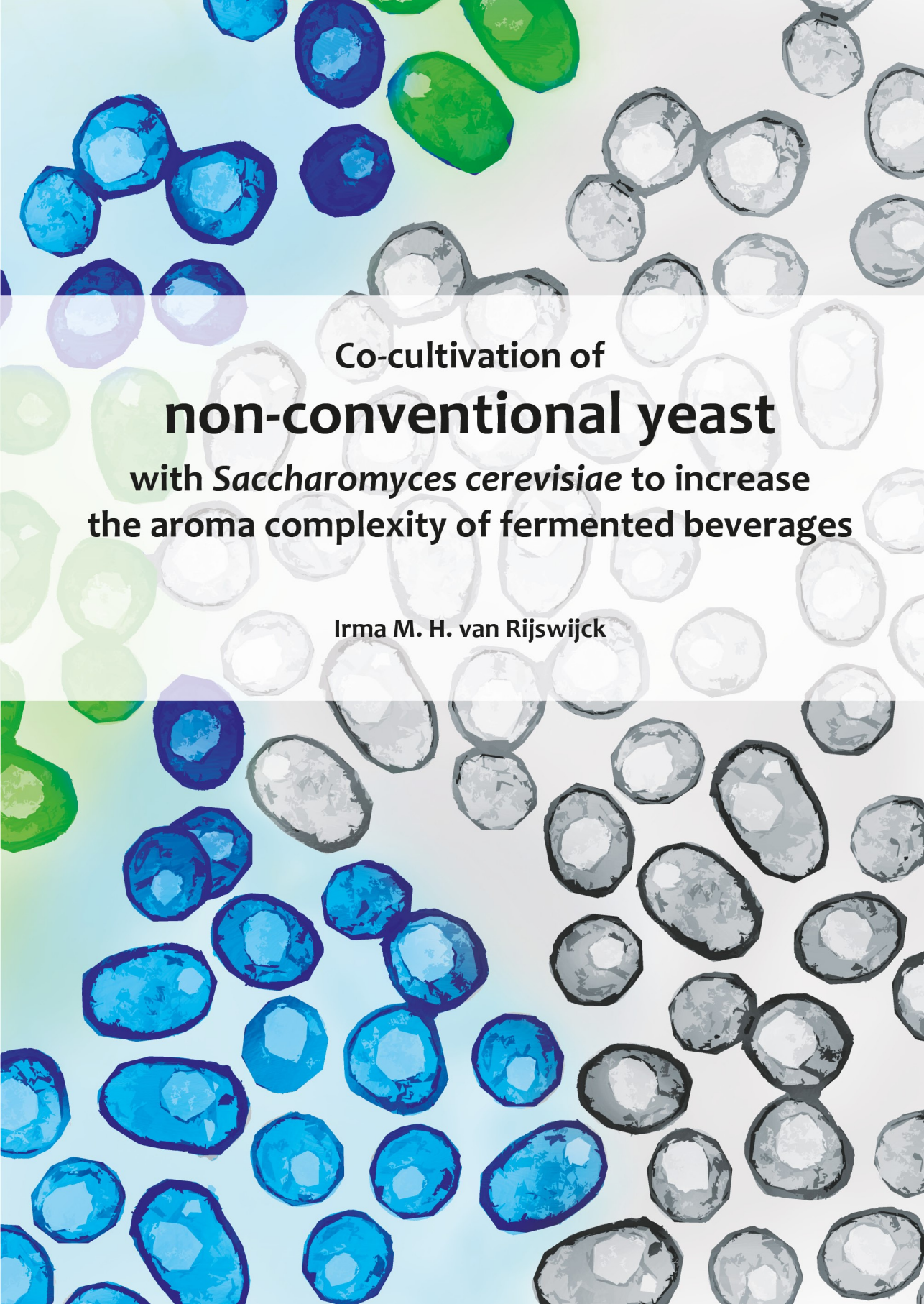


Co-cultivation of non-conventional yeast with *Saccharomyces cerevisiae* to increase the aroma complexity of fermented beverages

Irma M. H. van Rijswijck

Propositions

1. Nutrient limitation induces multicellular behaviour in yeasts, enabling penetrative growth on semi-solid (agar) medium.
   (this thesis)

2. A higher dose of *Cyberlindnera fabianii* in co-cultivation with brewers' yeast results in a more complex aroma bouquet of fermented wort.
   (this thesis)

3. The recent finding that truffle microbiome determines the truffle aroma (Vahdatzadeh et al. 2015. The role of the microbiome of truffles in aroma formation: a meta-analysis approach. *Appl Environ Microbiol* **81,** 6946-6952), makes it even more difficult to develop a proper *in vitro* production system for truffles.

4. Practical skills can only be obtained by practicing, not by watching videos of instructors demonstrating techniques.

5. Optimism and perseverance are needed to conquer disabilities.

6. Regulations stimulate smart marketing of food products which may mislead consumers.

Propositions belonging to the thesis, entitled:

"**Co-cultivation of non-conventional yeast with *Saccharomyces cerevisiae* to increase the aroma complexity of fermented beverages**"

Irma M. H. van Rijswijck

Wageningen, 8 September 2017

Co-cultivation of non-conventional yeast with *Saccharomyces cerevisiae* to increase the aroma complexity of fermented beverages

Irma M. H. van Rijswijck

Thesis committee

Promotors
Prof. Dr E. J. Smid
Personal chair at the Laboratory of Food Microbiology
Wageningen University & Research

Prof. Dr T. Abee
Personal chair at the Laboratory of Food Microbiology
Wageningen University & Research

Other members
Prof. Dr R. van Kranenburg, Wageningen University & Research
Prof. Dr H. A. B. Wösten, Utrecht University
Dr J. Morrissey, University College Cork, Ireland
Dr L. A. Hazelwood, HEINEKEN Supply Chain B.V., Zoeterwoude

This research was conducted under the auspice of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences)

Co-cultivation of non-conventional yeast with *Saccharomyces cerevisiae* to increase the aroma complexity of fermented beverages

Irma M. H. van Rijswijck

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University & Research
by the authority of the Rector Magnificus,
Prof. Dr A. P. J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 8 September 2017
at 1:30 p.m. in the Aula.

Irma M. H. van Rijswijck
Co-cultivation of non-conventional yeast with *Saccharomyces cerevisiae* to increase the aroma complexity of fermented beverages,
146 pages.

PhD thesis, Wageningen University & Research, Wageningen, NL (2017)
With references, with summary in English

ISBN 978-94-6343-625-0
DOI http://dx.doi.org/10.18174/419524

Table of Contents

Chapter 1

General introduction

"Traditional" beer production

Evidence has been found that beer-like beverages are produced as early as 5000 BC (Wang, et al., 2016). Barley or wheat was moistened, germinated and dried, a process nowadays known as malting. Slurry was made with a mixture of wheat or barley, malted barley and water which was cooked and afterwards exposed to ambient air to induce fermentation by endogenous microorganisms. Conceivably, already in those early days, the so-called backslopping technique was developed that is based on the use of a small portion of the final fermented product to induce the fermentation of a new batch (Wang, et al., 2016). Only from the mid-17th century, scientists started to unravel the mystic behind fermentation processes (Barnett, 2003). In 1680 Antonie van Leeuwenhoek was the first to observe yeast cells in beer using his self-made microscope. More than a century later, in 1837, three scientists, Charles Cagniard-Latour, Friedrich Kützing and Theodor Schwann, independently described yeast as living organisms that could transform sugar into alcohol (Barnett, 2003; Cagniard-Latour, 1838; Kützing, 1837; Schwann, 1837). Pasteur (1857) actually proved that fermentation was a result of the yeast metabolism. Additionally, Eduard Buchner provided evidence in 1897 that enzymes were essential for fermentation (Buchner, 1897). These discoveries resulted in isolation techniques and the selection and use of pure starter cultures to induce the fermentation process. S*accharomyces cerevisiae* was regularly found to dominate alcoholic fermentations and it has become the commonly used starter culture in beer production, generally known as brewers' yeast.

Nowadays, the beer production process is well understood and a large variety of beer types is on the market. In general, these types can be subdivided in two main categories; lager beers (bottom-fermented) and ale type beers (top-fermented). For ale fermentations *S. cerevisiae* is used as a starter culture whereas *S. pastorianus* is used for lager fermentations. Recently, Libkind, et al. (2011) and Hebly, et al. (2015) reported that *S. pastorianus* is a natural hybrid of *S. cerevisiae* and *S. eubayanus*. The term brewers' yeast refers to both ale and lager yeasts.

Brewers' yeast

Brewers' yeast were selected over the past decades based on three criteria; (i) efficient ethanol formation, (ii) active fermentative pathway due to the Crabtree effect (repression of respiration by glucose) and (iii) a high tolerance to environmental stresses such as high levels of ethanol (Basso, et al., 2016; Steensels and Verstrepen, 2014). Additionally, selected yeast strains should produce a balanced mixture of secondary metabolites, which largely impacts flavour characteristics of fermented beverages (Table 1.1).

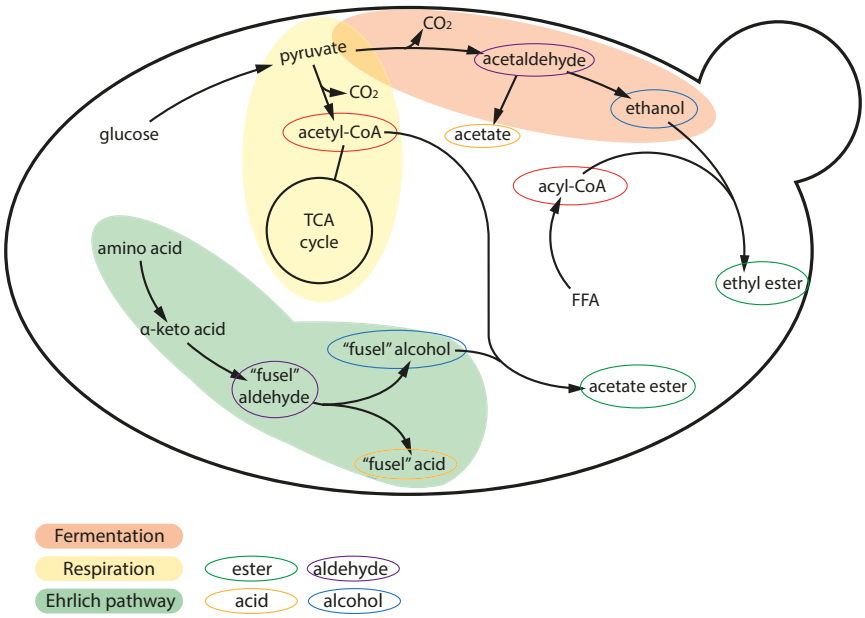


Figure 1.1: Overview of yeast metabolic pathways involved in the production of acetate and ethyl esters.

Key metabolic pathways

Metabolic pathways allowing the yeast to ferment wort sugars into ethanol, carbon dioxide and important flavour compounds are crucial for their overall performance in beer production. Yeast cells derive their metabolic energy (in the form of adenosine triphosphate (ATP)) from the breakdown of sugars and use inorganic nitrogen sources (ammonia or ammonium) to synthesize amino acids and proteins (Magasanik, 2003). Yeast cells convert glucose into pyruvate via the glycolytic pathway (Figure 1.1). Depending on the oxygen and sugar availability, pyruvate is either converted into ethanol and carbon dioxide (fermentative pathway) or it enters the tricarboxylic acid (TCA) cycle (Figure 1.1). Simple organic nitrogen sources such as amino acids are transported into the cells and subsequently converted to fusel alcohols and acids via the Ehrlich pathway (Figure 1.1) (reviewed by Hazelwood, et al. (2008)). These fusel alcohols and the esters thereof, are aromatic compounds that constitute the so-called flavour fingerprint of the fermented beverage. Together with flavour compounds (smell) the mouthfeel (texture) and taste will determine the sensorial perception, and therefore also the characteristics and quality of the beer (Adams and Taylor, 2012).

Volatile organic compounds

Wort contains many flavour compounds as well as precursors for flavour production by yeast, which originate from the hops and malted barley. Fermentation by yeast goes along

with degradation of these wort compounds to secondary metabolites including flavour compounds. The most important aroma compounds determining the flavour of beer are listed in Table 1.1. The sulphuric - and carbonyl compounds, phenols, esters and terpene alcohols all have a low odour threshold, which means that these compounds can be sensed by the human olfactory system at very low concentrations and thus have a high impact on the overall flavour perception. Some of these compounds can be formed by chemical reactions but the most relevant compounds result from yeast metabolism. Among these, esters constitute the most important flavour products of yeast metabolism due to their low-odour threshold and fruity or flowery aroma (Table 1.1).

Esterases, hemiacetal dehydrogenase and alcohol acetyltransferases are enzymes associated with ester formation by yeast (Kruis, et al., 2017). However, the thermodynamic equilibrium strongly favours ester hydrolysis instead of formation. A condensation reaction of an alcohol with ac(et)yl CoA is catalysed by alcohol acetyl transferases (AATases) and has a negative thermodynamic equilibrium and is therefore more likely biologically formed (Figure 1.1) (Kruis, et al., 2017; Nordström, 1963a; Nordström, 1963b; Nordström, 1964). Esters can be divided into two subgroups: medium chain fatty acid (MCFA) ethyl esters and acetate esters. MCFA ethyl esters consist of ethanol as ethyl group and MCFA as acid group. Whereas acetate esters consist of ethanol or a fusel alcohol as alcohol group and acetate as acid group. Acetate esters have drawn most attention since they generally appear in higher amounts and are more important for the flavour of the fermented product. For the ester yield, the AATase enzyme activity and substrate availability are of importance. An additional crucial factor that may affect the final level of esters is, as already mentioned earlier, the ester hydrolysis reaction catalysed by esterases (Kruis, et al., 2017). Taken all the above given considerations into account, it is obvious that aroma profiles as such can be very complex in composition and highly variable at different stages in the fermentation process. Moreover, the balance of aroma compounds determines the actual sensorial properties, where a too low or too high concentration of (a) particular compound(s) can result in off-flavour perception.

Table 1.1: Secondary metabolites produced by yeast and of importance for beer flavour. Compound name, flavour description and aroma threshold (Maarse, 1991).

compound name	aroma	threshold (mg/L)
Sulphuric compounds		
Sulphur dioxide	sulphurous	25
Hydrogen sulphide	rotten-egg	0.005
Undesirable carbonyl compounds		
Acetaldehyde	green apple-like or grassy	10
Diacetyl	buttery	0.1-0.15
2,3-pentanedione	toffee-like	~0.9
Phenols		
4-vinylguaiacol	phenolic, bitter, clove	0.3
4-vinylphenol	phenolic, smoky	0.2
4-ethylguaiacol	phenolic, sweet	0.13
4-ethylphenol	phenolic, astringent	0.9
Organic acids		
Acetic acid	vinegar	175
Caprylic acid	goaty	15
Capric acid	waxy	10
Lauric acid	soapy	6.1
Oxalic acid	salty, oxidised	500
Citric acid	sour	400
Malic acid	apple	700
Fumaric acid	sour, acedic	400
Succinic acid	acidic	220
Lactic acid	sour, acedic	400
Pyruvic acid	salty, forage	300
Higher alcohols		
n-propanol	sweet alcoholic	600
Isobutanol	solvent-like	100
Amyl alcohol	solvent-like	50-70
Isoamyl alcohol	banana, alcoholic	50-65
2-phenylethanol	gummy bear, rose-like	40
Esters		
Ethyl acetate	solvent-like	33
Isoamyl acetate	banana	1.6
Isobutyl acetate	fruity, sweet	1.6
Phenylethyl acetate	rose, apple, honey	3.8
Ethyl hexanoate	apple, aniseed	0.23
Ethyl octanoate	sour apple	0.9
Monoterpene alcohols		
Linalool	lavender	0.005
α-terpineol	lilac	2
β-citronellol	lemon, lime	0.008
Geraniol	rose	0.006
Nerol	rose, citrus	0.001

Trends in Brewing

Consumers are nowadays more aware of their health and therefore low-alcohol and alcohol-free beers are more trending than ever before. Moreover, consumers tend to look for more diversity in beer types, especially those richer in fruity flavours linked to the presence of esters. To fulfil these consumer demands, beer innovation is needed. For beer innovation various factors can be modulated such as the use of raw materials (i.e. barley, hops, and water), process parameters during brewing and fermentation conditions (e.g. temperature, fermentation time, oxygen, type of yeast) (Figure 1.2). In this thesis, we will focus on the possible routes to introduce beer innovation through choice and handling of the starter yeast(s). Two major routes are discussed here; (i) classical approach: *Saccharomyces* strain optimization, and (ii) the novel approach: co-cultivation with non-conventional yeast.

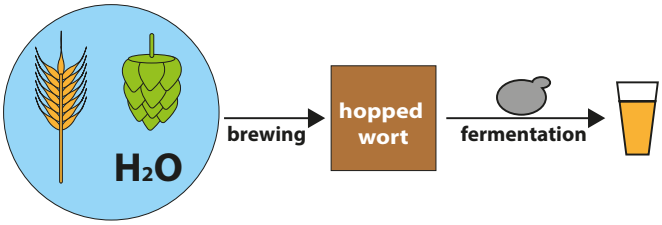


Figure 1.2: Schematic presentation of the beer production process. The raw materials to produce beer are malted barley, hops and water. During brewing the malt/barley sugars are degraded into fermentable sugars and aroma pre-cursors are derived from the hops. The hopped wort is fermented by yeast.

Classical approach: *Saccharomyces* strain optimization

The performance of brewers' yeast can be improved by changing fermentation parameters or generation of strain diversity. Brewers' yeast performance can be affected by variations in the composition of the hopped wort (i.e. carbon/nitrogen ratio, flavour pre-cursors) and the fermentation parameters (i.e. temperature, fermentation time, pitching rate). However, these approaches can be very costly or result in unwanted side effects. Therefore, other approaches to add traits to industrial yeasts have been evaluated, such as genetic engineering and the creation of artificial diversity (reviewed by Steensels, et al. (2014)). Genetic engineering has not been implemented in industrial yeasts used as starters for fermentations based on presumed consumers disapproval (Hammond, 1995; Steensels, et al., 2014). The second approach, creation of diversity, entails hybridization, mutagenesis and evolutionary engineering (Figure 1.3, adapted from Steensels, et al. (2014)). The latter methods require high-throughput screening methods to select for variants with the desired traits. Also, the variation that can be reached is limited by the genetic potential present within one genus/species.

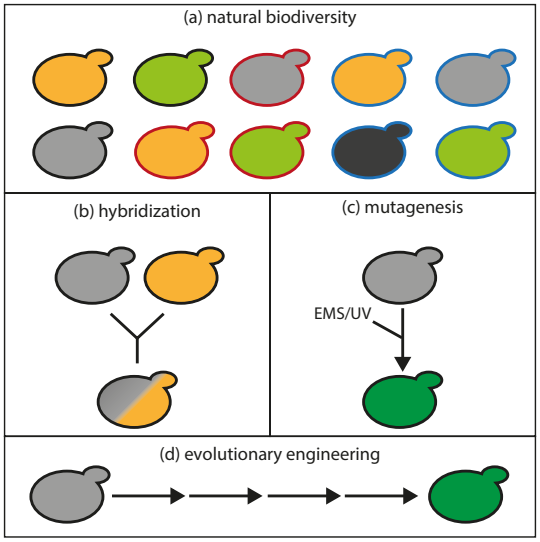


Figure 1.3: (non-GMO) strategies to optimize yeast performance. (a) Selection of a strain using the natural biodiversity of yeast, (b) creation of a new strain using hybridization, (c) and selection for new phenotypes following mutagenesis or (d) evolutionary engineering.

Novel approach: use of non-conventional yeast

In nature, there are about 1500 yeast species identified (Kurtzman, et al., 2011; Kurtzman and Piskur, 2006). A broad range of diversity is found among these 1500 species, therefore there is an increasing interest to explore and harness the biodiversity of new yeast species, the so-called non-conventional yeasts (Kurtzman, et al., 2011; Kurtzman and Piskur, 2006).

Replacement of the traditional brewers' yeast by non-conventional yeast attracts increasing attention. Recently, an increasing number of research papers and reviews have been published on the performance and possibilities to use non-conventional yeast in beer production (Basso, et al., 2016; Gamero, et al., 2016; Michel, et al., 2016b; Saerens and Swiegers, 2014; Saerens and Swiegers, 2016; Steensels, et al., 2015; Steensels and Verstrepen, 2014). Examples of strains that produced beers appreciated by consumers are described by Michel, et al. (2016b), among which strains of the species: *Torulaspora delbrueckii*, *Brettanomyces anomalus*, *Bretanomyces bruxellensis*, *Candida shehatae*, *Candida tropicalis*, *Saccharomycodes ludwigii*, *Zygosaccharomyces rouxii* and *Pichia kluyverii* (Alloue-Boraud, et al., 2015; Canonico, et al., 2016; Crauwels, et al., 2015; De Francesco, et al., 2015; Gamero, et al., 2016; Lentz, et al., 2014; Michel, et al., 2016a; N'Guessan, et al., 2009; Saerens and Swiegers, 2014; Saerens and Swiegers, 2016). Most strains have only been used in laboratory-scale trials and larger-scale pilots still have to be performed. Recently, Gamero, et al. (2016) screened a collection of 143 yeast species on their fermentation performance and their potential for aroma formation. This study

revealed some interesting aroma producing species, however their first selection criteria was based on fermentation performance, whereby some novel aroma producing species with poor fermentation capacities have been excluded.

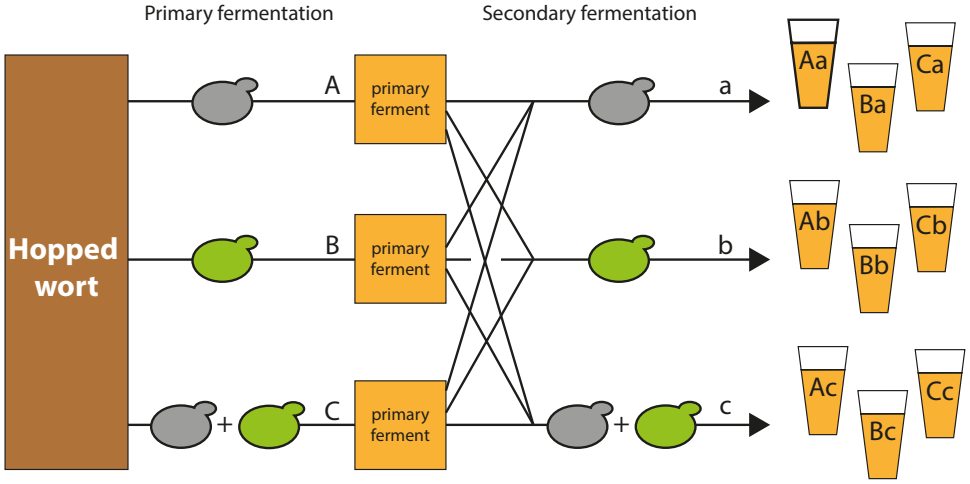


Figure 1.4: Strategies to ferment wort using brewers' yeast (grey) and/or a non-conventional yeast species (green) either as mono- or co-culture in primary (route A, B, C) or secondary fermentation (route a, b, c) resulting in nine different end-products. (Adapted from Vanderhaegen, et al. (2003))

Notably, some developments for example in dairy fermentations and in wine production may provide leads for innovations in beer production. In dairy fermentations it is common to add a so-called "adjunct-culture" which is for instance a lactic acid bacterium that is able to add specific aroma compounds to the final product, but does not contribute to the fermentation of the milk (El Soda, et al., 2000; Spus, et al., 2017). In the wine industry adjunct strains are added together with the starter culture such as *Oenococcus oeni* or *Pichia kudriavzevii* that have the capacity to de-acidify the wine by malic acid degradation (Del Monaco, et al., 2014; Nielsen and Richelieu, 1999). Such approaches are however not commonly used in the brewing industry and therefore options involving modulations in inoculation strategies will be discussed.

Traditionally, brewers' yeast is inoculated to induce the primary fermentation and consequently viable cells are still present in the secondary fermentation (route Aa in Figure 1.4). Vanderhaegen, et al. (2003), described different strategies for primary and secondary fermentation using either a brewers' yeast or another bacterium or yeast. An adapted strategy can be envisioned in Figure 1.4, where two different yeast species either as mono- or co-culture in primary or secondary fermentation can yield up to nine different fermented products. Additionally, the pitching rate (inoculation amount) is of importance for the formation of aroma compounds, growth yield and storage of fatty acids (Erten, et al., 2007; Verbelen, et al., 2009). Obviously, changing the pitching rate and/or other parameters in the brewing and/or fermentation stages will lead to variations in the composition of the fermented end product.

Yeast species used in this thesis

The yeast species used in this thesis project originate from the microbiota of spontaneously fermented masau (*Ziziphus mauritiana*) fruits in Zimbabwe (Nyanga, et al., 2007; Nyanga, et al., 2013). Here we focused on the wild isolates belonging to the species *S. cerevisiae*, *Cyberlindnera fabianii* and *Pichia kudriavzevii*.

Saccharomyces cerevisiae

S. cerevisiae is known as the "conventional yeast". It has been intensively used as eukaryotic model organism and it was the first eukaryote of which the complete genome was sequenced (Goffeau, et al., 1996; Kurtzman, et al., 2011). It is the most commonly used yeast species in the food industry and obtained the "Generally Recognized as Safe" (GRAS) status by the Food and Drug Authority (FDA, United states) and is listed as a biological agent with a qualified presumption of safety (QPS) that can be intentionally added to food and feed by the European Food Safety Authority (EFSA) (Bourdichon, et al., 2012; EFSA, 2016). *S. cerevisiae* representatives have been found to dominate a variety of environmental niches, since this species can grow in the presence and absence of oxygen in a broad range of temperatures and it can metaboliZe a wide range of carbohydrates (glucose, sucrose, maltose, raffinose, trehalose but also ethanol, Table 1.2) (Kurtzman, et al., 2011). Other synonyms for *S. cerevisiae* are brewers' yeast, bakers' yeast and wine-makers' yeast, each mentioned is optimized for the indicated fermentations since it has been domesticated for decades in these man-made environments (Gallone, et al., 2016).

Cyberlindnera fabianii

C. fabianii, previously known as *Lindnera fabianii*, *Hansenula fabianii*, *Pichia fabianii* and *Candida fabianii (Kurtzman, et al., 2011; Minter, 2009)*, is involved in various food fermentations such as the production of rice wine (Jeyaram, et al., 2008; Kurtzman, et al., 2008; Lv, et al., 2013; Minter, 2009), cereal beverages such as Obushera (Uganda) (Mukisa, et al., 2012), Fura (West-Africa) (Pedersen, et al., 2012), Korean traditional Nuruks (Ji Ho, et al., 2013) and Chinese soy sauce (Yan, et al., 2013). Despite its use in various fermented food products, this yeast species has not yet obtained the GRAS-status from the FDA and is neither listed as QPS by EFSA. To the authors' knowledge, it has also not been submitted for a QPS or GRAS qualification. *C. fabianii* can grow on various substrates (Table 1.2) including soluble starch. The latter phenomenon may explain why it has also been found as a contaminant in industrial soluble starch fermentations. *C. fabianii* is known for its high ester production thereby contributing to a fruity flavour of fermented products (Meersman, et al., 2016; Nyanga, et al., 2013).

Pichia kudriavzevii

P. kudriavzevii, previously known as *Issatchenkia orientalis*, has obtained the GRAS status by the FDA (Bourdichon, et al., 2012; Kurtzman, et al., 2011; Kurtzman, et al., 2008). It occurs often in soil, on fruits and therefore in various spontaneous fermentations. Recently, it has gained additional attention as a potential probiotic strain (Greppi, et al., 2017), as a promising strain for various (food) fermentations such as olives (Golomb, et al., 2013), for degradation of phytate (Hellstrom, et al., 2012) and for deacidification of wine (Del Monaco, et al., 2014). Notably, *P. kudriavzevii* can only ferment glucose, but grows on various other substrates in the presence of oxygen (Table 1.2) at temperatures as high as 40 °C.

Table 1.2: Comparison of the fermentation and growth characteristics of *S. cerevisiae*, *C. fabianii* and *P. kudriavzevii (Kurtzman, et al., 2011).*

	S. cerevisiae	*C. fabianii*	*P. kudriavzevii*
Fermentation of			
Glucose	+	+	+
galactose	v	-	-
Sucrose	+	+	-
Maltose	+	w/s	-
Raffinose	+	w	-
Growth on			
glucose	+	+	+
Sucrose	+	+	-
Raffinose	+	+	-
galactose	v	-	-
Trehalose	+	+	-
Maltose	+	+	-
Melezitose	v	+	-
Methyl-α-D-glucoside	-	+	-
Soluble starch	-	+	-
Cellobiose	-	+	-
Salicin	-	+	-
D-Xylose	-	+	-
Ethanol	+	+	+
Glycerol	v	+	+
DL-lactate	v	+	+
Succinate	-	+	+
Citrate	-	+	+
D-Gluconate	v	+	-
D-Glucosamine	-	-	+
N-Acetyl-D-glucosamine	-	-	+
Nitrate	-	+	-
Vitamin-free	-	-	+

v= versatile

w= weak

s= positive but slow

Scope of this thesis

This thesis mainly focusses on three yeast isolates: *S. cerevisiae* 131, *C. fabianii* 65 and *P. kudriavzevii* 129, that each show distinct characteristics concerning sugar utilization, aroma production and alcohol production (Nyanga, et al., 2007; Nyanga, et al., 2013). Knowledge on the aroma formation by the two non-conventional yeast species was gained by comparison of their behaviour in different environments. The three isolates were exposed to nitrogen limitation on defined semi-solid agar plates, to study their morphological and metabolic response (Chapter 2). Clear morphological differences were observed among the strains and analysis of volatile organic compounds revealed their metabolic response towards nitrogen limitation.

In Chapter 3, the alcohol dehydrogenase and esterase activities in relation to aroma formation in the three yeast species were compared with the focus on the production of esters. Significant differences in acetate ester yields could not be linked to alcohol dehydrogenase activity but appeared inversely correlated with the acetate-ester hydrolysing activity of the selected yeasts.

The knowledge obtained in Chapter 2 and 3 has been applied in Chapter 4, where *C. fabianii* and *P. kudriavzevii* isolates were used in single culture and in co-cultures for beer innovation. Various inoculation strategies (Figure 1.4) were applied to yield a beer with lower alcohol and higher ester levels. The best approach appeared to be co-cultivation of *C. fabianii* 65 with brewers' yeast. With this study, the feasibility of using non-conventional yeast species in co-cultivation with traditional brewers' yeast to tailor aroma profiles as well as the final ethanol content of beer is demonstrated.

The relationship between brewers' yeast and *C. fabianii* in co-cultivation is further explored in Chapter 5. Here, dynamic modelling was used to describe the crucial parameters in the co-culture behaviour of *C. fabianii* 65 and brewers' yeast during wort fermentation. This model can support optimization of the optimal inoculation dose of brewers' yeast and *C. fabianii* 65 and to predict the final product characteristics.

Finally, a synthesis of the results described in the experimental chapters has been made (Chapter 6) and combined with relevant research published in scientific literature in order to provide perspectives for future fundamental and applied research that may steer innovations in yeast driven fermentations.

References

Adams, S. and Taylor, A. J. (2012). Oral processing and flavour sensing mechanisms In Chen, J. and Engelen, L. (Eds), *Food oral processing: Fundamentals of eating and sensory perception*, Blackwell Publishing Ltd., 177-202.

Alloue-Boraud, W. A., N'Guessan, K. F., Djeni, N. T., Hiligsmann, S., Dje, K. M. and Delvigne, F. (2015). Fermentation profile of *Saccharomyces cerevisiae* and *Candida tropicalis* as starter cultures on barley malt medium. *J Food Sci Technol* **52,** 5236-42.

Barnett, J. A. (2003). Beginnings of microbiology and biochemistry: the contribution of yeast research. *Microbiology* **149,** 557-67.

Basso, R. F., Alcarde, A. R. and Portugal, C. B. (2016). Could non-*Saccharomyces* yeasts contribute on innovative brewing fermentations? *Food Research International* **86,** 112-20.

Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J. C., Gerds, M. L., Hammes, W. P., Harnett, J., Huys, G., Laulund, S., Ouwehand, A., Powell, I. B., Prajapati, J. B., Seto, Y., Ter Schure, E., Van Boven, A., Vankerckhoven, V., Zgoda, A., Tuijtelaars, S. and Hansen, E. B. (2012). Food fermentations: Microorganisms with technological beneficial use. *Int J Food Microbiol* **154,** 87-97.

Buchner, H. (1897). Die Bedeutung der activen löslichen Zellprodukte für den Chemismus der Zelle. *Münch. med. Wochens* **44,** 299-302.

Cagniard-Latour, C. (1838). Mémoire sur la fermentation vineuse. *Annales de chimie et de physique*, pp. 206-223.

Canonico, L., Agarbati, A., Comitini, F. and Ciani, M. (2016). *Torulaspora delbrueckii* in the brewing process: A new approach to enhance bioflavour and to reduce ethanol content. *Food Microbiol* **56,** 45-51.

Crauwels, S., Steensels, J., Aerts, G., Willems, K. A., Verstrepen, K. J. and Lievens, B. (2015). *Brettanomyces bruxellensis*, essential contributor in spontaneous beer fermentations providing novel opportunities for the brewing industry. *BrewingScience* **68,** 110-21.

De Francesco, G., Turchetti, B., Sileoni, V., Marconi, O. and Perretti, G. (2015). Screening of new strains of *Saccharomycodes ludwigii* and *Zygosaccharomyces rouxii* to produce low-alcohol beer. *Journal of the Institute of Brewing* **121,** 113-21.

Del Monaco, S. M., Barda, N. B., Rubio, N. C. and Caballero, A. C. (2014). Selection and characterization of a Patagonian *Pichia kudriavzevii* for wine deacidification. *J Appl Microbiol* **117,** 451–64.

EFSA (2016). Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 4: suitability of taxonomic units notified to EFSA until March 2016. *EFSA Journal* **14,** 1-37.

El Soda, M., Madkor, S. A. and Tong, P. S. (2000). Adjunct cultures: Recent developments and potential significance to the cheese industry. *Journal of Dairy Science* **83,** 609-19.

Erten, H., Tanguler, H. and Cakiroz, H. (2007). The effect of pitching rate on fermentation and flavour compounds in high gravity brewing. *J. Inst. Brew.* **113,** 75-9.

Gallone, B., Steensels, J., Prahl, T., Soriaga, L., Saels, V., Herrera-Malaver, B., Merlevede, A., Roncoroni, M., Voordeckers, K., Miraglia, L., Teiling, C., Steffy, B., Taylor, M., Schwartz, A., Richardson, T., White, C., Baele, G., Maere, S. and Verstrepen, K. J. (2016). Domestication and divergence of *Saccharomyces cerevisiae* beer yeasts. *Cell* **166,** 1397-410.

Gamero, A., Quintilla, R., Groenewald, M., Alkema, W., Boekhout, T. and Hazelwood, L. (2016). High-throughput screening of a large collection of non-conventional yeasts reveals their potential for aroma formation in food fermentation. *Food Microbiology* **60,** 147-59.

Goffeau, A., Barrell, B. G., Bussey, H., Davis, R. W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J. D., Jacq, C., Johnston, M., Louis, E. J., Mewes, H. W., Murakami, Y., Philippsen, P., Tettelin, H. and Oliver, S. G. (1996). Life with 6000 Genes. *Science* **274,** 546+563-67.

Golomb, B. L., Morales, V., Jung, A., Yau, B., Boundy-Mills, K. L. and Marco, M. L. (2013). Effects of pectinolytic yeast on the microbial composition and spoilage of olive fermentations. *Food Microbiol* **33,** 97-106.

Greppi, A., Saubade, F., Botta, C., Humblot, C., Guyot, J. P. and Cocolin, L. (2017). Potential probiotic *Pichia kudriavzevii* strains and their ability to enhance folate content of traditional cereal-based African fermented food. *Food Microbiol* **62,** 169-77.

Hammond, J. R. M. (1995). Genetically-modified brewing yeasts for the 21st century. Progress to date. *Yeast* **11,** 1613-27.

Hazelwood, L. A., Daran, J. M., van Maris, A. J., Pronk, J. T. and Dickinson, J. R. (2008). The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. *Appl Environ Microbiol* **74,** 2259-66.

Hebly, M., Brickwedde, A., Bolat, I., Driessen, M. R., de Hulster, E. A., van den Broek, M., Pronk, J. T., Geertman, J. M., Daran, J. M. and Daran-Lapujade, P. (2015). *S. cerevisiae* x *S. eubayanus* interspecific hybrid, the best of both worlds and beyond. *FEMS Yeast Res* **15,** 1-14.

Hellstrom, A. M., Almgren, A., Carlsson, N. G., Svanberg, U. and Andlid, T. A. (2012). Degradation of phytate by *Pichia kudriavzevii* TY13 and *Hanseniaspora guilliermondii* TY14 in Tanzanian togwa. *Int J Food Microbiol* **153,** 73-7.

Jeyaram, K., Singh, W. M., Capece, A. and Romano, P. (2008). Molecular identification of yeast species associated with 'Hamei'--a traditional starter used for rice wine production in Manipur, India. *Int J Food Microbiol* **124,** 115-25.

Ji Ho, C., Hwan Yeo, S., Park, J.-H., Choi, H. S., Gang, J.-E., In Kim, S., Tae Jeong, S. and Ra Kim, S. (2013). Isolation of aromatic yeasts (non-*Saccharomyces cerevisiae*) from Korean traditional nuruks and identification of fermentation characteristics. *Agricultural Sciences* **4,** 136-40.

Kruis, A. J., Levisson, M., Mars, A. E., van der Ploeg, M., Garcés Daza, F., Ellena, V., Kengen, S. W. M., van der Oost, J. and Weusthuis, R. A. (2017). Ethyl acetate production by the elusive alcohol acetyltransferase from yeast. *Metabolic Engineering* **41,** 92-101.

Kurtzman, C. P., Fell, J. W. and Boekhorst, J. (2011). *The Yeasts, a taxonomic study*, Fifth edition edn. Elsevier.

Kurtzman, C. P. and Piskur, J. (2006). Taxonomy and phylogenetic diversity among the yeasts, *Comparative genomics, Using fungi as models*, Springer-Verlag Berlin Heidelberg, pp. 29-46.

Kurtzman, C. P., Robnett, C. J. and Basehoar-Powers, E. (2008). Phylogenetic relationships among species of *Pichia*, *Issatchenkia* and *Williopsis* determined from multigene sequence analysis, and the proposal of *Barnettozyma* gen. nov., *Lindnera* gen. nov. and *Wickerhamomyces* gen. nov. *FEMS Yeast Res* **8,** 939-54.

Kützing, F. (1837). Microscopische Untersuchungen über die Hefe und Essigmutter, nebst mehreren andern dazu gehörigen vegetabilischen Gebilden. *Journal für Praktische Chemie* **11,** 385-409.

Lentz, M., Putzke, T., Hessler, R. and Luman, E. (2014). Genetic and physiological characterization of yeast isolated from ripe fruit and analysis of fermentation and brewing potential. *Journal of the Institute of Brewing* **120,** 559-64.

Libkind, D., Hittinger, C. T., Valerio, E., Goncalves, C., Dover, J., Johnston, M., Goncalves, P. and Sampaio, J. P. (2011). Microbe domestication and the identification of the wild genetic stock of lager-brewing yeast. *Proc Natl Acad Sci U S A* **108,** 14539-44.

Lv, X.-C., Huang, X.-L., Zhang, W., Rao, P.-F. and Ni, L. (2013). Yeast diversity of traditional alcohol fermentation starters for Hong Qu glutinous rice wine brewing, revealed by culture-dependent and culture-independent methods. *Food Control* **34,** 183-90.

Maarse, H. (1991). Volatile compounds in foods and beverages. CRC PRESS: New York, US.

Magasanik, B. (2003). Ammonia Assimilation by *Saccharomyces cerevisiae*. *Eukaryotic Cell* **2,** 827-9.

Meersman, E., Steensels, J., Struyf, N., Paulus, T., Saels, V., Mathawan, M., Allegaert, L., Vrancken, G. and Verstrepen, K. J. (2016). Tuning chocolate flavor through development of thermotolerant *Saccharomyces cerevisiae* starter cultures with increased acetate ester production. *Appl Environ Microbiol* **82,** 732-46.

Michel, M., Kopecka, J., Meier-Dornberg, T., Zarnkow, M., Jacob, F. and Hutzler, M. (2016a). Screening for new brewing yeasts in the non-*Saccharomyces* sector with *Torulaspora delbrueckii* as model. *Yeast* **33,** 129-44.

Michel, M., Meier-Dörnberg, T., Jacob, F., Methner, F.-J., Wagner, R. S. and Hutzler, M. (2016b). Review: Pure non-*Saccharomyces* starter cultures for beer fermentation with a focus on secondary metabolites and practical applications. *Journal of the Institute of Brewing* **122,** 569-87.

Minter, D. W. (2009). *Cyberlindnera*, a replacement name for *Lindnera* Kurtzman et al., nom. illegit. *Mycotaxon* **110,** 473-6.

Mukisa, I. M., Porcellato, D., Byaruhanga, Y. B., Muyanja, C. M., Rudi, K., Langsrud, T. and Narvhus, J. A. (2012). The dominant microbial community associated with fermentation of Obushera (sorghum and millet beverages) determined by culture-dependent and culture-independent methods. *Int J Food Microbiol* **160,** 1-10.

N'Guessan, F. K., N'Dri, D. Y., Camara, F. and Djè, M. K. (2009). *Saccharomyces cerevisiae* and *Candida tropicalis* as starter cultures for the alcoholic fermentation of tchapalo, a traditional sorghum beer. *World Journal of Microbiology and Biotechnology* **26,** 693-9.

Nielsen, J. C. and Richelieu, M. (1999). Control of flavor development in winde during malolactic fermentation by *Oenococcus oeni*. *Appl Environ Microbiol* **65,** 740-5.

Nordström, K. (1963a). Formation of esters from acids by Brewer's yeast I. Kinetic theory and basic experiments. *J. Inst. Brew.* **69,** 310-22.

Nordström, K. (1963b). Formation of esters, acids and alcohols from a-keto acids by Brewer's yeast. *J. Inst. Brew.* **96,** 483-95.

Nordström, K. (1964). Formation of esters from alcohols by Brewer's yeast. *J. Inst. Brew.* **70,** 328-36.

Nyanga, L. K., Nout, M. J., Gadaga, T. H., Theelen, B., Boekhout, T. and Zwietering, M. H. (2007). Yeasts and lactic acid bacteria microbiota from masau (*Ziziphus mauritiana*) fruits and their fermented fruit pulp in Zimbabwe. *Int J Food Microbiol* **120,** 159-66.

Nyanga, L. K., Nout, M. J., Smid, E. J., Boekhout, T. and Zwietering, M. H. (2013). Fermentation characteristics of yeasts isolated from traditionally fermented masau (*Ziziphus mauritiana*) fruits. *Int J Food Microbiol* **166,** 426-32.

Pasteur, L. (1857). Mémoire sur la fermentation alcoolique. *Comptes Rendus Chimie* **45,** 1032-6.

Pedersen, L. L., Owusu-Kwarteng, J., Thorsen, L. and Jespersen, L. (2012). Biodiversity and probiotic potential of yeasts isolated from Fura, a West African spontaneously fermented cereal. *Int J Food Microbiol* **159,** 144-51.

Saerens, S. M. and Swiegers, H. (2014). Enhancement of beer flavor by a combination of *Pichia* yeast and different hop varieties: US2014/0234480, pp. 27.

Saerens, S. M. and Swiegers, H. (2016). Production of low-alcohol or alcohol-free beer with *Pichia kluyveri* yeast strains In Chr. Hansen A/S, H. D. (Ed), CHR. Hansen A/S, Hoersholm (DK): US2016/0010042, pp. 15.

Schwann, T. (1837). Vorläufige Mittheilung, betreffend Versuche über die Weingährung und Fäulniss. *Annalen der Physik* **117,** 184-93.

Spus, M., Liu, H., Wels, M., Abee, T. and Smid, E. J. (2017). Isolation and characterization of *Lactobacillus helveticus* DSM 20075 variants with improved autolytic capacity. *Int J Food Microbiol* **241,** 173-80.

Steensels, J., Daenen, L., Malcorps, P., Derdelinckx, G., Verachtert, H. and Verstrepen, K. J. (2015). *Brettanomyces* yeasts--From spoilage organisms to valuable contributors to industrial fermentations. *Int J Food Microbiol* **206,** 24-38.

Steensels, J., Snoek, T., Meersman, E., Picca Nicolino, M., Voordeckers, K. and Verstrepen, K. J. (2014). Improving industrial yeast strains: exploiting natural and artificial diversity. *FEMS Microbiol Rev* **38,** 947-95.

Steensels, J. and Verstrepen, K. J. (2014). Taming wild yeast: potential of conventional and nonconventional yeasts in industrial fermentations. *Annu Rev Microbiol* **68,** 61-80.

Vanderhaegen, B., Neven, H., Coghe, S., Verstrepen, K. J., Derdelinckx, G. and Verachtert, H. (2003). Bioflavoring and beer refermentation. *Appl Microbiol Biotechnol* **62,** 140-50.

Verbelen, P. J., Dekoninck, T. M., Saerens, S. M., Van Mulders, S. E., Thevelein, J. M. and Delvaux, F. R. (2009). Impact of pitching rate on yeast fermentation performance and beer flavour. *Appl Microbiol Biotechnol* **82,** 155-67.

Wang, J., Liu, L., Ball, T., Yu, L., Li, Y. and Xing, F. (2016). Reavealing a 5,000-y-old beer recipe in China. *Proc Natl Acad Sci U S A* **113,** 6444-8.

Yan, Y. Z., Qian, Y. L., Ji, F. D., Chen, J. Y. and Han, B. Z. (2013). Microbial composition during Chinese soy sauce koji-making based on culture dependent and independent methods. *Food Microbiol* **34,** 189-95.

Chapter 2

Nutrient limitation leads to penetrative growth into agar and affects aroma formation in *Cyberlindnera fabianii*, *Pichia kudriavzevii* and *Saccharomyces cerevisiae*

Irma M. H. van Rijswijck[1], Jan Dijksterhuis[2],
Judith C. M. Wolkers-Rooijackers[1],
Tjakko Abee[1] and Eddy J. Smid[1]

Published in Yeast, Volume 32, Issue 1, January 2015, Pages: 89–101,
Version of Record online : 14 NOV 2014, DOI: 10.1002/yea.3050

Affiliations:
1. Wageningen University & Research, Laboratory of Food Microbiology, PO Box 17, 6700 AA Wageningen, The Netherlands
2. CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands.

Abstract

Among fermentative yeast species, *Saccharomyces cerevisiae* is most frequently used as model organism, although other yeast species may have special features that make them interesting candidates to apply in food fermentation processes. In this study, we used three yeast species isolated from fermented *masau* (*Ziziphus mauritiana*) fruit, *S. cerevisiae* 131, *Cyberlindnera fabianii* 65 and *Pichia kudriavzevii* 129, and determined the impact of nitrogen and/or glucose limitation on surface growth mode and production of volatile organic compounds (VOCs). All three species displayed significant changes in growth mode in all nutrient-limited conditions, signified by the formation of metafilaments or pseudohyphae. The timing of the transition was found to be species specific. Transition in growth mode is suggested to be linked to the production of certain fusel alcohols (such as phenylethyl alcohol) which serve as quorum sensing molecules (Chen and Fink, 2006). Interestingly, we did not observe concomitant increased production of phenylethyl alcohol and filamentous growth. Notably, a broader range of esters was found only for *C. fabianii* and *P. kudriavzevii* grown on nitrogen limited agar for 21 days compared to nutrient rich agar, and when grown on glucose and glucose plus nitrogen limited agar. Our data suggests that for the *C. fabianii* and *P. kudriavzevii*, the formation of esters may play an important role in the switch in growth mode upon nitrogen limitation. Further biological or ecological implications of ester formation will be discussed.

Introduction

Saccharomyces cerevisiae is the most extensively studied and industrially used eukaryotic microorganism. In addition to *S. cerevisiae* there are many other fermentative yeast species involved in fermentation processes. Nyanga et al., (2007) isolated various microorganisms from fermented *masau* (*Ziziphus mauritiana*) fruit. Within this niche various species of yeast and lactic acid bacteria were found. The dominating yeast species were *S. cerevisiae*, *Cyberlindnera fabianii* and *Pichia kudriavzevii*. Strains belonging to these yeast species were phenotypically characterized. *C. fabianii* 65 and *P. kudriavzevii* 129 were selected as interesting candidates to study the aroma production, based on their relatively high fusel acid, alcohol and ester formation and low ethanol production. *S. cerevisiae* 131 was chosen for comparison with the non-*Saccharomyces* strains (Nyanga, et al., 2013).

S. cerevisiae has been used in various studies as model organism for research on aroma formation (Dickinson, et al., 2003; Gamero, et al., 2011; Gamero, et al., 2013a; Hazelwood, et al., 2008; Perpete, et al., 2006). The formation of aroma compounds on basis of amino acid degradation is mainly driven by enzymes of the Ehrlich pathway (reviewed by Hazelwood et al., (2008)). The first step of this pathway is transamination of amino acids into α-keto acids, followed by decarboxylation into the corresponding fusel aldehydes. Subsequently, the fusel aldehydes can be either reduced or oxidized into fusel alcohols or fusel acids, respectively (reviewed by Hazelwood et al., (2008)). Next, aromatic esters can be formed from fusel alcohols and acids (Saerens, et al., 2010). The bouquet of fusel alcohols, volatile (aromatic) esters, organic acids, carbonyl compounds, sulphur-containing molecules and phenolic molecules constitutes the fingerprint of the characteristic aroma and taste of the final fermented product (Cordente, et al., 2012; Saerens, et al., 2010). The major contribution of this profile is determined by the volatile esters, because of their low odour detection threshold. This means that only trace amounts of these compounds are required for perception by the human olfactory senses (Saerens, et al., 2010).

Although *S. cerevisiae* metabolism has been studied intensively, the role of the Ehrlich metabolites remains to be unravelled (Hazelwood, et al., 2008). Fusel alcohols, and in particular tyrosol, tryptophol, phenylethanol and isoamyl alcohol, were suggested to be involved in quorum sensing with their production being induced in nitrogen-limited conditions (Chen and Fink, 2006; Lorenz, et al., 2000; Zupan, et al., 2013). Under these conditions, *S. cerevisiae* produces relatively more of these fusel alcohols coinciding with entering of the stationary phase (in liquid medium) or invasive pseudohyphal growth (on solid medium) (Chen and Fink, 2006; Zupan, et al., 2013). Also the role of esters in signalling invasive growth during amino acid starvation has been discussed (Dumlao, et al., 2008). The change in growth mode as result of nutrient (nitrogen or carbon) limitation or other stress factors is a fascinating behaviour which does not only occur in food associated fermentative yeasts, but also in pathogenic yeast species where invasive growth into tissue contributes to

virulence (Granek, et al., 2011; Granek and Magwene, 2010; Hornby, et al., 2004; Hornby, et al., 2001; Kugler, et al., 2000; Sprague and Winans, 2006). The change in growth mode involves regulation by appropriate signalling pathways depending on the environmental stresses encountered (Cullen and Sprague, 2012; Gimeno, et al., 1992; Wuster and Babu, 2010).

This paper describes the behaviour of three *masau* fruit isolates, *C. fabianii* 65, *P. kudriavzevii* 129 and *S. cerevisiae* 131, under conditions of nitrogen and carbohydrate excess and limitation. A change in growth mode was observed for all species when grown on nutrient limited agar. The cell structure within a colony is studied by Cryo Scanning Electron Microscopy (Cryo-SEM) and linked with penetrative growth and volatile organic compounds (VOCs) production, measured by Headspace Gas Chromatography – Mass Spectrometry (HS GC-MS). Species-specific VOCs profiles were established and high level production of esters was found specifically for the *C. fabianii* and *P. kudriavzevii*, only when grown on nitrogen limited agar for 21 days. Interestingly, no increased level of the quorum sensing associated volatile phenylethyl alcohol was found for any of the species, which is not in line with the studies on laboratory *S. cerevisiae* strains. The results found for these wild yeast isolates are compared with knowledge obtained from years of research on *S. cerevisiae* lab strains and industrial implications for these findings are discussed.

Materials and methods

Yeast strains

The three yeast strains used in this study (*S. cerevisiae* 131, *C. fabianii* 65 and *P. kudriavzevii* 129) were isolated by Nyanga et al., (2007). The correct species identification was confirmed by sequencing the internal transcribed spacer (ITS) and the Large Subunit (LSU) regions (Nyanga, et al., 2007).

Culture conditions

Each strain was separately streaked onto Malt Extract Agar (MEA) (Oxoid Limited, Basingstroke, England) and incubated overnight at 30 °C. A single colony of each strain was picked and separately inoculated into Malt Extract Broth (MEB) (Oxoid Limited, Basingstroke, England), overnight incubated at 160 rpm, 30 °C. To obtain single colonies, a 10^{-6} dilution was made in peptone physiological salt solution of which 100 µl was spread plated onto the appropriate agar plates. Plates where incubated at 30 ˚C for 21 days to monitor the colony morphology.

Composition of the agar plates used in the experiments are displayed in table 2.1.

Table 2.1: Composition agar plates

Agar types	YNB[(1)]	Agar[(2)]	Glucose[(3)]	$(NH_4)_2SO_4$ [(4)]
Rich	1 x	2 %	2 %	45.4 mM
Nitrogen limited	1 x	2 %	2 %	0.045 mM
Glucose limited	1 x	2 %	0.00125 %	45.4 mM
Nitrogen and Glucose limited	1 x	2 %	0.00125 %	0.045 mM

[(1)] Difco™ Yeast Nitrogen Base w/o amino acids and ammonium sulphate (*Beckton, Dickinson and Company Sparks, MD 21152*)

[(2)] Agar Bacteriological, No. 1, LP0011 (Oxoid Limited, Basingstoke, England)

[(3)] D(+)- glucose, anhydrous for biochemistry (Merck KGaA, 64293 Darmstadt, Germany)

[(4)] Ammonium sulphate, for analysis (Merck KGaA, 64293, Darmstadt, Germany)

Determination of the colony morphology

Each strain was inoculated as stated above onto each agar type (nutrient rich, nitrogen limited, glucose limited and nitrogen plus glucose limited). Of all combination, the colonies were followed for 21 days. Pictures were taken with a digital camera (Nikon S3600) through the lens of a stereo microscope (objective 4x, ocular 10x, Nikon).

Invasive growth experiment

Invasive growth was determined by washing of the agar plate. Colony material and cells that have not invaded into the agar will be washed off. Plates were washed with running water and carefully rubbing with a glove onto the surface for 1 minute (Chen and Thorner, 2010). Afterwards, a picture was taken through a stereo microscope (objective 4x, ocular 10x, Nikon).

Cryo Scanning Electron Microscopy (cryo-SEM)

Colonies or sections of colonies, depending on the size, were selected by means of a stereo microscope (Nikon SMZ800) cut out by means of a stainless razor blade (GEM products, Orange Park, Florida, USA). Small cubes of agar (4x4x4 mm) were glued with KP-Cryoblock (Klinipath, Duiven, the Netherlands) in a 1 cm ø copper cup and investigated with a JEOL 5600LV scanning electron microscope (JEOL, Tokyo, Japan) via an Oxford CT1500 Cryostation as described by Wyatt et al., (2014).

Headspace Gas Chromatography – Mass Spectrometry analysis (HS GC-MS)

Volatile Organic Compounds (VOCs) produced by each strain on 4 types of agar were measured according to the following procedure.

Samples for each strain – agar type combination were taken as follows. At time points 7, 14 and 21 days, 2 colonies were cut out of the agar plate and put into a GC-MS vial. A control for each agar type was determined by a piece of agar (without growth) in a GC-MS vial. GC-MS Vials were stored at -20 °C until GC-MS analysis was performed. No significant difference in aroma profile was found between the controls (different agar types without growth).

VOCs were extracted and detected by headspace solid-phase micro extraction GC-MS, using a HS-SPME fibre (carboxen/polydimethylsiloxane, CAR/PDMS, Supelco Inc. USA) (Gamero, et al., 2013b).

Samples were pre-incubated at 60 °C for 2 minutes without agitation. Followed by exposure of the fibre to the sample headspace for 5 minutes at 60 °C. Volatile compounds were injected into the GC column by desorption of the fibre for 10 minutes.

The Finnigan Trace GC Ultra (Thermo Fisher Scientific, USA) equipped with a Stabilwax®-DA-Crossband®-Carbowax®-polyethylene-glycol column (30 m length, 0.32 mm internal diameter, 1 µm internal thickness) (Restek, Bellefonte, PA, USA) was used for GC-MS analysis.

The injection device was a TriPlus™ autosampler (Thermo Fisher Scientific, USA) in PTV Splitless mode (5 minutes) at 250 °C. Helium was used as carrier gas at a constant flow of 10 ml/min. The GC oven was initially at 40 °C for 2 minutes, raised to 250 °C (10 °C/min) and kept at 250 °C for 5 minutes. The total runtime was 28 minutes.

Mass spectral data was collected over a range of m/z 33-250 in full scan mode, scan time 0.5 seconds.

HS GC-MS data and statistical analysis

HS GC-MS data was analysed using AMDIS software (NIST, Gaithersburg, MD, USA). Xcalibur software package (Thermo Scientific, Austin, TX, USA) was used for automated peak integration by using the m/z model and specific retention times.

Presence of compounds is expressed in peak areas. To obtain equal distribution of the values we transformed them into $\log_{10}$ values. These values were used to analyse if there is significant difference between the treatments by ANOVA (using SPSS version 20).

Results and discussion

The effect of carbon and nitrogen limitation on colony morphology

Some yeast species develop pseudohyphae and penetrate into a substrate upon carbon or nitrogen limitation. To determine whether nutrient status evokes a different growth mode in our isolates, we studied their morphology on nutrient rich, nitrogen limited, glucose limited and nitrogen plus glucose limited agar plates (Table 2.1). The isolates were grown for 21 days at 30 °C and colony morphology was determined after 7, 14 and 21 days of incubation (Figure 2.1).

On rich agar, the colonies of *S. cerevisiae* 131 and *C. fabianii* 65 were circular with smooth edges and retained this shape during the 21 days of incubation. A slight irregular edge was established for *P. kudriavzevii* 129 colonies grown on nutrient rich agar plates.

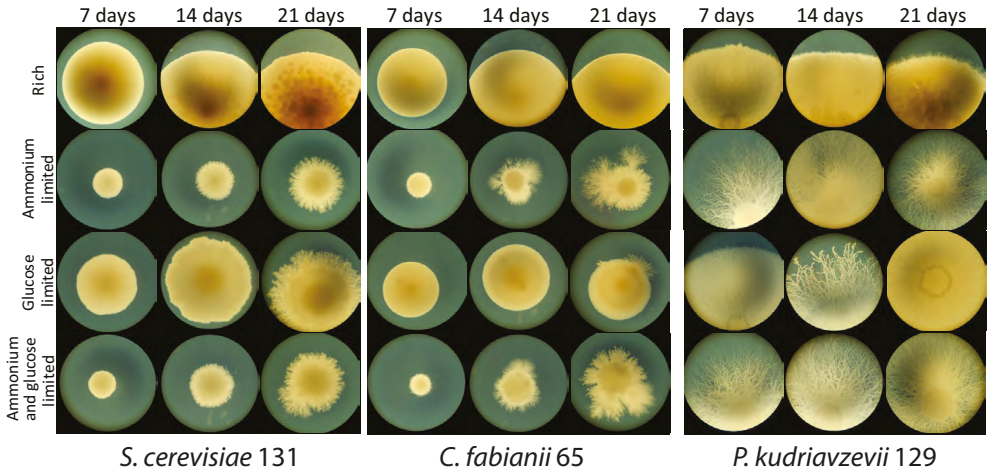


Figure 2.1: Colony morphology of *S. cerevisiae* 131, *C. fabianii* 65 and *P. kudriavzevii* 129 over 21 days on four types of media; nutrient rich, nitrogen limited, glucose limited and nitrogen plus glucose limited. Pictures were taken through a stereo microscope (magnification: 40 x)

Under either nitrogen, glucose or nitrogen plus glucose limitation all three species show a differentiation in growth mode. Noteworthy, the timing and colony morphology is species and condition specific (Figure 2.1). Contradictory to the study of Granek et al. (2010), we did not observe complex colony growth mode when grown on glucose limited agar and neither did we observe sporulation when nitrogen plus glucose were limited (Figure 2.1).

For *S. cerevisiae* 131 similar growth modes were observed when cultivated on either nitrogen limited or nitrogen plus glucose limited agar. Under all limiting conditions the colony size of *S. cerevisiae* 131 is smaller compared to the colony size on nutrient rich media. The first few

days colonies are smooth edged, but after 7 days finger-like structures are observed. These structures are regularly distributed around the edge of the colony. *S. cerevisiae* 131 grown on glucose limited agar demonstrates slightly different behaviour. The colony size is bigger and has an irregular shape at day 14, whereas at day 21 appearances of finger-like structures can be observed (Figure 2.1).

C. fabianii 65 demonstrated similar behaviour as *S. cerevisiae* 131 when grown on either nitrogen limited or nitrogen plus glucose limited agar, although the finger-like structures are less regular distributed around the edge of the colony. *C. fabianii* 65 grown on glucose limited agar retained smooth colony morphology up to 14 days, but as for *S. cerevisiae* 131, at day 21 finger-like structures appeared (Figure 2.1).

P. kudriavzevii 129 exhibits extremely long finger-like structures, regularly distributed around the edge of a small colony when grown on either nitrogen limited or nitrogen plus glucose limited agar. When grown on glucose limited agar, a fuzzy colony structure was observed at day 7, after which appearance of finger-like structures was noted. This morphology is similar to the colonies grown on either nitrogen limited or nitrogen plus glucose limited agar, although the edges of the finger-like structures are more clear (Figure 2.1).

One remarkable phenomenon was seen for finger-like structures from all species grown in nutrient limitation. When two colonies were located closely to each other, the structures avoided to grow into the direction of the other (Figure S 2.1). Such interactions could be directed by sensing depletion of nutrients in the agar (chemotaxis) and/or production of inhibitory compounds by the cells.

While colonies that consist of unicellular yeasts are considered not to penetrate into substrates such as agar, filamentous cells do. For example, filaments of *Magnaporthe grisea* (rice blast), *Ustilago maydis* (corn smut) and *Candida albicans* actively penetrate into rice, corn and human tissue (Palecek, et al., 2002). Penetration of tissue by yeast is related to virulence and therefore of specific interest for biomedical applications. Control of penetration of fungal pathogens into human tissue may assist in curing disease (Klingberg, et al., 2008). To study if our isolates do penetrate the agar, we performed the invasive growth assay (Chen and Thorner, 2010). Interestingly, for *C. fabianii* 65 and *P. kudriavzevii* 129, grown on nutrient limited agar, this revealed that invasive growth starts at specific locations underneath the smooth round shaped colony. For *S. cerevisiae* 131 colonies grown on nutrient limited agar were barely washed of the plates, this suggests that a major fraction of the colony had invaded into the agar (Figure S 2.2).

Stereo microscopy of the colonies revealed irregular boundaries and finger-like structures that penetrate into agar for all species under nutrient limitation. However, the finger-like structures of *P. kudriavzevii* 129 appeared to be much longer than the structures of *C. fabianii* 65 and *S. cerevisiae* 131. Therefore we used Cryo-SEM to study the cell organization in the finger-like structures of *C. fabianii* and *P. kudriavzevii* grown for 21 days on nitrogen

limited agar.

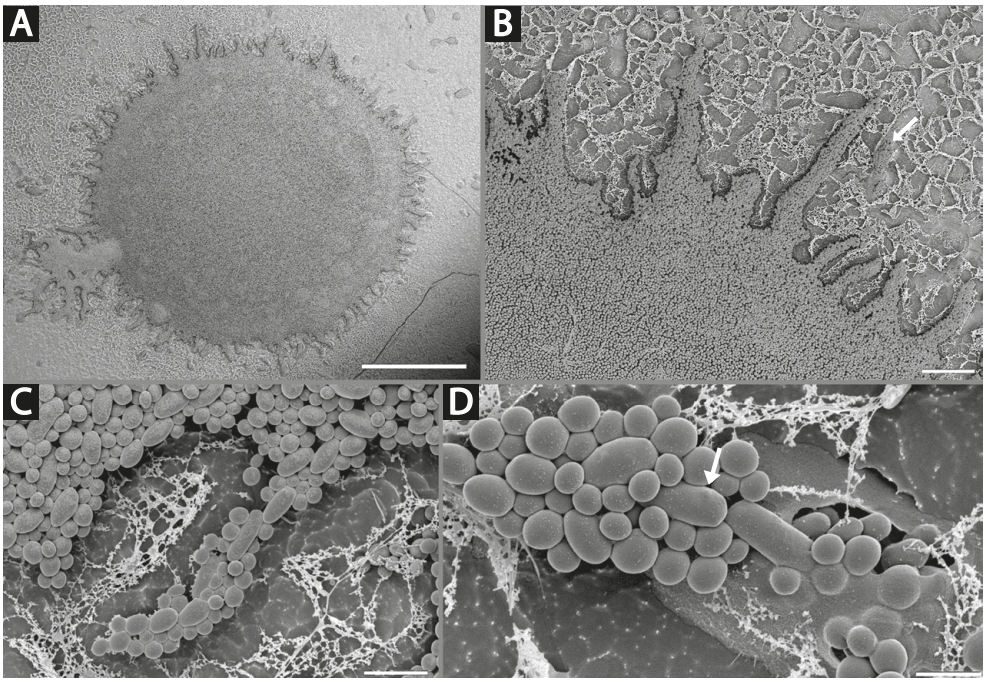


Figure 2.2: Cryo-SEM pictures of *C. fabianii* 65 grown on nitrogen limited agar for 21 days at 30°C. (A) whole colony (bar = 500 µm); (B) edge of the colony including "islands" indicated with arrow (bar = 50 µm); (C) one of the finger-like structures with elongated cells located in the middle (bar = 10 µm); (D) one of the "islands", with budding scars indicated by an arrow at the side of one elongated cell (bar = 5 µm).

Figure 2.2A represents one complete colony of *C. fabianii* 65. Here, the irregular boundary of the colony is clearly visible. In the lower-left part in the colony the boundary is disrupted and part of the colony extends further on the agar surface. Figure 2.2B and C zoom further in on the finger-like structures and shows that they are composed of two types of cells, namely elongated cells that are present in the middle of the structure and rounded cells that are attached to these cells. The structure resembles so-called metafilaments, which are structures that consist of round and elongated yeast cells. On the backbone cell, different budding scars can be seen (see arrows in Figure 2.2D). The rounded cells seem to remain firmly attached to the elongated cells judged by the flattened cell walls, remarkable, the elongated cells are not firmly attached to each other (Figure 2.4). The power of cell-cell adhesion could provide the metafilaments the rigidity to penetrate the agar by holding the elongated cells together. Supported by Figure 2.2D, which clearly illustrates that the yeast metafilaments originate from underneath the agar surface (at the right side of the picture). Regularly, these structures appear on the agar surface again, leading to the formation of an "island" colony (Figure 2.2B, designated by arrow). This provides a unique example of how

a culture that consists of unicellular yeast cells is able to colonize and penetrate a substrate.

Figure 2.3 represents only a small part of a colony of *P. kudriavzevii* 129 grown for 21 days on nitrogen limited agar. At the rim of the culture individual elongated cells (to > 30 µm) that are connected to each other (pseudohyphae) reach out over the substrate (Figure 2.3C). At both ends of these cells shorter (but still elongated) cells are continuously formed (see Figure 2.3D) as is also illustrated by the buds present on these sites. A number of these cells will become elongated which results in further extension of the filament and also in the formation of novel "branches" (Figure 2.3B). As this process continues, more yeast cells accumulate near the junctions of the cells, which results in numerous microcolonies (Figure 3B), that merge in more mature and centrally located parts of the colony (Figure 2.3A). As a result of this way of development open spaces exist within the colony (see arrow in Figure 2.3A).

Most studies established the ability of yeast to change in growth mode within 3 days of incubation (Gimeno, et al., 1992). At such a short time scale, we would have concluded that only *P. kudriavzevii* 129 changes in growth mode and that *S. cerevisiae* 131 and *C. fabianii* 65 do not show this phenomenon, which is obviously not the case, since prolonged incubation up to 21 days does show changes in growth mode.

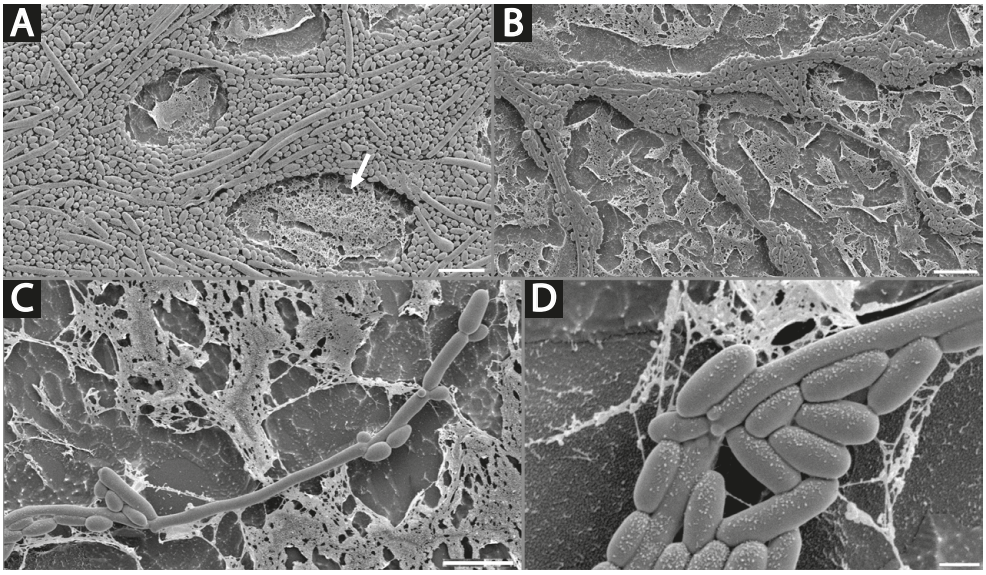


Figure 2.3: Cryo-SEM pictures of *P. kudriavzevii* 129 grown on nitrogen limited agar for 21 days at 30°C. (A) Dense part of colony, with open spaces indicated with an arrow (bar = 20 µm); (B) finger-like structure (bar = 20 µm); (C) end of one pseudohyphae (bar = 10 µm); (D) buddingsites at the end of the pseudohyphae (bar = 10 µm).

In literature, filamentous growth is an umbrella term for pseudohyphal growth and invasive growth (filamentation phenomenon by haploids, since they can invade into the agar). Cullen et al., (2012), described three major events needed to change in growth mode. First, the

cells remain attached to each other. This is explained by induced expression of the cell adhesion molecule (flocculin) Flo11 (Cullen and Sprague, 2012). The Cryo-SEM pictures of *C. fabianii* 65 and *P. kudriavzevii* 129 confirmed the attachment of the cells to each other by the flattened cell walls (Figure 2.4B). Secondly, a switch in polarity with axial (haploid) or bipolar (diploid) budding patterns switching to distal-unipolar budding patterns, which means that new cells are only formed at the opposite site from the mother cell (Cullen and Sprague, 2012). This is most clearly seen in the Cryo-SEM pictures of *P. kudriavzevii* 129 (Figure 2.3D). Noteworthy, some elongated cells also show budding scars at both sides of elongated cells (see arrow Figure 2.4D), but of course the cells also form new yeast cells on their own. Thirdly, elongation of the cells, which can be explained by two mechanisms. One is polarized growth, by only growth at the apex. The other, is when one phase of the cell cycle is extended, this can tip the balance towards apical growth over isotropic growth (Cullen and Sprague, 2012; Pruyne and Bretscher, 2000). The Cryo-SEM pictures of *P. kudriavzevii* 129 demonstrate that the cells at the end of the pseudohyphae are shorter (Figure 2.3C). This would suggest that the cells do elongate more at the apex (Figure 2.3).

For some *S. cerevisiae* strains it has been described that nutrient limitation can result in a change in growth mode in connection with up-regulation of a large group of genes responsible for signalling pathways, including mitogen-activated protein kinase (MAPK), Ras/protein kinase A (Ras/PKA), sucrose nonfermentable (SNF) and target of rapamycin (TOR) (Banuett, 1998; Cullen and Sprague, 2012; Gagiano, et al., 2002; Granek, et al., 2011; Madhani and Fink, 1998; Wuster and Babu, 2010). It is hypothesized that induction of the expression of hundreds of genes is needed for yeast cells to change in growth mode (Chen and Fink, 2006; Wuster and Babu, 2010). Fusel alcohols (tyrosol, tryptophol and phenylethanol) are suggested to activate the signalling pathways regulating the change in growth mode. In *S. cerevisiae*, ammonium represses regulatory genes (*Aro8*, *Aro9* and *Aro10*) responsible for production of these fusel alcohols (Chen and Fink, 2006; Wuster and Babu, 2010). Additionally, tryptophol induces expression of *Aro80* which induces *Aro8*, *Aro9* and *Aro10* (positive feedback loop) (Wuster and Babu, 2010). Therefore, under nitrogen limited conditions, higher levels of tyrosol, tryptophol and phenylethanol are found in *S. cerevisiae*. These fusel alcohols are suggested to act as quorum sensing molecules for yeast to change in growth mode, although the quorum sensing detection mechanism is unknown (Chen and Fink, 2006; Cottier and Muhlschlegel, 2012; Hogan, 2006; Leeder, et al., 2011; Wuster and Babu, 2010; Zupan, et al., 2013). Additionally, knowledge on the regulation and role of fusel alcohol formation by yeast is of major interest to the industry, since these compounds are important aroma compounds in many processes, such as production of beer and wine. Therefore, in the next section, production of aroma compounds is determined for the selected yeasts in nutrient excess and nutrient-limited conditions and linked to differences in growth mode.

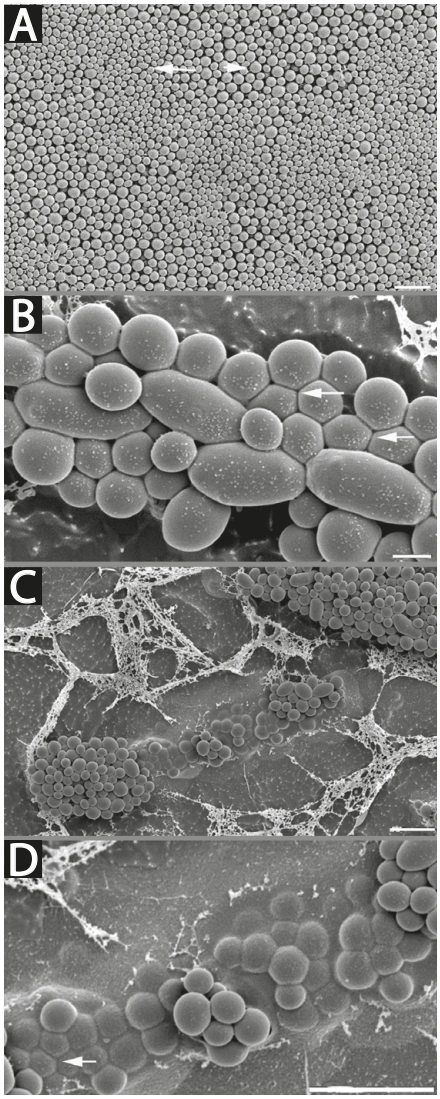


Figure 2.4: Growth of *C. fabianii* 65 into agar. (A) Micrograph taken from the central part of a colony. The yeast cells appear rounded, although differentiation in size readily occurs in certain areas (arrow and arrowhead) (bar = 10 µm); (B) Ultrastructure of a metafilament containing elongated cells and smaller, rounded cells, which at the location of contact appear flattened (arrows), which suggest an intimate contact between the cells (bar = 2 µm); (C) A metafilament growing from inside out the agar forming a crop of rounded cells (bar = 10 µm); (D) Detail of C, illustrating the presence of firmly connected cells (arrow) (bar = 10 µm).

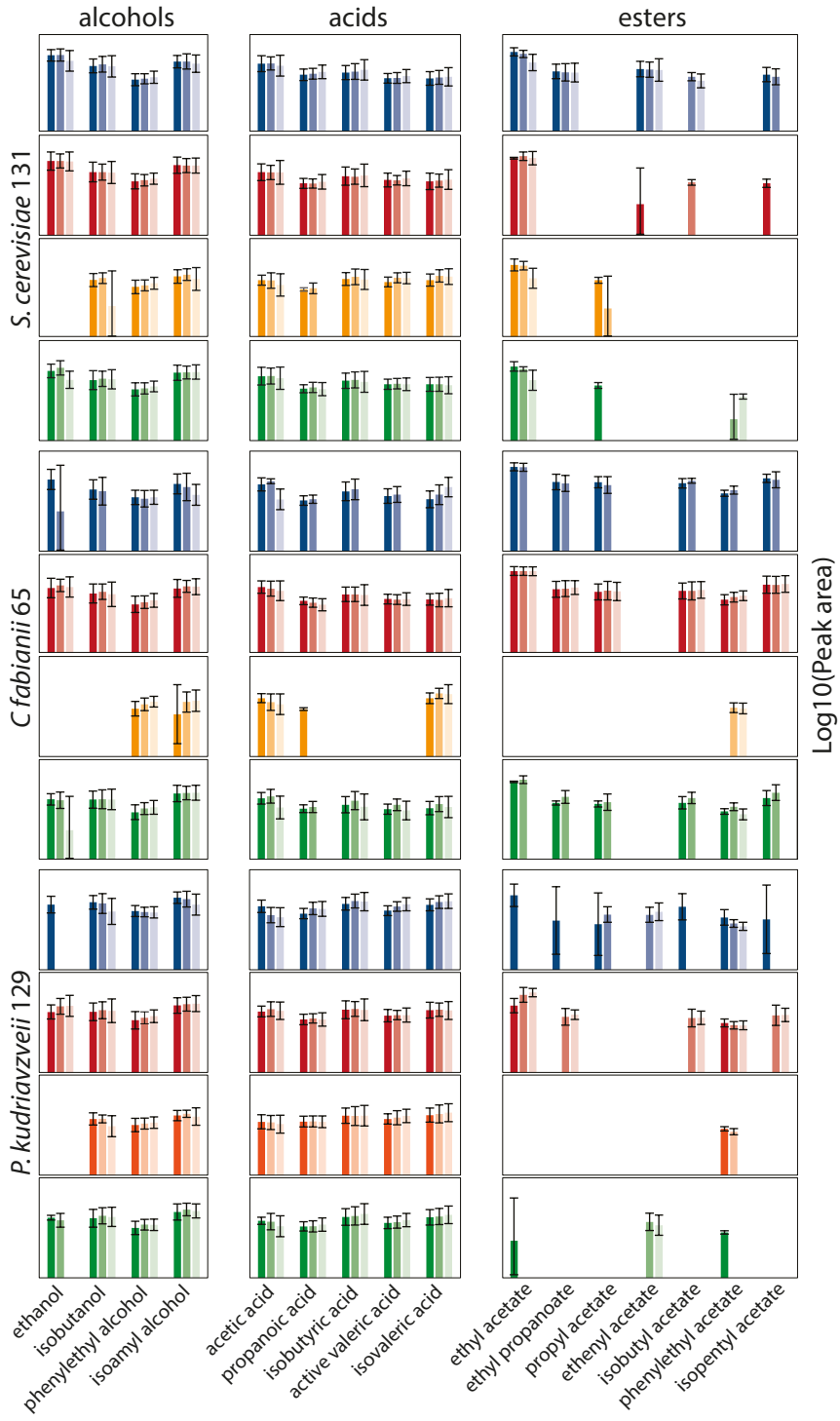
Detection of volatile organic compounds

We used HS GC-MS to determine the volatile organic compounds (VOCs) produced by all three yeast species after 7, 14 and 21 days of incubation on 4 types of agar (see Figure 2.5). For each sample, the VOCs were annotated and classified into three groups (alcohols, acids and esters) (see Table 2.2).

Table 2.1: Alcohols, acids and esters produced by *S. cerevisiae* 131, *C. fabianii* 65 or *P. kudriavzevii* 129 on agar plates

Alcohols		
Ethyl alcohol	Ethanol	
Isobutyl alcohol	Valine fusel alcohol	
Phenylethyl alcohol	Phenylalanine fusel alcohol	
Isoamyl alcohol	Leucine fusel alcohol	
Acids		
Acetic acid		
Propanoic acid		
Isobutyric acid	Valine fusel acid	
Butanoic acid, 2-methyl	Leucine fusel acid	
Isopentanoic acid	Isoleucine fusel acid	
Esters	**Alcohol – Acid**	**Flavour description**
Ethyl acetate	Ethanol – acetate	Nail polish, fruity
Ethyl propionate	Ethanol – propionic acid	Pineapple
Propyl acetate	1-propanol – acetate	Pears
Vinyl acetate	Ethylene – acetic acid	Pungent
Isobutyl acetate	Valine fusel alcohol – acetate	Banana, fruity
Phenylethyl acetate	Phenylalanine fusel alcohol – acetate	Roses, flowery
Isoamyl acetate	Leucine fusel alcohol - acetate	Banana, pear

Figure 2.5: Production of alcohols, acids and esters by either *S. cerevisiae* 131, *C. fabianii* 65 or *P. kudriavzevii* 129 on 4 types of media; nutrient rich (blue), nitrogen limited (red), glucose limited (orange) or nitrogen and glucose limited (green). The colour gradient represents the time of sampling (dark = 7 days, medium = 14 days, light = 21 days). The height of the bars corresponds to the log10 of the peak area. No bar, represents compound below detection limit. Error bars represent standard deviation of 4 replicates.


alcohols
acids
esters
S. cerevisiae 131
C fabianii 65
P. kudriavzveii 129
Log10(Peak area)
ethanol
isobutanol
phenylethyl alcohol
isoamyl alcohol
acetic acid
propanoic acid
isobutyric acid
active valeric acid
isovaleric acid
ethyl acetate
ethyl propanoate
propyl acetate
ethenyl acetate
isobutyl acetate
phenylethyl acetate
isopentyl acetate

Species specific VOCs profiles produced on rich media

The production of alcohols, acids and esters of yeasts depends on the metabolic capacities of the organism and the environmental conditions. The profile of VOCs produced by colonies of *S. cerevisiae* 131, *C. fabianii* 65 and *P. kudriavzevii* 129 grown on rich media were compared in order to establish differences in alcohol, acid and ester production.

All detected alcohols (ethanol, isobutanol, phenylethyl alcohol and isoamyl alcohol) and acids (acetic acid, propanoic acid, isobutyric acid, active valeric acid and isovaleric acid), were initially found for all species, although the timeframe differs for some compounds .

More variation between the species was seen for the esters. Ethyl acetate, ethyl propanoate, isobutyl acetate and isopentyl acetate was initially found for all species. Ethenyl acetate was only found for *S. cerevisiae* 131 and *P. kudriavzevii* 129 and not for *C. fabianii* 65. The esters propyl acetate and phenylethyl acetate was only found for the two non-*Saccharomyces* species and not for *S. cerevisiae* 131.

As expected, the volatile metabolite profiles were species specific on nutrient rich agar. Interestingly, among the species, we observed the most extreme differences for the esters.

The effect of nutrient limitation on the VOC profiles

Next, the effect of nutrient limitation on the production of VOCs was investigated for the three yeast species (Figure 2.5). The results will be discussed per group of VOCs (alcohols, acids and esters).

No ethanol was found for all three species when grown on glucose limited agar. This is in line with the fact that yeast are known to respire under glucose limitation. Additionally no isobutanol was found for *C. fabianii* 65 grown on glucose limited agar. The other alcohols (phenylethyl alcohol and isoamyl alcohol) were detected in similar amounts for all species and media types.

All acids were detected for all species grown on the 4 media types, except for *C. fabianii* 65 grown on glucose limited agar. No isobutyric acid and active valeric acid was detected at any of the time points when *C. fabianii* 65 was grown on glucose limited agar.

More variation was seen for the esters when the species were grown on nutrient limited agar. For *S. cerevisiae* 131, ethyl acetate was found regardless the media type. Propyl acetate was only found when *S. cerevisiae* 131 was grown on either glucose limited or nitrogen plus glucose limited agar, and was not detected when grown on rich agar. Ethenyl acetate, isobutyl acetate and isopentyl acetate was only found at one time point when grown on nitrogen limited agar, and was not found at any of the other nutrient limited agars. Phenylethyl acetate was only found when nitrogen plus glucose was limited.

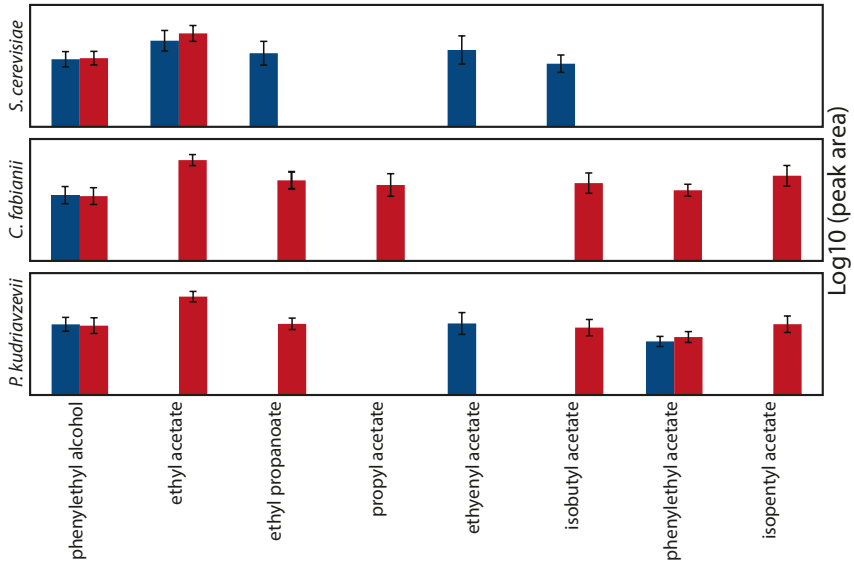


Figure 2.6: Phenylethyl alcohol and esters produced at day 21 by *S. cerevisiae* 131, *C. fabianii* 65 and *P. kudriavzevii* 129 grown in either nutrient rich (blue) or nitrogen limited (orange) conditions. Error bars represent standard deviation of 4 duplicates. Star indicates significant difference (< 0.05) using ANOVA.

For *C. fabianii* 65 the same range of esters was found when grown on either nitrogen or nitrogen plus glucose limited agar compared to nutrient rich agar. Noteworthy, that this range of esters was only found up to 21 days when grown on nitrogen limited agar. Only phenylethyl acetate was found when *C. fabianii* was grown on glucose limited agar.

For *P. kudriavzevii* 129 phenylethyl acetate was found on all nutrient limited types. When grown on nitrogen limited agar, ethyl acetate, ethyl propanoate, isobutyl acetate and isopentyl acetate were also detected. On nitrogen plus glucose limited agar ethyl acetate, ethenyl acetate and phenylethyl acetate were detected additionally to phenylethyl acetate.

In summary, nearly no differences were observed between alcohols and acids produced by the three yeasts under all conditions, except for ethanol. This suggests that the production of these compounds is not influenced by the nutrient availability (ammonium and glucose) and the growth mode of the yeast.

No general relationship between nutrient limitation, shift in growth mode and VOC profiles could be established. Although, for the non-*Saccharomyces* species, prolonged ester production up to 21 days was observed when grown on nitrogen limited agar (Figure 2.6). Remarkably, production of these esters is energy expensive and it is conceivable that these compounds have a biological role during growth under nitrogen limitation. Saerens et al., (2010), reviewed the biological function of esters produced by *S. cerevisiae*. Possible roles of ester formation include; detoxification of the cellular metabolism and safe-guarding optimal

membrane fluidity during fermentation by serving as unsaturated fatty acid analogue in the cell membrane. It could also have an ecological function, such as attracting insects to fermenting fruits to ensure effective distribution of the micro-organisms (Palanca, et al., 2013; Saerens, et al., 2010). Additionally Dumlao et al., (2008), presented evidence that esters could also function as auto-inductive molecules to signal haploid invasive growth. Upon amino acid starvation, methylation of 3-isopropylmalate was induced. They also showed that the ability for invasive growth of haploid yeast strains was enhanced when purified 3-isopropylmalate methyl ester was present. Therefore, the production and role of specific esters identified in the current study warrants further research and will provide insights in their possible role in signalling and formation of filamentous forms of the non-*Saccharomyces* species.

This research shows the value of colony morphology studies by revealing the power of cell-cell adhesion for penetrative growth and formation of metafilaments or pseudohyphae to extend the surface area. Many other studies progress understanding of the molecular mechanism behind this change in growth mode in *S. cerevisiae* research strains (Cottier and Muhlschlegel, 2012; Granek and Magwene, 2010; Hauser, et al., 2007; Johnson, et al., 2014; Leeder, et al., 2011; Torbensen, et al., 2012; Veelders, et al., 2010; Zhang, et al., 2013). Our study has been performed on wild yeast isolates. Our results do not correspond to the finding by various research groups on *S. cerevisiae* laboratory strains, since they suggest that phenylethyl alcohol is a quorum sensing molecule triggering filamentous growth upon nitrogen limitation. Nevertheless, we do find a prolonged production of esters for *C. fabianii* and *P. kudriavzevii* when grown for 21 days which corresponds with the finding of ester formation upon amino acid starvation (Dumlao, et al., 2008). For industrial relevance, the change in growth mode can increase the efficacy of yeast by penetration of fermenting fruits and substrate in solid state fermentation processes. Additionally, the importance of control over cell morphology and cell-cell adhesion in industrial microorganisms is emphasized by the self-clearing of beers at the end of fermentation by flocculation and settling of ale yeast (Palecek, et al., 2002). Moreover, understanding of the relation between nitrogen limitation and prolonged ester formation would give more insight in the ability to steer ester formation by nutrient availability. For the biomedical industry, understanding of the change in growth mode can possibly help to cure penetration of pathogenic yeast in human tissue.

Acknowledgements

This research was financially supported by the Graduate School VLAG, Wageningen University & Research, Wageningen, The Netherlands. The authors would like to acknowledge Geert Meijer (department of Food Quality Design, Wageningen University & Research, The Netherlands) for the technical assistance of the HS GC-MS analyses.

Supplementary materials

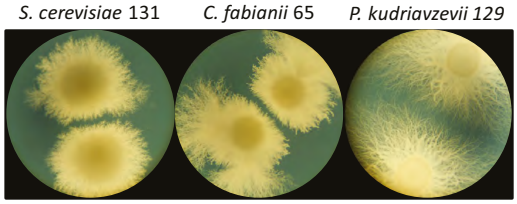


Figure S2.1: *S. cerevisiae* 131, *C. fabianii* 65 and *P. kudriavzevii* 129 grown on nitrogen limited agar for 21 days at 30 °C. Demonstrating the fact that somehow they do not grow filamentous in the direction of another colony.

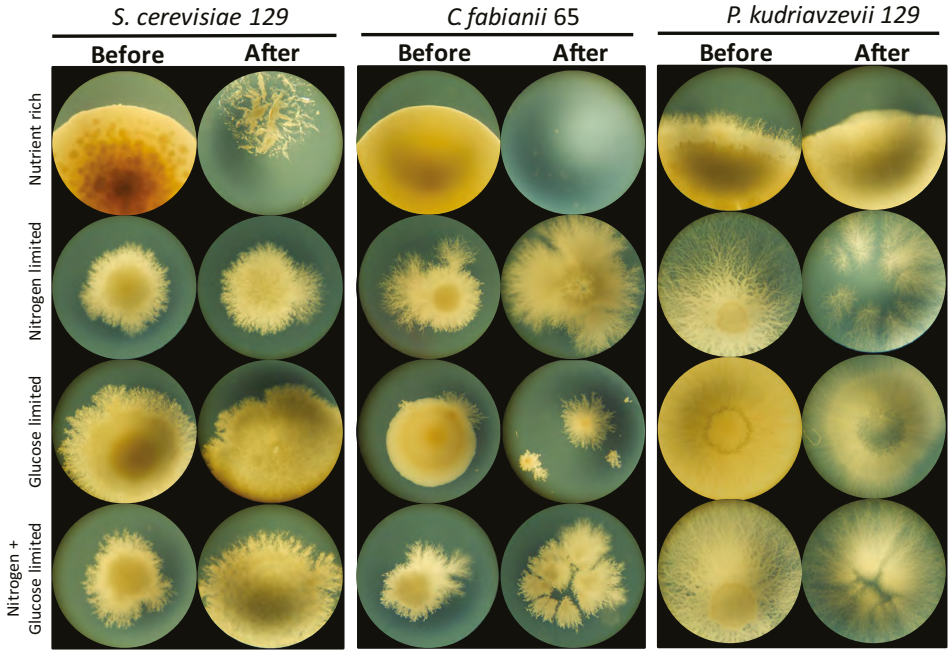


Figure S2.2: Invasive growth experiment. Colony before washing and after.

References

Banuett, F. (1998). Signalling in the Yeasts: An Informational Cascade with Links to the Filamentous Fungi. *Microbiology and Molecular Biology Reviews* **62,** 249-74.

Chen, H. and Fink, G. R. (2006). Feedback control of morphogenesis in fungi by aromatic alcohols. *Genes Dev* **20,** 1150-61.

Chen, R. E. and Thorner, J. (2010). Systematic epistasis analysis of the contributions of protein kinase A- and mitogen-activated protein kinase-dependent signaling to nutrient limitation-evoked responses in the yeast *Saccharomyces cerevisiae*. *Genetics* **185,** 855-70.

Cordente, A. G., Curtin, C. D., Varela, C. and Pretorius, I. S. (2012). Flavour-active wine yeasts. *Appl Microbiol Biotechnol* **96,** 601-18.

Cottier, F. and Muhlschlegel, F. A. (2012). Communication in fungi. *Int J Microbiol* **2012,** 1-9.

Cullen, P. J. and Sprague, G. F., Jr. (2012). The regulation of filamentous growth in yeast. *Genetics* **190,** 23-49.

Dickinson, J. R., Salgado, L. E. and Hewlins, M. J. (2003). The catabolism of amino acids to long chain and complex alcohols in *Saccharomyces cerevisiae*. *J Biol Chem* **278,** 8028-34.

Dumlao, D. S., Hertz, N. and Clarke, S. (2008). Secreted 3-Isoproylmalate Methyl Ester Signals Invasive Growth during Amino Acid Starvation in *Saccharomyces cerevisiae*. *Biochemistry* **47,** 698-709.

Gagiano, M., Bauer, F. F. and Pretorius, I. S. (2002). The sensing of nutritional status and the relationship to filamentous growth in *Saccharomyces cerevisiae*. *FEMS Yeast Res* **2,** 433-70.

Gamero, A., Hernandez-Orte, P., Querol, A. and Ferreira, V. (2011). Effect of aromatic precursor addition to wine fermentations carried out with different *Saccharomyces* species and their hybrids. *Int J Food Microbiol* **147,** 33-44.

Gamero, A., Tronchoni, J., Querol, A. and Belloch, C. (2013a). Production of aroma compounds by cryotolerant *Saccharomyces* species and hybrids at low and moderate fermentation temperatures. *J Appl Microbiol* **114,** 1405-14.

Gamero, A., Wesselink, W. and de Jong, C. (2013b). Comparison of the sensitivity of different aroma extraction techniques in combination with gas chromatography-mass spectrometry to detect minor aroma compounds in wine. *J Chromatogr A* **1272,** 1-7.

Gimeno, C. J., Ljungdahl, P. O., Styles, C. A. and Fink, G. R. (1992). Unipolar cell division in the Yeast *S. cerevisiae* Lead to Filamentous Growth: Regulation by Starvation and RAS. *Cell* **68,** 1077-90.

Granek, J. A., Kayikci, O. and Magwene, P. M. (2011). Pleiotropic signaling pathways orchestrate yeast development. *Curr Opin Microbiol* **14,** 676-81.

Granek, J. A. and Magwene, P. M. (2010). Environmental and Genetic Determinants of Colony Morphology in Yeast. *PloS Genetics* **6**, e1000823.

Hauser, M., Horn, P., Tournu, H., Hauser, N. C., Hoheisel, J. D., Brown, A. J. and Dickinson, J. R. (2007). A transcriptome analysis of isoamyl alcohol-induced filamentation in yeast reveals a novel role for Gre2p as isovaleraldehyde reductase. *FEMS Yeast Res* **7,** 84-92.

Hazelwood, L. A., Daran, J. M., van Maris, A. J., Pronk, J. T. and Dickinson, J. R. (2008). The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. *Appl Environ Microbiol* **74,** 2259-66.

Hogan, D. A. (2006). Quorum sensing: alcohols in a social situation. *Curr Biol* **16,** R457-8.

Hornby, J. M., Jacobitz-Kizzier, S. M., McNeel, D. J., Jensen, E. C., Treves, D. S. and Nickerson, K. W. (2004). Inoculum size effect in dimorphic fungi: extracellular control of yeast-mycelium dimorphism in Ceratocystis ulmi. *Appl Environ Microbiol* **70,** 1356-9.

Hornby, J. M., Jensen, E. C., Lisec, A. D., Tasto, J. J., Jahnke, B., Shoemaker, R., Dussault, P. and Nickerson, K. W. (2001). Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Appl Environ Microbiol* **67,** 2982-92.

Johnson, C., Kweon, H. K., Sheidy, D., Shively, C. A., Mellacheruvu, D., Nesvizhskii, A. I., Andrews, P. C. and Kumar, A. (2014). The yeast sks1p kinase signaling network regulates pseudohyphal growth and glucose response. *PLoS Genet* **10,** e1004183.

Klingberg, T. D., Lesnik, U., Arneborg, N., Raspor, P. and Jespersen, L. (2008). Comparison of *Saccharomyces cerevisiae* strains of clinical and nonclinical origin by molecular typing and determination of putative virulence traits. *FEMS Yeast Res* **8,** 631-40.

Kugler, S., Schurtz Sebghati, T., Groppe Eissenberg, L. and Goldman, W. E. (2000). Phenotypic variation and intracellular parasitism by histoplasma Capsulatum. *Proc Natl Acad Sci U S A* **97,** 8794-8.

Leeder, A. C., Palma-Guerrero, J. and Glass, N. L. (2011). The social network: deciphering fungal language. *Nat Rev Microbiol* **9,** 440-51.

Lorenz, M. C., Cutler, N. S. and Heitman, J. (2000). Characterization of alcohol-induced filamentous growth in *Saccharomyces cerevisiae*. *Mol Biol Cell* **11,** 183-99.

Madhani, H. D. and Fink, G. R. (1998). The control of filamentous differentiation and virulence in fungi. *Trends in Cell Biology* **8,** 348-53.

Nyanga, L. K., Nout, M. J., Gadaga, T. H., Theelen, B., Boekhout, T. and Zwietering, M. H. (2007). Yeasts and lactic acid bacteria microbiota from masau (*Ziziphus mauritiana*) fruits and their fermented fruit pulp in Zimbabwe. *Int J Food Microbiol* **120,** 159-66.

Nyanga, L. K., Nout, M. J., Smid, E. J., Boekhout, T. and Zwietering, M. H. (2013). Fermentation characteristics of yeasts isolated from traditionally fermented masau (*Ziziphus mauritiana*) fruits. *Int J Food Microbiol* **166,** 426-32.

Palanca, L., Gaskett, A. C., Günther, C. S., Newcomb, R. D. and Goddard, M. R. (2013). Quantifying Variation in the Ability of Yeasts to Attract *Drosophila melanogaster*. *PLoS One* **8,** e75332.

Palecek, S. P., Parikh, A. S. and Kron, S. J. (2002). Sensing, signalling and integrating physical processes during *Saccharomyces cerevisiae* invasive and filamentous growth. *Microbiology* **148,** 893-907.

Perpete, P., Duthoit, O., De Maeyer, S., Imray, L., Lawton, A. I., Stavropoulos, K. E., Gitonga, V. W., Hewlins, M. J. and Dickinson, J. R. (2006). Methionine catabolism in *Saccharomyces cerevisiae*. *FEMS Yeast Res* **6,** 48-56.

Pruyne, D. and Bretscher, A. (2000). Polarization of cell growth in yeast. *Journal of Cell Science* **113,** 365-75.

Saerens, S. M., Delvaux, F. R., Verstrepen, K. J. and Thevelein, J. M. (2010). Production and biological function of volatile esters in *Saccharomyces cerevisiae*. *Microb Biotechnol* **3,** 165-77.

Sprague, G. F., Jr. and Winans, S. C. (2006). Eukaryotes learn how to count: quorum sensing by yeast. *Genes Dev* **20,** 1045-9.

Torbensen, R., Moller, H. D., Gresham, D., Alizadeh, S., Ochmann, D., Boles, E. and Regenberg, B. (2012). Amino acid transporter genes are essential for FLO11-dependent and FLO11-independent biofilm formation and invasive growth in *Saccharomyces cerevisiae*. *PLoS One* **7,** e41272.

Veelders, M., Bruckner, S., Ott, D., Unverzagt, C., Mosch, H. U. and Essen, L. O. (2010). Structural basis of flocculin-mediated social behavior in yeast. *Proc Natl Acad Sci U S A* **107,** 22511-6.

Wuster, A. and Babu, M. M. (2010). Transcriptional control of the quorum sensing response in yeast. *Mol Biosyst* **6,** 134-41.

Wyatt, T. T., van Leeuwen, M. R., Wosten, H. A. and Dijksterhuis, J. (2014). Mannitol is essential for the development of stress-resistant ascospores in Neosartorya fischeri (Aspergillus fischeri). *Fungal Genet Biol* **64,** 11-24.

Zhang, Y., Kweon, H. K., Shively, C., Kumar, A. and Andrews, P. C. (2013). Towards systematic discovery of signaling networks in budding yeast filamentous growth stress response using interventional phosphorylation data. *PLoS Comput Biol* **9,** e1003077.

Zupan, J., Avbelj, M., Butinar, B., Kosel, J., Sergan, M. and Raspor, P. (2013). Monitoring of Quorum-Sensing Molecules during Minifermentation Studies in Wine Yeast. *J Agric Food Chem***,** 2496-505.

Chapter 3

Linking acetate ester hydrolysing activities to aroma profiles of *Cyberlindnera fabianii*, *Pichia kudriavzevii* and *Saccharomyces cerevisiae*

Irma M. H. van Rijswijck[1], Judith C. M. Wolkers – Rooijackers[1], Tjakko Abee[1] and Eddy J. Smid[1]

Manuscript in preparation

Affiliations:

1. Laboratory of Food Microbiology, Wageningen University & Research, P.O. Box 17, 6700 AA Wageningen, The Netherlands

Abstract

Esters constitute an important class of aroma compounds and contribute significantly to the aroma of yeast-fermented beverages. Ester formation is well studied in *Saccharomyces cerevisiae*, while production of aroma compounds by non-conventional yeasts has received limited attention. The selection of such strains for co-culturing with *S. cerevisiae* may offer opportunities for product innovations. Therefore, we performed a comparative analysis of the diversity in ester production in broth by *Cyberlindnera fabianii* 65 (Cf65), *Pichia kudriavzevii* 129 (Pk129) and *S. cerevisiae* 131 (Sc131). For all three species distinct aroma profiles were identified, with Cf65 producing the highest amount of acetate esters. Since esters are formed from alcohols and acyl or acetyl CoA, we also analysed alcohol dehydrogenase activities in those three yeasts and found no correlation with ester formation. In contrast, a clear inverse correlation between the acetate ester hydrolysis (esterase) activity and acetate ester yield was found for the three yeasts. Our study indicates that esterases play a key role in determination of the final amount of acetate esters in fermentation broths.

Introduction

Increasing interest in non-conventional yeast applications is caused by their unique growth and aroma formation characteristics compared to conventional *Saccharomyces cerevisiae* (Contreras, et al., 2014; Gamero, et al., 2016). Currently, they are also used in various fermentation processes in the biotech and food industry (Belda, et al., 2016; Contreras, et al., 2015; Del Monaco, et al., 2014; Steensels, et al., 2015). Recently, van Rijswijck, et al. (2017b) described the application of *Cyberlindnera fabianii* and *Pichia kudriavzevii* in a co-culture with brewers' yeast for beer production. Especially, a co-culture of brewers' yeast with *C. fabianii* resulted in a beer with increased ester levels (fruity flavour) (van Rijswijck, et al., 2017b). Their ability to produce distinct aroma profiles with different ratios of esters and alcohols make them ideal candidates for a more detailed investigation of the underlying mechanisms affecting ester formation and hydrolysis.

Esters are important aromatic compounds produced by yeasts with a fruity characteristic, a low odour threshold and therefore noticeable in very low amounts (Saerens, et al., 2010; Suomalainen, 1981; Verstrepen, et al., 2003a). Esters can be divided in two groups; Medium chain fatty acid (MCFA) ethyl esters and acetate esters. In this study the focus will be mainly on acetate esters since those are usually present in higher levels in fermented beverages. Acetate esters can be formed intracellularly by alcohol acyltransferases (AATs) that convert an alcohol and acetyl-CoA to the corresponding ester (Mason and Dufour, 2000; Nordström, 1963a; Nordström, 1964) or by $NADP^+$-dependent alcohol dehydrogenases by oxidation of hemiacetals (formed by alcohols and aldehydes mixtures)(Kruis, et al., 2017; Park, et al., 2009). The ester yield depends on the amount of substrates and the activities of the enzymes involved in ester synthesis and hydrolysis (Fukuda, et al., 1998; Inoue, et al., 1997; Nordström, 1964; Saerens, et al., 2010; Verstrepen, et al., 2003a). Additionally, all parameters involved will contribute to the final aroma profile signifying the complexity of this product characteristic.

Either ethanol or a higher alcohol (fusel alcohol) can be used as substrate for AATs. Ethanol is the main product of the alcoholic fermentation pathway, whereas fusel alcohols are products of the Ehrlich pathway (reviewed by Hazelwood, et al. (2008)). Both pathways use alcohol dehydrogenases (ADHs) with NAD(P)H as co-factor to reduce aldehydes to the corresponding alcohols. Genes encoding alcohol dehydrogenases in *S. cerevisiae* are *ADH1-7*, of which *ADH1-5* use NADH and *ADH6-7* NADPH as co-factor (reviewed by de Smidt, et al. (2008)). The function and localization of ADHs has been extensively studied and reviewed for *S. cerevisiae* (de Smidt, et al., 2008; Larroy, et al., 2002; Raj, et al., 2014; van Iersel, et al., 1997). Also ADH encoding genes of *Pichia stipitis*, *Kluyveromyces lactis*, *Yarrowia lipolitica* have been studied (Cho and Jeffries, 1998; Gatter, et al., 2016; Saliola, et al., 1990). But for *C. fabianii* and *P. kudriavzevii*, no genetic information is available and there is no record on the localization and function of putative ADHs.

The second substrate, acetyl-CoA, can either be formed by oxidative decarboxylation of pyruvate by a pyruvate dehydrogenase using CoA as a co-factor or by direct activation of acetate using ATP and CoA. The pyruvate dehydrogenase is located in the mitochondria; therefore the only source for cytosolic acetyl-CoA in *S. cerevisiae* is via direct activation of acetate. In this respect, it is relevant to note that *S. cerevisiae* does not contain ATP-citrate lyase, an enzyme that plays a major role in translocation of acetyl-CoA across the mitochondrial membrane in mammalian cells and in several non-*Saccharomyces* yeasts (Boulton and Ratledge, 1981).

Together with the substrate availability, the balance between AATs and esterase activity is of key importance for the overall ester yield (Fukuda, et al., 1998; Lilly, et al., 2006; Lilly, et al., 2000). The AATs and esterase activity and substrate specificity is determined by expression of the specific genes encoding these enzymes and the final protein levels. Genes encoding AATs in *S. cerevisiae* are *ATF1* and *ATF2* (Fujii, et al., 1994; Nagasawa, et al., 1998). Isoamyl acetate hydrolase is considered to act as the key acetate esterase and is encoded by *IAH1* (Fukuda, et al., 2000; Fukuda, et al., 1998; Inoue, et al., 1997). These genes are well studied for *S. cerevisiae*, but no information is available on the *ATF1*, *ATF2* and *IAH1* encoding genes in *C. fabianii* and *P. kudriavzevii*.

In this study we focused on three strains with distinct aroma profiles; *S. cerevisiae* 131 (Sc131), *C. fabianii* 65 (Cf65) and *P. kudriavzevii* 129 (Pk129) (Nyanga, et al., 2007; Nyanga, et al., 2013; van Rijswijck, et al., 2017a; van Rijswijck, et al., 2015; van Rijswijck, et al., 2017b). We investigated their specific enzyme activities (ADHs and esterases) and correlated these to the distinct aroma profiles produced. Additionally, the genomes of these isolates have been sequenced, annotated and a comparative analysis of the encoded putative ADHs and esterases was performed (van Rijswijck, et al., 2017a). By comparison of representative strains of these three yeast species, we revealed that the acetate ester hydrolysing activity is the crucial step that determines the different amounts of acetate ester found in the fermentation broths.

Materials and methods

Yeast strains

Three isolates from fermented masau fruit were used in this study (Nyanga, et al., 2007; Nyanga, et al., 2013): *Saccharomyces cerevisiae* 131, *Cyberlindnera fabianii* 65 (previously known as *Pichia fabianii* 65) and *Pichia kudriavzevii* 129 (previously known as *Issatchenkia orientalis* 129). Glycerol stocks (15 % v/v) of the strains were kept at -80 °C until use.

Media

Malt Extract Agar (MEA) and Malt Extract Broth (MEB) were used as culture media and prepared according manufactures recipe (Oxoid).

Culture conditions

Glycerol stocks were streaked on MEA plates and incubated at 20 °C for 3 days. A single colony was inoculated into 10 mL MEB and incubated for 24 hours at 30 °C, 200 rpm. 1 % (v/v) overnight culture was transferred to 150 mL MEB and incubated at 30 °C for 48 hours (static), after which cells were collected for cell free extract preparation.

Cell free extract preparation

Cells were collected by centrifugation (OD_{600nm} = 60 for *C. fabianii* and OD_{600nm} = 50 for *S. cerevisiae* and *P. kudriavzevii*) at 13.000 x g for 15 minutes. Cells were washed 3 times in Peptone Buffered Saline (PBS) (pH = 7.4), and finally re-suspended in 700 µl PBS and transferred to a screwcap-tube containing 300 mg sterilized lysing matrix C (MP Biomedicals). Samples were cooled for at least 5 minutes on ice before cell disruption. Cells were disrupted by 9 beadbeating intervals: 20 seconds (4.5 m/s), 1 minute on ice. Cell material was removed by centrifugation for 5 minutes at 11.300 x g at 4 °C. The supernatant was transferred to a pre-cooled tube and kept on ice.

Total protein determination

The total protein concentration was determined using the Pierce™ Coomassie (Bradford) Protein Assay Kit. The assay was performed according manufactures protocol in 96-well plates using Spectramax® M2 (Molecular devices).

Specific alcohol dehydrogenase activity

10 µl or 40 µl (for NAD- or NADP-dependent ADH respectively) cell free extract was combined with reaction buffer up to a total volume of 200 µl in an UV-transparent 96-

well plate (Greiner). The reaction buffer for NAD-dependent ADH activity consisted of; 0.1 M Tris-HCL buffer (pH 8.5), 1 % Triton X-100, 1 % substrate (ethyl alcohol, butyl alcohol, phenylethyl alcohol, isoamyl alcohol or isobutyl alcohol) and 1.25 mg/mL NAD^+. For the $NADP^+$-dependent ADH activity assay the reaction buffer consisted of; 0.1 M Tris-HCl buffer (pH 9), 1 % substrate (ethyl alcohol, butyl alcohol, phenylethyl alcohol, isoamyl alcohol or isobutyl alcohol) and 0.5 mg/ml $NADP^+$. The reaction kinetics were measured at λ_{340nm} every 2 minutes for 40 minutes at 25 °C using Spectramax® M2 (Molecular devices).

Specific esterase activity

Specific esterase activity assay was adapted from Breeuwer, et al. (1995). 40 µl cell free extract was combined with 160 µl Mc Ilvaine buffer (100 mM citric acid, 200 mM disodium hydrogen phosphate set at pH 7.3) containing 50 µM cFDA (5- (and 6-) carboxyfluorescein diacetate(Sigma, zwijndrecht, the Netherlands)). Hydrolysis of cFDA was measured using Spectramax® M2 (Molecular devices) (excitation λ_{490nm} emission λ_{515nm}) every 2 minutes for 40 minutes at 40 °C using black 96-well plates (Greiner).

3

Volatile organic compounds (VOCs) analysis

At the same time as cells were collected for preparation of the cell free extract, also 2 x 2 ml was frozen in a GC-MS vial and stored at -20 °C upon analysis.

Volatile organic compounds present in the sample were determined by headspace solid phase micro extraction gas chromatography mass spectrometry (HS-SPME GC-MS) analysis.

Samples were kept at -1 °C in the TriPlus RSH auto-sampler (Interscience) until analysis. Until analysis, samples were incubated for 10 minutes at 60 °C, after which the volatile compounds were extracted for 20 minutes at 60 °C using a SPME fiber (Car/DVB/PDMS Suppelco). The compounds are desorbed from the fiber for 5 minutes on a Stabilwax®-DA-Crossband®-Carbowax®-polyethylene-glycol column (30 m length, 0.25 mmID, 0.5 µm df).

The Trace 1300 gas chromatograph (Interscience) settings were: PTV Split-less mode (5 minutes) at 240 °C. Helium was used as carrier gas at a constant flow of 1.5 ml/min. The GC oven was initially at 35 °C for 2 minutes, raised to 240 °C (10 °C/min) and kept at 240 °C for 5 minutes.

Mass spectral data was collected by an ISQ QD single quadrupole MS (Interscience) over a range of m/z 33-250 in full scan mode with 3.0030 scans/sec seconds.

Peaks were annotated using Chromeleon® 7.2. The ICIS algorithm was used for peak integration and the NIST mainlib to match the mass spectral profiles with the profiles of NIST. Peak areas were calculated using the MS quantitation peak area (highest m/z peak per compound).

For Figure 3.1, the following equation was used to determine the abundance per compound:

$$relative\ abundance\ (y)\ of\ compound\ (x) = log_2\left(\frac{MSquantitation_{xy}}{median\left(MSquantitation_x\right)}\right)$$

Comparative genomics

The genome sequences and predicted genes of Sc131, Cf65 and Pk129 are publicly available on NCBI under the bioproject numbers: PRJNA353176 (*S. cerevisiae* 131), PRJNA353175 (*C. fabianii* 65) and PRJNA353174 (*P. kudriavzevii* 129) (van Rijswijck, et al., 2017a). Relevant predicted genes are used for comparative genomics using clustal W with the software package DNASTAR lasergene 11 MegAlign. Unrooted phylogenetic tree was visualized using FigTree (version 1.4.2 2006-2014, Andrew Rambaut Institute of Evolutionary Biology, University of Edinburgh).

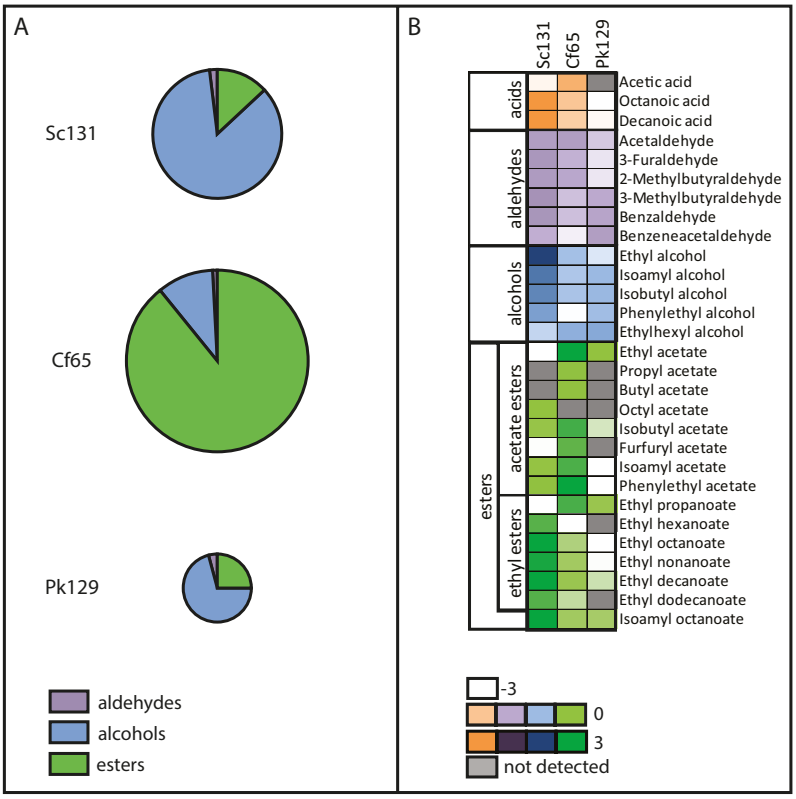


Figure 3.1: Box A (left): Pie chart of volatile aldehydes (purple), alcohols (blue) and esters (green) produced by Sc131, Cf65 and Pk129 on malt extract broth (the size indicates the sum peak area (MSquantitation) per compound group). Box B (Right): Relative abundance per compound (colour indicates abundance compared to median per compound of the three strains (3 = above median, 0 = median, -3 = below median and grey = not detected)). The compounds are grouped in acids (orange), aldehydes (purple), alcohols (blue) and esters (green).

Results

The link between aroma formation, specific enzyme activities (ADHs and esterase) and substrate specificity (ADHs) was studied in *S. cerevisiae* 131 (Sc131), *C. fabianii* 65 (Cf65) and *P. kudriavzevii* 129 (Pk129). First, the distinct aroma profiles of each yeast strain are presented, followed by the ADH substrate specificity and specific esterase activity. After which the link between the aroma profiles and enzyme activities is made. Finally, the translated nucleotide sequences of predicted genes involved in the formation of alcohols and hydrolysis of acetate esters are compared.

Distinct aroma profiles

Sc131, Cf65 and Pk129 were grown on Malt Extract Broth (MEB) for 48 hours at 30 °C (static incubation), after which the aroma profiles were determined using HS-SPME GC-MS. A clear difference in relative abundance as well as amount of each compound group (volatile aldehydes, alcohols and esters) was observed (Figure 3.1A). The size of the pie indicates the amount (sum peak area (MSquantitation)) and each part of the pie chart the relative abundance of a compound group. The aroma profile of Sc131 consists for 83 % of volatile alcohols, compared to 13 % for Cf65 and 72 % for Pk129. Whereas 86 % of the VOCs produced by Cf65 are esters, and only 16 % and 24 % by Sc131 and Pk129 respectively (Figure 3.1A). However, a closer look at the relative abundance per compound between the strains (See M&M for calculations), reveals that Cf65 produces most acetate esters and Sc131 most ethyl esters as well as alcohols and acids under the applied circumstances (Figure 3.1B). The distinct aroma profiles make these strains ideal candidates to study the activities of enzymes involved in alcohol (ADHs) and ester production as well as ester hydrolysis (esterases).

ADH substrate specificity

ADHs use NADH or NADPH as co-factor to convert aldehydes into alcohols. Here we tested the substrate specificity of Sc131, Cf65 and Pk129 cell free extracts of a culture grown for 48 hours on MEB (static at 30 °C). The substrates were chosen upon analysis of the aroma profiles and include ethyl alcohol, butyl alcohol, phenylethyl alcohol, isoamyl alcohol and isobutyl alcohol. The specific ADH-activity is expressed in nmol/minute*mg protein^{-1} with either NAD^+ or $NADP^+$ as co-factor (Table 3.1). Clear differences are observed between the three strains, as well as for the substrates and co-factors.

Table 3.1: Specific alcohol dehydrogenase activity in nmol/minute*mg protein^{-1} (average ± standard deviation, n=9). n.d. = not detectable. Between the brackets a different letter (a, b or c) is used to indicate significant difference ($p < 0.05$) between the three strains per substrate and a different number (1, 2, 3 or 4) is used to indicate significant difference ($p < 0.05$) between substrates for one strain with the same co-factor used (NAD^+ or $NADP^+$).

NAD+ - dependent ADH	**Sc131**	**Cf65**	**Pk129**
ethyl alcohol	2237 ± 326 (a,1)	977 ± 88 (b,1)	403 ± 71 (c,1)
butyl alcohol	188 ± 147 (a,2)	572 ± 40 (b,2)	591 ± 28 (c,2)
phenylethyl alcohol	n.d.	n.d.	34 ± 5 (c,3)
isoamyl alcohol	n.d.	n.d.	n.d.
isobutyl alcohol	n.d.	n.d.	n.d.

NADP+-dependent ADH	**Sc131**	**Cf65**	**Pk129**
ethyl alcohol	1.76 ± 0.98 (a,1)	2.91 ± 0.63 (b,1)	n.d.
butyl alcohol	2.50 ± 0.63 (a,2)	19.80 ± 1.11 (b,2)	11.24 ± 1.36 (c,1)
phenylethyl alcohol	1.32 ± 0.51 (a,1,3)	2.48 ± 0.93 (b,1)	4.79 ± 0.52 (c,2)
isoamyl alcohol	2.75 ± 0.52 (a,2)	12.28 ± 0.74 (b,3)	12.56 ± 1.76 (b,1)
isobutyl alcohol	1.04 ± 0.32 (a,3)	5.66 ± 0.84 (b,4)	8.42 ± 1.30 (c,3)

Sc131 specific ADH-activity using NAD^+ as co-factor is 11.9 times higher for ethyl alcohol compared to butyl alcohol (2237 ± 326 and 188 ± 147 nmol/min*mg protein^{-1} respectively, Table 3.1). For phenylethyl alcohol, isoamyl alcohol and isobutyl alcohol the activities are below the detection limit. The specific ADH-activity using $NADP^+$ as co-factor was, as expected, much lower than using NAD^+ as co-factor. Significantly higher ADH-activities were found for the substrates butyl alcohol and isoamyl alcohol (2.50 ± 0.63 and 2.75 ± 0.52 nmol/minute*mg protein^{-1} respectively) compared to ethyl alcohol, phenylethyl alcohol and isobutyl alcohol (1.76 ± 0.98, 1.32 ± 0.51, 1.04 ± 0.32 nmol/minute*mg protein^{-1} respectively, Table 3.1).

The specific ADH-activity of Cf65 using NAD^+ as co-factor is 1.7 times higher for the substrate ethyl alcohol compared to butyl alcohol (Table 3.1). For all other substrates the enzyme activities were below detection limit using NAD^+ as co-factor. The highest specific ADH-activity using $NADP^+$ as co-factor was found for the substrates butyl alcohol and isoamyl alcohol (19.80 ± 1.11 and 12.28 ± 0.74 nmol/minute*mg protein^{-1} respectively), followed by isobutyl alcohol, ethyl alcohol and phenylethyl alcohol (5.66 ± 0.84, 2.91 ± 0.63 and 2.48 ± 0.93 nmol/minute*mg protein^{-1} respectively, Table 3.1).

NAD^+ dependent ADH-activity of Pk129 is 1.4 times higher for the substrate butyl alcohol compared to ethyl alcohol, also low activity levels using phenylethyl alcohol as substrate are observed (34 ± 5 nmol/minute*mg protein^{-1}, Table 3.1). $NADP^+$ as co-factor resulted in the highest specific ADH-activity for the substrates isoamyl alcohol and butyl alcohol (12.57 ± 1.76 and 11.25 ± 1.36 nmol/minute*mg protein^{-1} respectively), followed by isobutyl alcohol (8.42 ± 1.30) and phenylethyl alcohol (4.79 ± 0.52) (Table 3.1).

Different activities for various substrates within one strain are observed. As expected, due to the distinct aroma profiles, also differences in activities between the strains are found.

Specific esterase activity

The ester hydrolysing activity was measured by the hydrolysis of non-fluorescent 5- (and 6-) carboxyfluorescein diacetate (cFDA) to fluorescent 5- (and 6-) carboxyfluorescein (cF). The specific activities of Sc131 and Pk129 are significantly higher compared to Cf65 (0.0279 ± 0.0077, 0.0249 ± 0.0069 and 0.0040 ± 0.0013 nmol/minute*mg protein^{-1} respectively, Figure 3.2). cFDA is an acetate ester; therefore its hydrolysis conceivably correlates inversely with the amount of acetate esters.

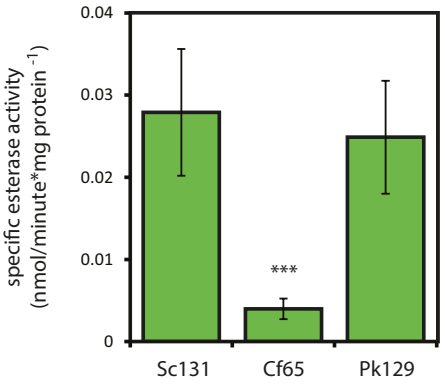


Figure 3.2: Specific cFDA hydrolysing activity in nmol/minute*mg protein^{-1} (average ± standard deviation (n=9)) of cell extracts from cultures of Sc131, Cf65 and Pk129 grown in MEB for 48 hours at 30 °C static. *** indicate significance difference ($p < 0.001$) of Cf65 with Sc131 and Pk129.

Link enzyme activities with aroma profiles

Combining the aroma profiles with the knowledge on ADH substrate specificity and esterase activity will reveal the critical step(s) determining the amount of acetate ester in the fermented broth. Figure 3.3 visualizes the correlation between aldehyde, alcohol, ester levels and specific ADH and esterase enzyme activities. The left box depicts the correlation between ethyl aldehyde, ethyl alcohol, ethyl acetate and derived enzyme activities, where the highest peak area (MSquantitation) was found for ethyl acetate by Cf65; this peak area is set as 100 % (indicated with colour). The relative enzyme activity is indicated with arrows, per enzyme activity the highest activity for the three strains was set as 100 % (indicated with colour). The blue arrow with black stroke represents the NAD^+-dependent ADH activity using ethyl alcohol as substrate, where a correlation is observed between the activity and the peak area of ethyl alcohol. This trend is not observed for the $NADP^+$-dependent ADH activity (blue arrow with grey stroke). However, a high esterase activity (green arrow) strongly correlates with low ethyl acetate area (peak area (MSquantitation)).

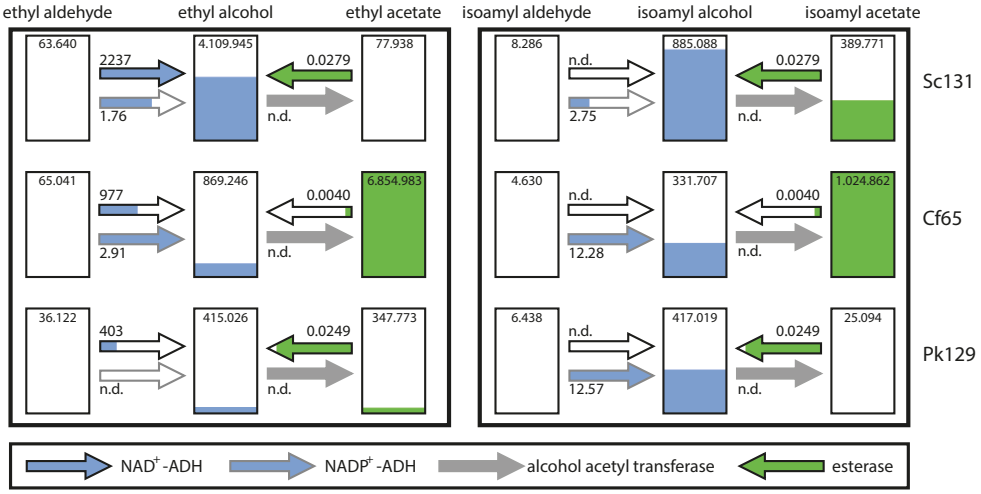


Figure 3.3: Visualization of the specific NAD(P)$^+$-dependent alcohol dehydrogenase (ADH), esterase activity and aldehyde, alcohol and ester levels of Sc131 (top), Cf65 (middle) and Pk129 (bottom). Left box: ethyl aldehyde -> ethyl alcohol -> ethyl acetate and right box isoamyl aldehyde -> isoamyl alcohol -> isoamyl acetate. The numbers in each rectangle represent the peak area (MS quantitation) of each compound and the colour indicates the relative abundance within one box. The numbers above/below each arrow represents the specific enzyme activity in nmol/minute*mg protein^{-1} and the colour represents the relative activity per enzyme assay within one box. n.d. = activity below detection limit, and the alcohol acetyl transferase activity was not determined.

The right box (Figure 3.3) envisions the correlation between the peak area (MSquantiation) of isoamyl aldehyde, isoamyl alcohol and isoamyl acetate and the corresponding enzyme activities. The highest peak area, isoamyl acetate by Cf65, was set to 100 % (indicated by colour). Interestingly, the NAD$^+$-dependent ADH enzyme activity of isoamyl alcohol was below the detection limit. The NADP$^+$-dependent ADH enzyme activity of isoamyl alcohol was significantly higher for Cf65 and Pk129 compared to Sc131, but no correlation with the peak area (MSquantiation) of isoamyl alcohol was found (Table 3.1).

For both ethyl acetate (left box) and isoamyl acetate (right box), an inverse correlation between the corresponding ester and esterase activity was observed. Additionally, the higher esterase activity also correlates positively with a higher peak area of isoamyl alcohol (Figure 3.3).

Comparative genomics

The genomes of Sc131, Cf65 and Pk129 were sequenced and potential genes were annotated (van Rijswijck, et al., 2017a). The translated nucleotide sequences of the predicted genes involved in ester metabolism were compared to the protein sequences available in NCBI database.

For Sc131 Adh1p, Adh2p, Adh3p, Adh4p, Adh6p and Adh7p were annotated and are highly similar to the corresponding ADH encoding genes found in the model strain *S. cerevisiae* S288c (99-100 %, Supplementary Table 3.1). As expected, a poor similarity was found for the predicted Adhp sequences of Cf65 and Pk129 towards the Adh1-7p sequences from *S. cerevisiae* S288c (Supplementary Table 3.1). For Cf65 six predicted NADH-dependent ADH encoding genes and no NADPH-dependent ADH encoding gene were found. It has to be noted that Cf65_ONH65852.1 is incomplete and Cf65_ONH65775.1 consists of a gene duplication, therefore only the first 350 amino acids of Cf65_ONH65775.1 was used for the alignment using clustal W. Only four NADH-dependent ADH encoding genes were predicted for Pk129. Interestingly, ten NADPH-dependent ADH encoding genes were predicted for Pk129, of which Pk129_ONH70384.1 is incomplete (Supplementary Table 3.1). Due to the low sequence similarity toward *S. cerevisiae* S288c it is difficult to annotate the functionality of each ADH encoding gene for Cf65 and Pk129.

The translated nucleotide sequences of predicted ADH genes in Sc131, Cf65 and Pk129 listed in Table 3.1 are clustered using clustal W (Figure 3.4), except Cf65_ONH65852.1 and Pk129_ONH75156.1 due to incompleteness and low similarity. A clear cluster of the NADH- and NADPH-dependent ADH was visible, indicated with grey and purple background respectively. Information available on protein sequences of ADHs predicts one amino acid residue, Asp238 or Ser246 to determine the NADH or NADPH-dependency respectively (Supplementary Figure 3.1, indicated with a star) (Larroy, et al., 2002). This strengthens the clustering in Figure 3.4.

For each of the strains (Sc131, Cf65 and Pk129) one isoamyl acetate hydrolysing (*IAH1*) encoding gene was predicted. The similarity of those genes towards *S. cerevisiae* S288c is, as expected, much lower for Cf65 and Pk129 than Sc131 (44 %, 39 %, 99 % respectively). Figure 3.5 reveals conserved regions and differences among the strains in the active centre of the enzyme.

The high number of NADPH-dependent ADH encoding genes found in Pk129, does correlate with an overall higher NADPH-dependent ADH enzyme activity compared to Sc131 (Table 3.1). However, for Cf65 also a higher NADPH-dependent ADH enzyme activity was found, whereas none NADPH-dependent ADH gene was annotated. It is therefore very likely that there are unknown NADPH-dependent ADH encoding genes which cannot be annotated due to the low similarities between the genomes of Cf65 and Pk129 compared to *S. cerevisiae*.

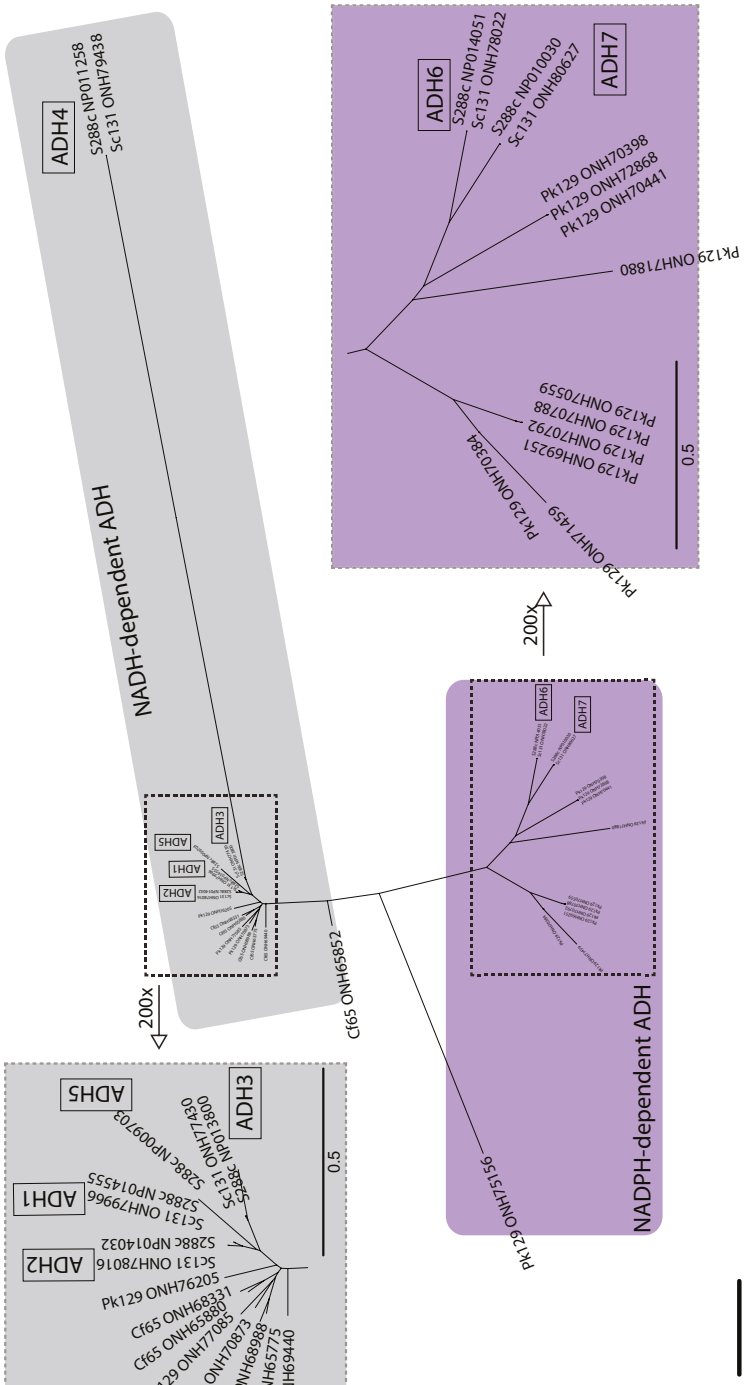


Figure 3.4: Phylogenetic clustering of all annotated alcohol dehydrogenase encoding genes of Sc131, Cf65 and Pk129. Untrimmed translated nucleotide sequences are clustered using Clustal W (DNASTAR MegAlign) and visualized using Figtree. The cluster of NADH-dependent ADHs is visualized with a grey background and NADPH-dependent ADHs with a purple background. The horizontal ruler indicates the amino acid substitution per 100 residues.

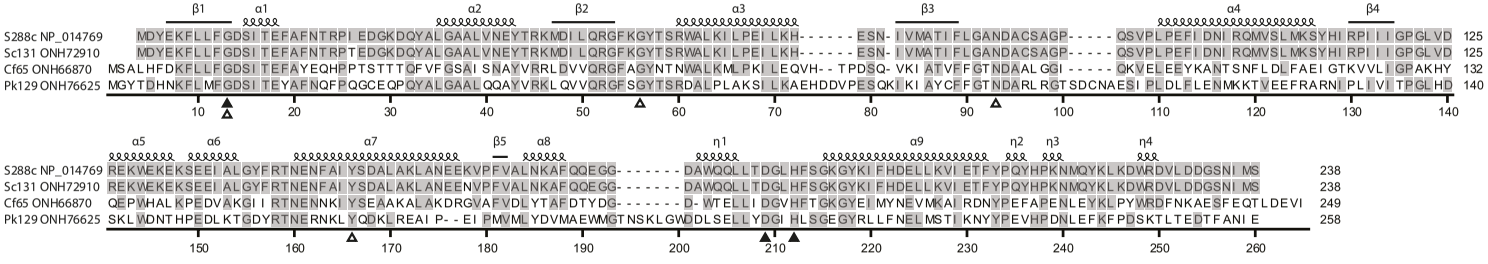


Figure 3.5: Clustal W alignment isoamyl acetate hydrolase (IAH1) translated nucleotide sequences of *S. cerevisiae* S288c (literature) and the wild isolates (Sc131, Cf65 and Pk129). Grey background indicates amino acids identical to literature strain (S288c). Above the amino acids the alpha- and beta-units are indicated and below the amino acid sequences a closed triangle indicates catalytic triad and an open triangle indicates oxyanion hole residues as described by Ma, et al. (2011)

Discussion

A fruity flavour and low odour threshold are characteristics of volatile esters that make them the most important group of flavour active compounds in fermented beverages. Volatile esters can be divided into two groups: acetate esters and medium chain fatty acid (MCFA) ethyl esters. Among the strains in this study representing three yeast species, major differences were found in acetate ester levels in the fermented products, therefore we will mainly focus on this category.

There is an ongoing demand for new yeast strains that produce a higher yield of acetate esters. Therefore, ester synthesis has been extensively studied and reviewed, mainly in the conventional yeast *S. cerevisiae* (Kruis, et al., 2017; Nordström, 1963b; Park, et al., 2009; Saerens, et al., 2010). The acetate ester yield depends on three factors or a combination thereof. First the alcohol acetyl transferase enzyme activity, which is encoded by the genes *ATF1* and *ATF2*. *S. cerevisiae* has been engineered to produce a single and double gene knockout of *ATF1* and *ATF2* (ΔATF1, ΔATF2, ΔATF1/ΔATF2) which revealed that *ATF2* only plays a minor and *ATF1* a major role in isoamyl acetate production. However, even in the double knockout still some isoamyl acetate was produced, which indicates that Atf1p and Atf2p are not the only enzymes responsible for the isoamyl acetate production (Verstrepen, et al., 2003b). Another engineered *S. cerevisiae* strain with increased ATF1/ATF2 expression resulted in 180-fold increase of isoamyl acetate production (Verstrepen, et al., 2003b). Similar results have also been found in a study by Lilly, et al. (2006) on increased ATF activity and the impact on flavour profiles of wine and distillates. Recently, Kruis, et al. (2017) revealed an new elusive alcohol acetyltransferase (*EAT1*) in *Wickerhamomyces anomalus* which belongs to the α/β hydrolase superfamily and is responsible for bulk ethyl acetate synthesis in various yeast species. A homolog for EAT1 was also found in *C. fabianii* and expressed in *S. cerevisiae*, which resulted in ethyl acetate and production (Kruis, et al., 2017). Kruis, et al. (2017) only tested the functionality of this gene for ethyl acetate production; however it would be very interesting to reveal the substrate specificity for this enzyme.

The second factor that can play a crucial role in acetate ester yield is the substrate availability (alcohol and acetyl-CoA). Volatile alcohols are products of the Ehrlich pathway (fusel alcohols) or glycolysis (ethanol) by reduction of the corresponding aldehyde (by an alcohol dehydrogenase (ADH)) (Reviewed by Hazelwood, et al. (2008)). It is therefore possible that the ADH enzyme activity is linked with the ester yield. The other substrate, acetyl-CoA, is a product of the TCA cycle. The third factor that can play a crucial role in the ester yield is ester hydrolysis (by esterases). Isoamyl acetate hydrolase (Iah1p) is the major acetate hydrolysing enzyme of *S. cerevisiae* (Fukuda, et al., 2000; Lilly, et al., 2006; Ma, et al., 2011). These studies all focus on the separate factors, by which the ester yield can be influenced. Remarkably, yet, no effort has been made to study the differences between *S. cerevisiae* and other yeasts on ester yield, enzyme activities and genes involved in ester production and

hydrolysis. By doing so, it is possible to unravel the crucial steps explaining the diversity in ester yield among different strains.

Here we investigated the relationship between key enzyme activities (ADH and esterase), the volatile compounds (alcohols and esters) and genes (*ADH1-7* and *IAH1*) annotated in Sc131, Cf65 and Pk129. The distinct aroma profiles of Sc131, Cf65 and Pk129 makes them ideal candidates to investigate the crucial step(s) that explain the different ester yields among the strains. Except for the substrate ethyl alcohol, we did not observe a correlation between the specific NAD(P)H-dependent ADH enzyme activity and the corresponding volatile alcohols or esters found using HS-SPME GC-MS. This suggests that the amount of volatile alcohols is not the limiting step for ester production.

However, a clear inverse correlation was found between the esterase activity (determined as hydrolysis rate of cFDA) and the amount of all acetate esters detected by HS-SPME GC-MS, suggesting that this is the crucial step determining the ester yield. As expected, no correlation was observed for the ethyl esters and the cFDA hydrolysing activity since ethyl esters are synthetized and hydrolysed by other esterases (encoded by *EEB1*/*EHT1*) (Saerens, et al., 2006). Notably, in our study the acetate esterase activity was determined using cFDA which offers an option for high throughput fluorescence screening of acetate esterase activity of (industrial) yeast strain collections and to determine the impact of growth history on this activity.

Comparison of translated nucleotide sequence of predicted ADHs (*ADH1-7*) and isoamyl acetate hydrolases (*IAH1*) in Sc131, Cf65 and Pk129 revealed that their similarity is poor (Figure 3.4 and 3.5, Supplementary Table 3.1 and Supplementary Figure 3.1). However, some conserved regions were found among the predicted NAD(P)H-dependent ADH encoding genes of all strains (Supplementary Figure 3.1). Larroy, et al. (2002) marked the amino acids involved in binding of the catalytic zinc and structural zinc as well as the amino acid residue characteristic for the NAD(P)H-dependent ADH activity. These are indicated in Supplementary Figure 3.1 and are all conserved among the various predicted ADH encoding genes of Sc131, Cf65 and Pk129.

Ma, et al. (2011) described the crystal structure of Iah1p (from the model strain *S. cerevisiae* S288c). The Iah1p protein sequence of *S. cerevisiae* S288c and Sc131 are 99 % similar (Figure 3.5). Comparison to the predicted Iah1p sequences of Cf65 and Pk129 revealed conserved regions, but also some interesting differences were found around the active site of the protein and therefore might influence the enzyme activity and substrate specificity (Figure 3.5). However, further investigations are needed to confirm this hypothesis.

In summary, comparison of Sc131, Cf65 and Pk129 revealed that the acetate ester hydrolysing activity is the key step that explains the variation in acetate ester yield among these strains. A low cFDA hydrolysing activity correlates with a high acetate ester yield.

Additionally, the cFDA hydrolysing assay described in this study is a powerful tool for high throughput screening to search for strains with low esterase activity or to study the effect of growth history on hydrolysis of acetate esters.

Acknowledgements

This research was financially supported by the Graduate School VLAG, Wageningen University & Research, Wageningen, The Netherlands and HEINEKEN Supply Chain B.V., Zoeterwoude, The Netherlands. The authors would like to acknowledge Jan-Maarten Geertman and Niels Kuijpers from HEINEKEN for sharing their knowledge and Wageningen University & Research thesis students Mengyuan Wang, Liang Xia and Wouter Ghering for the support of the experimental work.

Supplementary materials

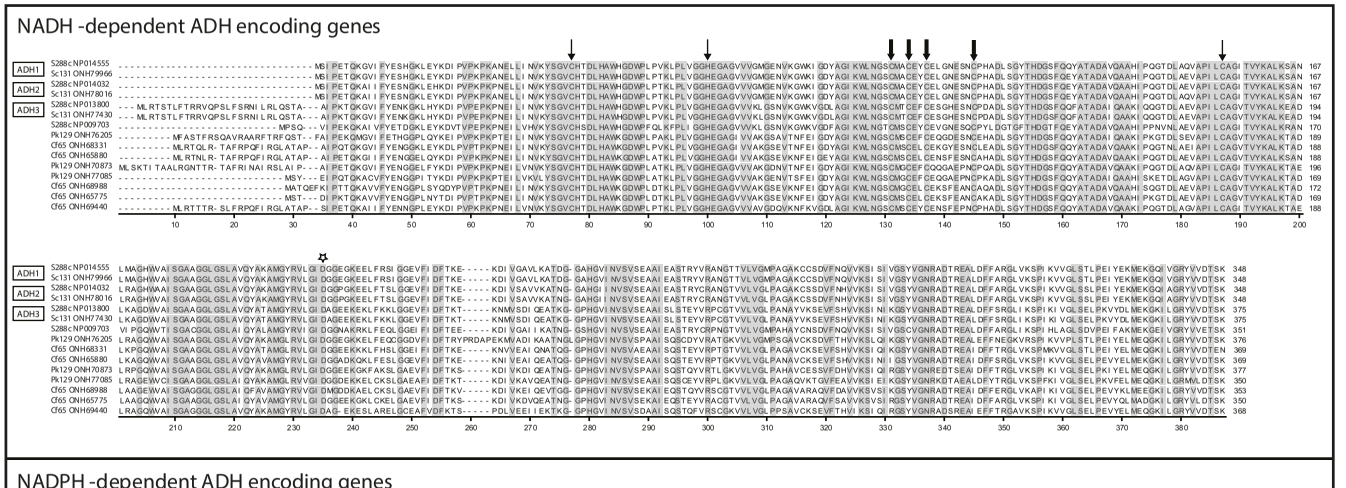
Supplementary table 3.1: Sequence distance towards *S. cerevisiae* S288c (literature strain). Protein sequences are aligned with Clustal W (DNASTAR metalign). 100 % = identical to literature protein sequence.

Strain	Strain_accession number	protein	accession number	amino acid length	sequence distance towards S. cerevisiae S288c (%)					
NADH-dependent Alcohol dehydrogenases					Adh1p	Adh2p	Adh3p	Adh4p	Adh5p	
S. cerevisiae S288c	S288c_NP_014555.1	Adh1p	NP_014555.1	348	**100.0**	93.1	79.3	13.6	76.4	
S. cerevisiae S288c	S288c_NP_014032.1	Adh2p	NP_014032.1	348	93.1	**100.0**	78.7	14.5	77.0	
S. cerevisiae S288c	S288c_NP_013800.1	Adh3p	NP_013800.1	375	79.3	78.7	**100.0**	12.7	70.7	
S. cerevisiae S288c	S288c_NP_011258.2	Adh4p	NP_011258.2	382	13.6	14.5	12.7	**100.0**	12.4	
S. cerevisiae S288c	S288c_NP_009703.3	Adh5p	NP_009703.3	351	76.4	77.0	70.7	12.4	**100.0**	
S. cerevisiae 131	Sc131_ONH79966.1	Adh1p	ONH79966.1	348	**99.1**	94.0	79.0	13.9	77.0	
S. cerevisiae 131	Sc131_ONH78016.1	Adh2p	ONH78016.1	348	94.3	**98.9**	78.7	14.5	77.0	
S. cerevisiae 131	Sc131_ONH77430.1	Adh3p	ONH77430.1	375	79.3	78.7	**100.0**	12.7	70.7	
S. cerevisiae 131	Sc131_ONH79438.1	Adh4p	ONH79438.1	382	13.6	14.5	12.7	**99.7**	12.4	
C. fabianii 65	Cf65_ONH69440.1		ONH69440.1	368	**76.0**	**77.2**	**74.1**	13.8	66.5	
C. fabianii 65	Cf65_ONH68988.1		ONH68988.1	353	**74.7**	**75.0**	**76.9**	14.1	66.1	
C. fabianii 65	Cf65_ONH68331.1		ONH68331.1	369	**78.7**	**77.5**	**78.3**	13.8	68.9	
C. fabianii 65	Cf65_ONH65880.1		ONH65880.1	369	**80.4**	**81.0**	**79.9**	14.9	70.0	
C. fabianii 65	Cf65_ONH65852.1		ONH65852.1	216	**56.9**	**56.5**	53.2	14.4	**56.9**	(1)
C. fabianii 65	Cf65_ONH65775.1		ONH65775.1	717	**76.9**	**77.2**	**79.7**	13.8	68.3	(2)
P. kudriavzevii 129	Pk129_ONH77085.1		ONH77085.1	350	**73.2**	**74.1**	**76.3**	14.4	65.1	
P. kudriavzevii 129	Pk129_ONH76205.1		ONH76205.1	376	**75.9**	**74.7**	**72.4**	14.0	64.1	
P. kudriavzevii 129	Pk129_ONH75156.1		ONH75156.1	368	20.8	21.1	22.6	9.7	20.8	
P. kudriavzevii 129	Pk129_ONH70873.1		ONH70873.1	377	**78.1**	**77.8**	**75.4**	15.8	67.4	

NADPH-dependent alcohol dehydrogenases					Adh6p	Adh7p	
S. cerevisiae S288c	S288c_NP_014051.3	Adh6p	NP_014051.3	360	**100.0**	63.6	
S. cerevisiae S288c	S288c_NP_010030.1	Adh7p	NP_010030.1	361	63.6	**100.0**	
S. cerevisiae 131	Sc131_ONH78022.1	Adh6p	ONH78022.1	360	99.7	63.3	
S. cerevisiae 131	Sc131_ONH80627.1	Adh7p	ONH80627.1	361	63.6	100.0	
P. kudriavzevii 129	Pk129_ONH72868.1		ONH72868.1	374	50.0	48.9	
P. kudriavzevii 129	Pk129_ONH71880.1		ONH71880.1	382	41.9	40.7	
P. kudriavzevii 129	Pk129_ONH71459.1		ONH71459.1	368	46.5	46.5	
P. kudriavzevii 129	Pk129_ONH70792.1		ONH70792.1	359	43.8	44.4	
P. kudriavzevii 129	Pk129_ONH70788.1		ONH70788.1	359	43.8	44.4	
P. kudriavzevii 129	Pk129_ONH70559.1		ONH70559.1	359	43.8	44.4	
P. kudriavzevii 129	Pk129_ONH70441.1		ONH70441.1	372	50.0	48.9	
P. kudriavzevii 129	Pk129_ONH70398.1		ONH70398.1	374	50.0	48.9	
P. kudriavzevii 129	Pk129_ONH70384.1		ONH70384.1	181	50.0	45.6	(1)
P. kudriavzevii 129	Pk129_ONH69251.1		ONH69251.1	343	42.5	43.7	

(1) amino acid sequence is incomplete

(2) gene duplication, for allignment only first 350 amino acids are used



Supplementary Figure 3.1: Clustal W alignment on translated nucleotide sequences for NADH-dependent ADH (top box) and NADPH-dependent ADH (bottom box) encoding genes. Grey background indicates conserved amino acid among all sequences. Star = NAD(P)H characteristic amino acid, small arrow = involved in binding catalytic zinc, Thick arrow = involved in binding structural zinc (Larroy, et al., 2002).

References

Belda, I., Navascues, E., Marquina, D., Santos, A., Calderon, F. and Benito, S. (2016). Outlining the influence of non-conventional yeasts in wine ageing over lees. *Yeast* **33,** 329-38.

Boulton, C. A. and Ratledge, C. (1981). Correlation of lipid accumulation in yeasts with possession of ATP: Citrate lyase. *Journal of General Microbiology* **127,** 169-76.

Breeuwer, P., Drocourt, J. L., Bunschoten, N., Zwietering, M. H., Rombouts, F. M. and Abee, T. (1995). Characterization of uptake and hydrolysis of fluorescein diacetate and carboxyfluorescein diacetate by intracellular esterases in *Saccharomyces cerevisiae*, which result in accumulation of fluorescent product. *Appl Environ Microbiol* **61,** 1614-9.

Cho, J. and Jeffries, T. W. (1998). *Pichia stipitis* genes for alcohol dehydrogenase with fermentative and respiratory functions. *Appl Environ Microbiol* **64,** 1350-8.

Contreras, A., Hidalgo, C., Henschke, P. A., Chambers, P. J., Curtin, C. and Varela, C. (2014). Evaluation of non-*Saccharomyces* yeasts for the reduction of alcohol content in wine. *Appl Environ Microbiol* **80,** 1670-8.

Contreras, A., Hidalgo, C., Schmidt, S., Henschke, P. A., Curtin, C. and Varela, C. (2015). The application of non-*Saccharomyces* yeast in fermentations with limited aeration as a strategy for the production of wine with reduced alcohol content. *Int J Food Microbiol* **205,** 7-15.

de Smidt, O., du Preez, J. C. and Albertyn, J. (2008). The alcohol dehydrogenases of *Saccharomyces cerevisiae*: a comprehensive review. *FEMS Yeast Res* **8,** 967-78.

Del Monaco, S. M., Barda, N. B., Rubio, N. C. and Caballero, A. C. (2014). Selection and characterization of a Patagonian *Pichia kudriavzevii* for wine deacidification. *J Appl Microbiol* **117,** 451–64.

Fujii, T., Nagasawa, N., Iwamatsu, A., Bogaki, T., Tamai, Y. and Hamachi, M. (1994). Molecular cloning, sequence analysis and expression of the yeast alcohol acetyltransferase gene. *Appl Environ Microbiol* **60,** 2786-92.

Fukuda, K., Kiyokawa, Y., Yanagiuchi, T., Wakai, Y., Kitamoto, K., Inoue, Y. and Kimura, A. (2000). Purification and characterization of isoamyl acetate-hydrolyzing esterase encoded by the *IAH1* gene of *Saccharomyces cerevisiae* from a recominant *Escherichia coli*. *Appl Microbiol Biotechnol* **53,** 596-600.

Fukuda, K., Yamamoto, N., Kiyokawa, Y., Yanagiuchi, T., Wakai, Y., Kitamoto, K., Inoue, Y. and Kimura, A. (1998). Balance of activities of alcohol acetyltransferase and esterase in *Saccharomyces cerevisiae* is important for production of isoamyl acetate. *Appl Environ Microbiol* **64,** 4076-8.

Gamero, A., Quintilla, R., Groenewald, M., Alkema, W., Boekhout, T. and Hazelwood, L. (2016). High-throughput screening of a large collection of non-conventional yeasts reveals their potential for aroma formation in food fermentation. *Food Microbiology* **60,** 147-59.

Gatter, M., Ottlik, S., Kovesi, Z., Bauer, B., Matthaus, F. and Barth, G. (2016). Three alcohol dehydrogenase genes and one acetyl-CoA synthetase gene are responsible for ethanol utilization in *Yarrowia lipolytica*. *Fungal Genet Biol* **95,** 30-8.

Hazelwood, L. A., Daran, J. M., van Maris, A. J., Pronk, J. T. and Dickinson, J. R. (2008). The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. *Appl Environ Microbiol* **74,** 2259-66.

Inoue, Y., Trevanichi, S., Fukuda, K., Izawa, S., Wakai, Y. and Kimura, A. (1997). Roles of esterase and alcohol acetyltransferase on production of isoamyl acetate in *Hansenula mrakii*. *J Agric Food Chem* **45,** 644-9.

Kruis, A. J., Levisson, M., Mars, A. E., van der Ploeg, M., Garcés Daza, F., Ellena, V., Kengen, S. W. M., Oost, J. v. d. and Weusthuis, R. A. (2017). Ethyl acetate production by the elusive alcohol acetyltransferase from yeast. *Genetic Engineering* **41**, *92-101*.

Larroy, C., Fernandez, M. R., Gonzalez, E., Pares, X. and Biosco, J. A. (2002). Characterization of the *Saccharomyces cerevisiae* YMR318C (ADH6) gene product as a broad specificity NADPH-dependent alcohol dehydrogenase: relevance in aldehyde reduction. *Biochem. J.* **361,** 163-72.

Lilly, M., Bauer, F. F., Lambrechts, M. G., Swiegers, J. H., Cozzolino, D. and Pretorius, I. S. (2006). The effect of increased yeast alcohol acetyltransferase and esterase activity on the flavour profiles of wine and distillates. *Yeast* **23,** 641-59.

Lilly, M., Lambrechts, M. G. and Pretorius, I. S. (2000). Effect of increased yeast alcohol acetyltransferase activity on flavour profiles of wine and distillates. *Appl Environ Microbiol* **66,** 744-53.

Ma, J., Lu, Q., Yuan, Y., Ge, H., Li, K., Zhao, W., Gao, Y., Niu, L. and Teng, M. (2011). Crystal structure of isoamyl acetate-hydrolyzing esterase from *Saccharomyces cerevisiae* reveals a novel active site architecture and the basis of substrate specificity. *Proteins* **79,** 662-8.

Mason, A. B. and Dufour, J. P. (2000). Alcohol acetyltransferases and the significance of esther synthesis in yeast. *Yeast* **16 ,** 1287 -98.

Nagasawa, N., Bogaki, T., Iwamatsu, A., Hamachi, M. and Kumagai, C. (1998). Cloning and nucleotide sequence of the alcohol acetyltransferase II gene (ATF2) from *Saccharomyces cerevisiae* Kyokai No. 7. *Biosci Biotechnol Biochem* **62,** 1852-7.

Nordström, K. (1963a). Formation of esters from acids by brewer's yeast I. Kinetic theory and basic experiments. *J. Inst. Brew.* **69,** 310-22.

Nordström, K. (1963b). Formation of esters, acids and alcohols from a-keto acids by brewer's yeast. *J. Inst. Brew.* **96,** 483-95.

Nordström, K. (1964). Formation of esters from alcohols by brewer's yeast. *J. Inst. Brew.* **70,** 328-36.

Nyanga, L. K., Nout, M. J., Gadaga, T. H., Theelen, B., Boekhout, T. and Zwietering, M. H. (2007). Yeasts and lactic acid bacteria microbiota from masau (*Ziziphus mauritiana*) fruits and their fermented fruit pulp in Zimbabwe. *Int J Food Microbiol* **120,** 159-66.

Nyanga, L. K., Nout, M. J., Smid, E. J., Boekhout, T. and Zwietering, M. H. (2013). Fermentation characteristics of yeasts isolated from traditionally fermented masau (*Ziziphus mauritiana*) fruits. *Int J Food Microbiol* **166,** 426-32.

Park, Y. C., Shaffer, C. E. and Bennett, G. N. (2009). Microbial formation of esters. *Appl Microbiol Biotechnol* **85,** 13-25.

Raj, S. B., Ramaswamy, S. and Plapp, B. V. (2014). Yeast alcohol dehydrogenase structure and catalysis. *Biochemistry* **53,** 5791-803.

Saerens, S. M., Delvaux, F. R., Verstrepen, K. J. and Thevelein, J. M. (2010). Production and biological function of volatile esters in *Saccharomyces cerevisiae*. *Microb Biotechnol* **3,** 165-77.

Saerens, S. M., Verstrepen, K. J., Van Laere, S. D., Voet, A. R., Van Dijck, P., Delvaux, F. R. and Thevelein, J. M. (2006). The *Saccharomyces cerevisiae* EHT1 and EEB1 genes encode novel enzymes with medium-chain fatty acid ethyl ester synthesis and hydrolysis capacity. *J Biol Chem* **281,** 4446-56.

Saliola, M., Shuster, J. R. and Falcone, C. (1990). The alcohol dehydrogenase system in the yeast *Kluyveromyces lactis*. *Yeast* **6,** 193-204.

Steensels, J., Daenen, L., Malcorps, P., Derdelinckx, G., Verachtert, H. and Verstrepen, K. J. (2015). *Brettanomyces* yeasts--From spoilage organisms to valuable contributors to industrial fermentations. *Int J Food Microbiol* **206,** 24-38.

Suomalainen, H. (1981). Yeast esterases and aroma esters in alcohol beverages. *J. Inst. Brew.* **87,** 296-300.

van Iersel, M. F., Eppink, M. H., van Berkel, W. J., Rombouts, F. M. and Abee, T. (1997). Purification and characterisation of a novel NADP-dependent branched-chain alcohol dehydrogenase from *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **63,** 4079-82.

van Rijswijck, I. M. H., Derks, M. F. L., Abee, T., de Ridder, D. and Smid, E. J. (2017a). Genome sequences of *Cyberlindnera fabianii* 65, *Pichia kudriavzevii* 129, and *Saccharomyces cerevisiae* 131, isolated from fermented masau fruits in Zimbabwe. *Genome Announcements* **5,** 1-3.

van Rijswijck, I. M. H., Dijksterhuis, J., Wolkers-Rooijackers, J. C. M., Abee, T. and Smid, E. J. (2015). Nutrient limitation leads to penetrative growth into agar and affects aroma formation in *Pichia fabianii*, *P. kudriavzevii* and *Saccharomyces cerevisiae*. *Yeast* **32,** 89-101.

van Rijswijck, I. M. H., Wolkers-Rooijackers, J. C. M., Abee, T. and Smid, E. J. (2017b). Performance of non-conventional yeasts in co-culture with brewers' yeast for steering ethanol and aroma production. *(Accepted) Microbial Biotechnology*.

Verstrepen, K. J., Derdelinckx, G., Dufour, J. P., Winderickx, J., Thevelein, J. M., Pretorius, I. S. and Delvaux, F. R. (2003a). Flavor-Active Esters: Adding Fruitiness to Beer. *Journal of Bioscience and Bioengineering* **96,** 110-8.

Verstrepen, K. J., Van Laere, S. D. M., Vanderhaegen, B. M. P., Derdelinckx, G., Dufour, J. P., Pretorius, I. S., Winderickx, J., Thevelein, J. M. and Delvaux, F. R. (2003b). Expression levels of the yeast alcohol acetyltransferase genes ATF1, Lg-ATF1, and ATF2 control the formation of a broad range of volatile esters. *Applied and Environmental Microbiology* **69,** 5228-37.

Chapter 4

Performance of non-conventional yeasts in co-culture with brewers' yeast for steering ethanol and aroma production

Irma M. H. van Rijswijck[1], Judith C.M. Wolkers – Rooijackers[1], Tjakko Abee[1] and Eddy J. Smid[1]

Accepted in Microbial Biotechnology (2017)

Affiliations:

1. Laboratory of Food Microbiology, Wageningen University & Research, P.O. Box 17, 6700 AA Wageningen, The Netherlands

Abstract

Increasing interest in new beer-types has stimulated the search for approaches to extend the metabolic variation of brewers' yeast. Therefore, we tested two approaches using non-conventional yeast to create a beer with lower ethanol content and a complex aroma bouquet. First, the mono-culture performance was monitored of 49 wild yeast isolates of *Saccharomyces cerevisiae* (16 strains), *Cyberlindnera fabianii* (9 strains) and *Pichia kudriavzevii* (24 strains). Interestingly, both *C. fabianii* and *P. kudriavzevii* isolates produced relatively more esters compared to *S. cerevisiae* isolates, despite their limited fermentation capacity. Next, one representative strain of each species (Sc131, Cf65 and Pk129) was applied as co-culture with brewers' yeast (ratio 1:1). Co-cultures with Cf65 and Pk129 resulted in a beer with lower alcohol content (3.5, 3.8 compared to 4.2 % v/v) and relatively more esters. At higher inoculum ratios of Cf65 over brewers' yeast, growth inhibition of brewers' yeast was observed, most likely caused by competition for oxygen between brewers' yeast and Cf65 resulting in a reduced level of ethanol and altered aroma profiles. With this study, we demonstrate the feasibility of using non-conventional yeast species in co-cultivation with traditional brewers' yeast to tailor aroma profiles as well as the final ethanol content of beer.

Introduction

The brewing industry traditionally uses *Saccharomyces* species as their workhorse to convert wort into beer. These species are extensively studied, have a long history of use and have the generally recognized as safe (GRAS) status (Bourdichon, et al., 2012). Nowadays, there is an increasing interest in a broad range of new beer-types. To create variation, most research focusses on the preparation of the unfermented wort with different carbon/nitrogen ratios, thereby controlling the supply of precursors for aroma compounds (Lei, et al., 2012). In addition, variation in fermentation conditions, (i.e. temperature, fermentation time) has been used to generate diversity in beers (Kaneda, et al., 1991; Landaud, et al., 2001; Saerens, et al., 2008).

Additional research has been done on strain performance, but remarkably this mostly entails single strain performance (Gallone, et al., 2016; Zhang, et al., 2013). Often, various approaches such as genome shuffling or adaptive evolution experiments are used to generate new variants of strains with desired traits (Snoek, et al., 2015; Steensels, et al., 2014a). Strain improvements can be obtained to a certain extent via these approaches. However, the degree of variation that can be achieved within one genus (*Saccharomyces*) is smaller compared to what can be obtained in terms of variation when crossing strains from different genera. Recently, Gamero, et al. (2016) studied the fermentation capacities of a large collection of non-conventional yeast. Additionally, Basso, et al. (2016), Steensels and Verstrepen (2014) and Steensels, et al. (2015) reviewed the use of various non-conventional yeasts for wort fermentation using various strategies. They all conclude that non-conventional yeast harbour features of interest for industrial fermentation processes, but the use of those species has to be explored further.

Strategies to apply these non-conventional yeast species need to be tailored based on their fermentation capacities, such as sugar utilization and aroma/alcohol formation. If the fermentation characteristics are good, selected non-conventional yeasts can be applied as single strain starter culture. Notably, isolates of non-conventional yeast with poor fermentation characteristics may also be applied for the introduction of specific traits, for example in co-cultures with a classical brewers' yeast with proven fermentation capacities. However, to the best of the authors' knowledge, the latter approach has only been reported once for beer production in a patent describing a sequential (and co-culture) inoculation of *Pichia* sp. and brewers' yeast (Saerens and Swiegers, 2014). For wine production, a range of co-culture approaches has been developed that are currently applied for example to de-acidify wine or to increase the complexity of the aroma bouquet (Andorrà, et al., 2012; Contreras, et al., 2015; Del Monaco, et al., 2014; Erten and Tanguler, 2010; Kim, et al., 2008; Quiros, et al., 2014).

In this paper, we describe the biodiversity and performance of 49 wild yeast isolates in wort

fermentation. These isolates originate from fermented masau fruits (Zimbabwe) and belong to the species *Saccharomyces cerevisiae*, *Cyberlindnera fabianii* and *Pichia kudriavzevii*. From this collection of isolates, one representative for each species was chosen (Sc131, Cf65 and Pk129) for application in co-culture with traditional brewers' yeast (*S. cerevisiae*). With this study, we demonstrate the feasibility of using non-conventional yeast species in co-cultivation with traditional brewers' yeast to tailor aroma profiles as well as the final ethanol content of beer.

Materials and methods

Yeast strains

49 yeast strains isolated from fermented masau fruit as described by Nyanga, et al. (2007) and were used in this study: 16 *Saccharomyces cerevisiae* strains (Sc38, Sc102, Sc116, Sc126, Sc131, Sc139, Sc141, Sc142, Sc143, Sc146, Sc148, Sc149, Sc153, Sc160, Sc165), 9 *Cyberlindnera fabianii* (formerly named *Pichia fabianii, Hansenula fabianii* and *Lindnera fabianii*) strains (Cf65, Cf145, Cf1a, Cf2a, Cf4a, Cf6a1, Cf6a2, Cf8a1, Cf8a2) and 24 *Pichia kudriavzevii (*formerly named *Issatchenkia orientalis)* strains (Pk5, Pk27, Pk32, Pk42, Pk52, Pk59, Pk91, Pk94, Pk100, Pk105, Pk110, Pk123, Pk125, Pk128, Pk129, Pk132, Pk137, Pk138, Pk140, Pk144, Pk150, Pk151, Pk152, Pk166). As reference strain, a brewers' yeast (*S. cerevisiae*) from Lallemand danstar called "Nottingham" was used. All strains were stored at -80 °C in 15 % (v/v) glycerol.

Wort preparation

Standardized wort concentrate (Brewferm Pils Hopped Malt extract) was used to avoid differences between batches of wort. One can (1.5 kg malt extract) was dissolved in 12 L water (12° Brix). The wort was heated for 15 min at 105 °C to avoid contamination and kept refrigerated until use.

Biodiversity 49 wild isolates on wort (100 mL cultures)

All strains were streaked from glycerol stock onto Malt Extract Agar (MEA, Oxoid) plates and incubated for 3 days at 20 °C. A single colony was inoculated in 15 mL wort and incubated for 22 hours at 30 °C and 160 rpm. The optical density (OD_{600nm}) of these cultures was measured in order to calculate the total amount of the inoculum to be used for the fermentation (starting OD_{600nm} = 0.5, total volume = 100 mL). The flasks were well shaken to introduce oxygen before closing the flasks with a plug and water lock and incubated at 20 °C for 7 days (primary fermentation). After primary fermentation the volatile organic compounds, ethanol formation and sugar utilization was measured.

Wort fermentation in Schott flasks for co-culture/dose-response experiment

Yeasts were pre-cultured starting with a streak on MEA plates from glycerol stocks and incubated for 24 hours at 30 °C. A single colony of each plate was inoculated in 250 mL Erlenmeyers containing 75 mL of wort, and incubated for 72 hours at 20 °C and 160 rpm. Then, 50 mL was transferred to a 1000 mL Erlenmeyer with 400 mL of wort and incubated for 72 hours at 20 °C under static conditions. The OD_{600nm} was measured and the appropriate

amount of culture was centrifuged and resuspended in wort to reach an OD_{600nm} of 0.5 in 450 mL wort in 500 mL Schott flasks. The flasks were shaken vigorously, closed with a plug and water lock and incubated for 7 days at 20 °C (mimicking primary fermentation). After 7 days the upper part of the ferment (250 mL) was transferred to beer bottles containing 3.5 mL 50 % sucrose solution (w/v) and locked with a cap (start secondary fermentation). These bottles were subsequently stored for 2 days at 20 °C followed by incubation for 47 days at 4 °C (end secondary fermentation).

The residual sugars and ethanol production (using HPLC), volatile organic compounds (HS -SPME GC-MS), pH, CO_2 production (Δ weight of bottles) and viable counts were monitored. Two types of agar plates were used to obtain viable counts; a non-selective plate for total viable counts and a selective plate to distinguish *C. fabianii* in co-cultivation. MEA was used as non-selective plate and incubated for 2 days at 30 °C. The selective plate contained 1 % sorbitol (w/v, Sigma-Aldrich), 1x yeast nitrogen base w/o amino acids and ammonium (Sigma-Aldrich), 45.4 mM $(NH_4)_2SO_4$, 2 % bacteriological agar (w/v, Oxoid) and was incubated at 30 °C for 2 days.

Test effect ergosterol supplementation (Infors bioreactors)

The effect of ergosterol supplementation was investigated using Infors HT 500 mL bioreactors. The bioreactors contained 450 ml wort which was stirred at 400 rpm and aerated till complete saturation of the wort and headspace was obtained. The wort was supplemented with antifoam (0.1 mL antifoam A, Sigma-Aldrich). Fermentation was executed in the absence or presence of 1 % (v/v) 100x ET80. 100x ET80 consists of 2.5 mg/mL ergosterol dissolved in ethanol and tween80 (ratio 1:1). Prior to inoculation the air inlet was closed and a water lock was attached to the air outlet. The bioreactors were stirred at 100 rpm to homogenize the wort and a sample port was connected to take samples during the fermentation run. The wort (either with or without ET80) was inoculated with 10^4 CFU/ml brewers' yeast + 10^6 CFU/ml *C. fabianii* as described in the section Wort fermentation in Schott flasks for co-culture/dose-response experiment. The fermentation performance was studied for 7 days (equal to primary fermentation). During the fermentation the pH, DO (dissolved oxygen), residual sugars and ethanol production, total viable plate counts, *C. fabianii* plate counts (see materials & methods wort fermentation in bottles (450 mL)) and the brewers' yeast viable plate counts were determined using MEA plates supplemented with 1 % ET80 (v/v, incubated anaerobically at 30 °C for 2 days).

Residual sugar and ethanol analysis (HPLC)

High Performance Liquid Chromatography (HPLC) was performed to quantify ethanol, glucose, maltose and maltotriose on an Ultimate 3000 HPLC (Dionex) equipped with an RI-101 refractive index detector (Shodex, Kawasaki, Japan), an autosampler and an

ion-exclusion Aminex HPX – 87H column (7.8 × 300 mm) with a guard column (Bio-Rad, Hercules, CA). As mobile phase, 5 mM H_2SO_4 was used at a flow rate of 0.6 mL/minute and the column was kept at 40 °C. Total run time was 30 minutes. The injection volume was 10 µl. Samples were deproteinated with 0.5 volume Carrez A (0,1 M potassium ferrocyanide trihydrate) and 0.5 volume Carrez B (0.2 M zinc sulfate heptahydrate) and 2x diluted with MilliQ. Diluted samples and standards (4-20 mM for glucose, 8-40 mM for maltose and 2-10 mM for maltotriose; 0.72-3.6 % (v/v) for ethanol) were injected onto the column.

Volatile organic compound (VOC) analysis (using HS-SPME GC-MS)

The final beer was filtered (0.45 µm) and 2 ml was transferred to a GC-MS vial. 10 µl of 0.4 mg/ml decane (dissolved in methanol) was added as internal standard. Samples were kept frozen (-20 °C) until analysis.

The following method was used to determine the volatile organic compounds present in the sample using headspace solid phase micro extraction gas chromatography mass spectrometry (HS-SPME GC-MS) analysis.

Samples were defrosted and incubated for 5 minutes at 60 °C, followed by extraction for 20 minutes at 60 °C using a Solid Phase Microextraction (SPME) fiber (Car/DVB/PDMS, Supelco). The compounds were desorbed from the fiber for 10 minutes on a Stabilwax®-DA-Crossband®-Carbowax®-polyethylene-glycol column (30 m length, 0.25 mmID, 0.5 µm df). The gas chromatograph settings were: PTV Split-less mode (5 minutes) at 250 °C. Helium was used as carrier gas at a constant flow of 1.5 ml/min. The GC oven temperature, initially at 40 °C for 2 minutes, raised to 240 °C (10 °C/min) and kept at 240 °C for 5 minutes. Total run time was 28 minutes. Mass spectral data was collected over a range of m/z 33-250 in full scan mode with 3.0030 scans/seconds.

Peaks were annotated using Chromeleon® 7.2. The ICIS algorithm was used for peak integration and the NIST main library to match the mass spectral profiles with the profiles of NIST. Peak areas were calculated using the MS quantitation peak (highest m/z peak per compound).

For hierarchical clustering, the peak areas were normalized per compound using Log_2(peak area/median (peak area all samples)). The hierarchical clustering was performed in MeV v4.8.1.

Shannon index was calculated to indicate the complexity of the compound composition. A higher index indicates a more complex aroma bouquet. The formula used was:

$$Shannon\ index = -\sum\left(P_i x Ln\left(P_i\right)\right)$$

$$P_i = \frac{Peak\ area\ (TIC)}{\sum Peak\ area's\ (TIC)}$$

Statistics

An initial inoculum of 100 % brewers' yeast was used as reference. A Students t-test was used to determine whether other inoculation strategies resulted in a significantly different end product. A *p*-value < 0.05 was used as cut off to indicate significance difference.

Only HS-SPME GC-MS data was first transferred to $\log_2$ values to obtain values that are equally distributed and have equal variance between the different treatments. After transformation of the values the students t-test was used to test for significant differences ($p < 0.05$).

Table 4.1: Residual wort sugars and ethanol production of all yeast isolates after 7 days of incubation at 20 °C (n=4). Sugars are the average in g/L ± standard deviation, ethanol in average % (v/v) ± standard deviation and relative abundance of all volatile alcohols, aldehydes, acids and esters ± standard deviation. Strains Sc131, Cf65 and Pk129 are highlighted in bold.

Results

Diversity in wort fermentation performance of non-conventional yeast isolates

The fermentation capacity of 49 wild yeast isolates belonging to the species *S. cerevisiae* (16 isolates), *C. fabianii* (9 isolates) and *P. kudriavzevii* (24 isolates), was tested on wort after 7 days of incubation at 20 °C (Figure 4.1). The peak areas of the volatile organic compounds (VOCs) were normalized and hierarchical clustering was applied. A clear clustering is observed per species, indicating that the diversity in VOCs between the three genera is higher than within one species. The relative abundance of the volatile esters compared to the total of volatile alcohols, aldehydes, acids and esters is higher for all *C. fabianii* and *P. kudriavzevii* isolates compared to all *S. cerevisiae* isolates (Table 4.1). It was demonstrated that all isolates of *C. fabianii* and *P. kudriavzevii* utilized all glucose, but only a limited amount of maltose was used (max 1 g/L). In addition, strains of both species used no maltotriose and produced only approximately 0.6 % (v/v) ethanol (Table 4.1). Glucose was depleted by all *S. cerevisiae* isolates, and all *S. cerevisiae* isolates utilized maltose with an amount varying between 18 and 46 g/L. About 1 g/L maltotriose was utilized by all *S. cerevisiae* isolates and only isolates Sc102 and Sc165 utilized more (3 and 6 g/L respectively). The ethanol formation for the *S. cerevisiae* isolates ranged between 1.9 - 3.7 % (v/v).

These results indicate that it is possible to tailor the final ethanol content and aroma profile by using non-conventional yeast species, such as *C. fabianii* and *P. kudriavzevii*. It also suggests that the composition of the final fermented product potentially can be influenced using a co-culture of brewers' yeast and one of the non-conventional yeast species. To investigate this concept further, one isolate per species was chosen: *S. cerevisiae* 131 (Sc131), *C. fabianii* 65 (Cf65) and *P. kudriavzevii* 129 (Pk129). As reference a commercial (Ale) brewers' yeast was used (*S. cerevisiae*, Lallemand danstar "Nottingham") to which we will refer to as brewers' yeast.

Table 4.1: Residual wort sugars and ethanol production of all yeast isolates after 7 days of incubation at 20 °C (n=4). Sugars are the average in g/L ± standard deviation, ethanol in average % (v/v) ± standard deviation and relative abundance of all volatile alcohols, aldehydes, acids and esters ± standard deviation. Strains Sc131, Cf65 and Pk129 are highlighted in bold.

	residual sugars (g/L)			ethanol	relative abundance VOCs per compound group			
Strain	**glucose**	**maltose**	**maltotriose**	**(%v/v)**	**alcohols**	**aldehydes**	**acids**	**esters**
uninoculated wort	10.4±0.6	48.3±2.5	17.3±0.8	0.0±0.0				
Cf145	2.1±0.2	45.1±1.7	15.9±0.7	0.6±0.0	0.53±0.03	0.02±0.00	0.01±0.00	0.44±0.03
Cf1a	1.8±0.1	44.1±0.8	15.3±0.6	0.6±0.0	0.54±0.01	0.02±0.00	0.02±0.00	0.43±0.01
Cf2a	1.9±0.1	45.1±0.6	15.9±0.6	0.6±0.0	0.57±0.01	0.02±0.00	0.02±0.00	0.40±0.01
Cf4a	1.9±0.1	44.5±1.0	15.7±0.7	0.6±0.0	0.55±0.01	0.02±0.00	0.01±0.00	0.42±0.01
Cf65	**2.0±0.3**	**43.7±0.8**	**15.4±0.5**	**0.6±0.0**	**0.56±0.05**	**0.02±0.01**	**0.01±0.00**	**0.41±0.05**
Cf6a1	2.1±0.1	44.9±0.2	16.0±0.4	0.6±0.0	0.56±0.01	0.02±0.00	0.02±0.00	0.41±0.01

Strain	residual sugars (g/L)			ethanol (%v/v)	relative abundance VOCs per compound group			
	glucose	maltose	maltotriose		alcohols	aldehydes	acids	esters
Cf6a2	2.1±0.0	44.7±1.1	15.8±0.6	0.6±0.0	0.55±0.01	0.02±0.00	0.01±0.00	0.42±0.01
Cf8a1	2.1±0.4	45.5±1.0	16.0±0.5	0.6±0.0	0.65±0.04	0.02±0.00	0.02±0.00	0.31±0.04
Cf8a2	2.3±0.1	45.9±2.5	16.2±0.8	0.6±0.0	0.62±0.07	0.02±0.00	0.02±0.00	0.35±0.08
Pk100	0.9±0.1	47.0±3.2	17.1±1.1	0.8±0.5	0.46±0.04	0.01±0.00	0.02±0.01	0.50±0.04
Pk105	0.8±0.2	46.9±3.2	16.9±1.0	0.6±0.0	0.45±0.05	0.02±0.00	0.02±0.01	0.52±0.05
Pk110	0.8±0.1	46.5±2.4	16.8±0.9	0.6±0.1	0.51±0.05	0.02±0.00	0.01±0.01	0.46±0.05
Pk123	0.9±0.2	46.8±2.5	17.0±0.7	0.6±0.0	0.48±0.03	0.02±0.00	0.02±0.01	0.49±0.03
Pk125	1.2±0.2	49.2±0.8	18.0±0.2	0.6±0.0	0.43±0.02	0.02±0.00	0.02±0.01	0.53±0.02
Pk128	1.1±0.1	46.7±2.9	16.9±1.3	0.5±0.0	0.40±0.03	0.01±0.00	0.01±0.00	0.57±0.02
Pk129	**0.8±0.1**	**46.0±2.8**	**16.6±1.2**	**0.6±0.1**	**0.48±0.02**	**0.02±0.00**	**0.02±0.01**	**0.48±0.02**
Pk132	0.8±0.1	46.1±2.3	16.9±0.7	0.6±0.0	0.46±0.03	0.02±0.00	0.02±0.00	0.51±0.03
Pk137	0.8±0.1	46.2±2.6	16.9±1.0	0.6±0.0	0.43±0.01	0.01±0.00	0.01±0.00	0.54±0.01
Pk138	0.9±0.1	46.1±2.0	16.7±0.9	0.6±0.1	0.52±0.03	0.02±0.00	0.02±0.01	0.44±0.02
Pk140	1.2±0.2	45.3±1.8	16.6±0.5	0.5±0.0	0.42±0.02	0.02±0.00	0.02±0.01	0.55±0.02
Pk144	0.8±0.1	46.5±2.5	16.9±1.3	0.6±0.0	0.48±0.02	0.02±0.00	0.01±0.01	0.49±0.02
Pk150	0.8±0.1	46.5±2.3	17.1±0.6	0.6±0.0	0.47±0.03	0.01±0.00	0.01±0.01	0.51±0.02
Pk151	0.8±0.1	45.9±1.6	16.6±0.6	0.6±0.0	0.48±0.02	0.01±0.00	0.01±0.01	0.49±0.02
Pk152	0.8±0.0	46.8±3.2	16.9±1.0	0.6±0.0	0.47±0.02	0.01±0.00	0.01±0.00	0.50±0.02
Pk166	0.8±0.1	46.2±2.3	16.7±0.7	0.6±0.0	0.48±0.02	0.02±0.00	0.02±0.01	0.48±0.02
Pk27	0.8±0.1	46.1±2.2	16.8±0.5	0.6±0.0	0.49±0.02	0.01±0.00	0.02±0.01	0.48±0.02
Pk3	0.8±0.1	46.1±1.2	16.6±0.3	0.6±0.0	0.48±0.03	0.01±0.00	0.02±0.01	0.48±0.02
Pk32	0.8±0.1	45.9±1.0	16.6±0.2	0.6±0.0	0.51±0.02	0.02±0.00	0.02±0.01	0.46±0.02
Pk42	0.8±0.0	45.7±1.8	16.3±0.6	0.6±0.0	0.56±0.02	0.02±0.00	0.03±0.01	0.40±0.02
Pk52	0.8±0.0	45.8±2.1	16.8±0.8	0.6±0.0	0.53±0.05	0.02±0.00	0.03±0.01	0.42±0.04
Pk59	0.9±0.2	45.2±1.7	16.2±0.4	0.5±0.1	0.47±0.06	0.02±0.01	0.02±0.01	0.49±0.06
Pk91	0.9±0.1	46.3±2.5	16.9±0.9	0.6±0.0	0.44±0.03	0.01±0.00	0.02±0.01	0.53±0.03
Pk94	0.8±0.1	45.8±2.1	16.6±0.6	0.6±0.0	0.45±0.03	0.02±0.00	0.02±0.01	0.52±0.03
Sc102	0.2±0.0	1.9±0.3	14.1±1.0	3.7±0.1	0.82±0.01	0.01±0.00	0.04±0.00	0.13±0.01
Sc116	0.6±0.1	3.2±0.0	15.9±0.9	3.4±0.1	0.76±0.01	0.01±0.00	0.07±0.00	0.16±0.00
Sc126	0.7±0.0	7.0±2.0	16.6±0.8	3.1±0.1	0.80±0.01	0.01±0.00	0.06±0.00	0.12±0.01
Sc131	**0.6±0.1**	**3.1±0.2**	**16.3±1.1**	**3.4±0.1**	**0.78±0.01**	**0.01±0.00**	**0.07±0.01**	**0.14±0.01**
Sc135	0.2±0.0	4.7±0.1	16.1±0.8	3.4±0.1	0.77±0.02	0.01±0.00	0.05±0.00	0.17±0.01
Sc139	0.3±0.0	19.9±0.6	16.5±0.6	2.4±0.0	0.79±0.00	0.01±0.00	0.07±0.01	0.13±0.01
Sc141	0.2±0.0	4.9±0.5	16.1±0.6	3.4±0.1	0.76±0.01	0.01±0.00	0.04±0.00	0.18±0.01
Sc142	0.7±0.1	3.0±0.1	16.3±0.8	3.5±0.1	0.76±0.01	0.01±0.00	0.07±0.00	0.16±0.01
Sc143	0.7±0.1	3.3±0.1	16.1±0.4	3.4±0.1	0.78±0.01	0.01±0.00	0.07±0.00	0.15±0.01
Sc146	0.6±0.0	3.2±0.1	16.1±0.5	3.4±0.1	0.77±0.02	0.01±0.00	0.07±0.00	0.16±0.02
Sc148	0.6±0.0	3.3±0.1	16.0±0.6	3.4±0.1	0.75±0.01	0.01±0.00	0.08±0.00	0.17±0.01
Sc149	0.7±0.1	3.4±0.4	16.0±0.6	3.4±0.1	0.76±0.01	0.01±0.00	0.07±0.01	0.16±0.01
Sc153	0.3±0.0	20.5±1.2	16.3±0.6	2.4±0.1	0.74±0.02	0.01±0.00	0.08±0.00	0.17±0.01
Sc160	0.3±0.0	17.9±0.4	16.3±0.8	2.5±0.1	0.72±0.01	0.01±0.00	0.09±0.00	0.18±0.01
Sc165	0.3±0.0	30.7±1.2	11.2±0.5	1.9±0.0	0.75±0.01	0.01±0.00	0.05±0.00	0.18±0.01
Sc38	0.2±0.0	21.7±2.8	16.3±0.7	2.2±0.0	0.78±0.01	0.01±0.00	0.07±0.00	0.14±0.01

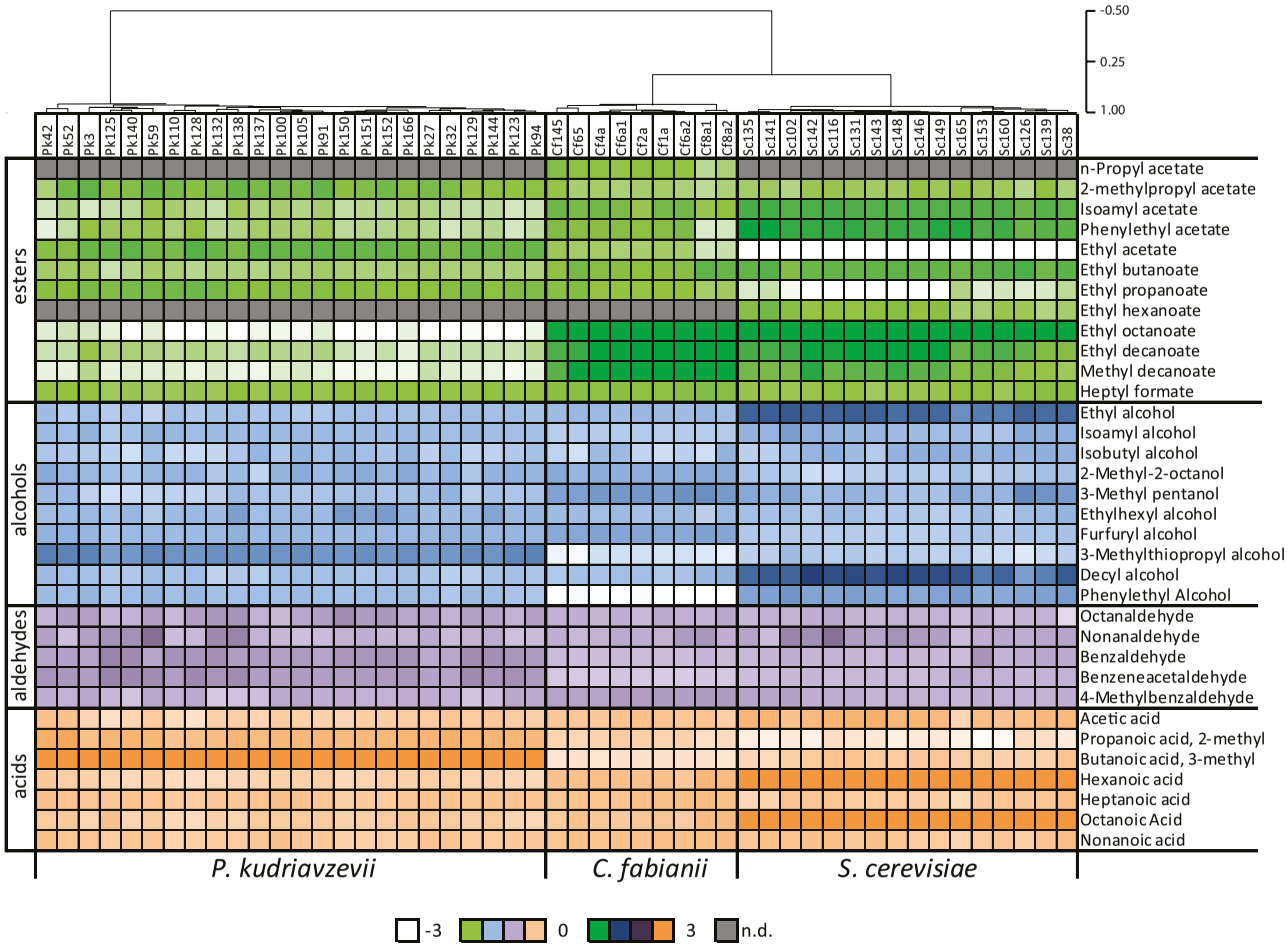


Figure 4.1: Hierarchical clustered aroma profiles of all 49 wild yeast isolates. A colour gradient is used to indicate the intensity of the compound (0= median, -3 = below median and 3=above median); (log2(compound/median compound all samples)). The compounds are grouped as esters (green), alcohols (blue), aldehydes (purple) and acids (orange). Grey means not detected.

Wort fermentation using co-cultures

The three representative isolates (Sc131, Cf65 and Pk129) were applied as co-culture with brewers' yeast in a ratio 1:1. Various parameters were analysed (i.e. residual sugars, ethanol and volatile organic compounds (VOCs)) after 1 week of primary fermentation and 6 weeks of secondary fermentation (final product). Subsequently, the performance of the co-cultures was compared with the performance of brewers' yeast as mono-culture (Figure 4.2, Table 4.2). Similar results were found for brewers' yeast in mono-culture and in co-culture with Sc131 except for the maltotriose consumption, which was significantly less for the co-culture with Sc131. Significantly less maltose and maltotriose was consumed by the co-cultures with Cf65 and Pk129 corresponding with significantly less ethanol formation (3.8 ± 0.1 % and 3.5 ± 0.1 % (v/v) respectively compared to 4,2 ± 0.1 % (v/v) by brewers' yeast in mono-culture, Figure 4.2, Table 4.2). Interestingly, no significant difference was found for the volatile esters and alcohols for the co-culture of brewers' yeast with Cf65 or Pk129. However, if we compare the ratio of each compound group per fermentation, it is clear that the co-culture of brewers' yeast with Cf65 produces relatively more esters compared to the mono-culture of brewers' yeast (fraction of 0.28 for the co-culture with Cf65 and 0.19 for the mono-culture

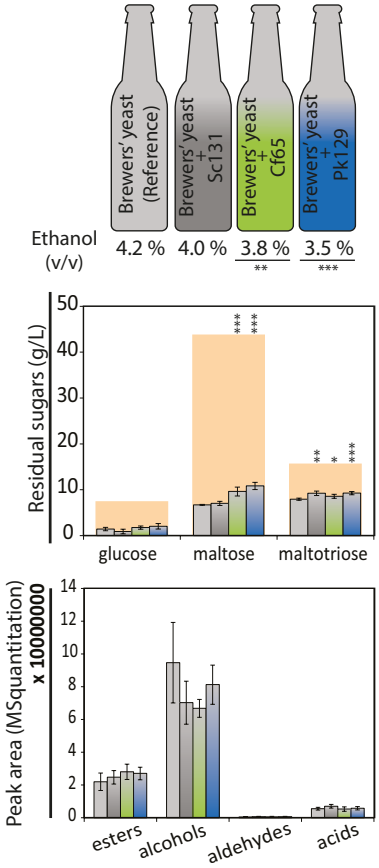


Figure 4.2: Characteristics of the wort fermented (t= end) with a mono-culture of brewers' yeast (reference), or a co-culture of brewers' yeast with Sc131, Cf65 and Pk129. Top bottles explain the colour code for each variant: brewers' yeast (light grey), Sc131 (dark grey), Cf65 (green), Pk129 (blue). For the co-cultures there is a gradient of the colours indicating the 1:1 ratio of the 2 strains. The ethanol content (% v/v) is listed below the bottles. Residual sugars are displayed in g/L (middle), orange indicates the amount of sugars present at t=0. The sum of peak area's (MS quantitation) for all esters, alcohols, aldehydes and acids is displayed on the right. Statistical differences (students t-test) compared to the mono-culture of brewers' yeast are indicated with stars; *= $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$.

Table 4.2: Parameters at the end of secondary fermentation of all mono- and co-cultures (n=4). The average ± standard deviation of residual sugars (glucose, maltose and maltotriose), ethanol % (v/v), CO_2 (mmol), relative abundance VOCs per compound group (esters, alcohols, aldehydes and acids) and the Shannon index are listed. Significant difference against brewers' yeast (bold) is indicated with a grey background ($p < 0.05$).

	Residual sugars (g/L)			ethanol % (v/v)	CO2 produced (mmol)	Relative abundance VOCs per compound group				Shannon index
	glucose	maltose	maltotriose			esters	alcohols	aldehydes	acids	
mono- cultures										
Brewers' yeast	**1.4±0.4**	**6.7±0.1**	**7.9±0.2**	**4.19±0.15**	**0.297±0.005**	**0.19±0.06**	**0.76±0.08**	**0.00±0.00**	**0.05±0.01**	**1.72±0.23**
Sc131	1.9±0.0	12.1±1.1	13.5±0.3	3.18±0.06	0.261±0.031	0.19±0.03	0.71±0.05	0.01±0.00	0.09±0.01	1.84±0.16
Cf65	6.4±0.1	39.2±1.2	13.7±0.3	0.49±0.02	0.013±0.001	0.43±0.08	0.55±0.08	0.01±0.01	0.01±0.00	1.86±0.08
Pk129	2.7±0.7	45.3±1.6	14.4±0.9	0.53±0.02	0.015±0.001	0.60±0.02	0.38±0.03	0.01±0.01	0.01±0.00	1.38±0.07
co-cultures (ratio 1:1)										
Brewers' yeast : Sc131	0.9±0.5	7.0±0.4	9.2±0.5	4.02±0.15	0.286±0.018	0.24±0.05	0.68±0.05	0.01±0.00	0.07±0.01	1.97±0.12
Brewers' yeast : Cf65	1.8±0.4	9.6±1.0	8.6±0.4	3.80±0.12	0.273±0.008	0.28±0.03	0.66±0.04	0.01±0.00	0.05±0.01	2.05±0.07
Brewers' yeast : Pk129	2.0±0.6	10.8±0.8	9.3±0.4	3.55±0.12	0.249±0.007	0.24±0.03	0.71±0.03	0.01±0.00	0.05±0.01	1.89±0.03
Dose response (Brewers' yeast : Cf65)										
200 : 1	1.6±0.4	6.8±0.5	8.1±0.1	4.19±0.20	0.297±0.010	0.18±0.02	0.77±0.02	0.00±0.00	0.05±0.00	1.67±0.13
100 : 1	1.4±0.3	6.6±0.5	7.9±0.3	4.11±0.18	0.299±0.006	0.20±0.07	0.74±0.08	0.01±0.00	0.05±0.02	1.77±0.31
10 : 1	1.3±0.7	6.7±0.7	7.9±0.3	4.09±0.21	0.292±0.012	0.22±0.07	0.72±0.08	0.01±0.00	0.05±0.01	1.84±0.17
1 : 1	1.8±0.4	9.6±1.0	8.6±0.4	3.80±0.12	0.273±0.008	0.28±0.03	0.66±0.04	0.01±0.00	0.05±0.01	2.05±0.07
1 : 10	1.9±0.4	17.8±0.9	10.9±0.4	2.97±0.18	0.198±0.011	0.31±0.03	0.61±0.03	0.01±0.00	0.07±0.02	2.14±0.08
1 : 100	2.2±0.4	26.6±1.3	12.3±0.2	1.99±0.04	0.118±0.008	0.36±0.06	0.54±0.04	0.01±0.01	0.09±0.02	2.26±0.18
1 : 200	2.4±0.3	30.9±2.9	13.0±0.9	1.75±0.13	0.097±0.008	0.35±0.03	0.55±0.06	0.01±0.01	0.08±0.03	2.20±0.25

of brewers' yeast, Table 4.2). The complexity of the volatile organic compounds is indicated by the Shannon diversity index. A significant higher index number was found for the co-cultures of brewers' yeast with either Cf65 or Pk129, this means that composition of the VOCs is more complex for these co-cultures and that this approach influenced the end product.

Often, cultures that are used for additional functionalities such as aroma formation and not for their fermentative capabilities are added at a higher dose compared to the culture with fermentation capabilities (El Soda, et al., 2000). Therefore we tested whether there is a dose-response relationship linked to the addition of Cf65 to brewers' yeast that affects final metabolite and aroma profiles.

Dose-response effect of *C. fabianii* in co-cultivation

Various ratios of brewers' yeast to Cf65 were tested (1:0, 200:1, 100:1, 10:1, 1:1, 1:10, 1:100, 1:200 and 0:1). In the final fermented beverage, a clear dose response relationship was observed regarding the sugar utilization, ethanol production (% v/v), the formation of VOCs and CO_2 production (Figure 4.3 and Table 4.2).

Interestingly, a negative correlation was observed between the amount of Cf65 inoculated and sum of all volatile alcohols produced, while a positive correlation was observed for the sum of all volatile esters (up to an initial inoculum ratio of 1:10) and no difference was found for the volatile alcohols and acids (Figure 4.3A, Table 4.2). This results also in a change in relative abundance per compound group (Table 4.2), which is very important for the sensorial characteristic of the final product. Figure 4.3B displays the dose-response relationship per compound. Phenylethyl acetate, methylpropyl acetate, ethyl butanoate, 3-methylbutyl acetate, ethyl hexanoate and all volatile alcohols showed a negative correlation towards the increasing initial inoculum ratio of Cf65 over brewers' yeast. In contrast, a positive correlation towards increasing initial ratios Cf65 was found for ethyl acetate, ethyl octanoate, methyl octanoate, furfuryl acetate, ethyl nonanoate, methyl decanoate, ethyl decanoate, ethyl 9-decenoate, methyl dodecanoate and ethyl dodecanoate (Figure 4.3B). The Shannon diversity index of the VOCs composition was calculated. The index increased with increasing Cf65 amounts and is significantly higher for the initial inoculum ratio's (brewers' yeast : Cf65) 1:1, 1:10, 1:100 and 1:200 compared to the mono-culture of brewers' yeast, which indicates a more complex aroma bouquet when Cf65 is most abundant in the initial inoculum (Table 4.2).

Remarkably, brewers' yeast does not dominate the fermentation in all tested conditions despite the presence of residual sugars in the end-product. This indicates that brewers' yeast is either inhibited by compounds produced by Cf65 (e.g. killer toxins) or that compounds needed for growth of brewers' yeast are utilized by Cf65. Since no evidence was found for the production of inhibitory compounds by Cf65 (Suppl. Fig. 1) the second option was investigated.

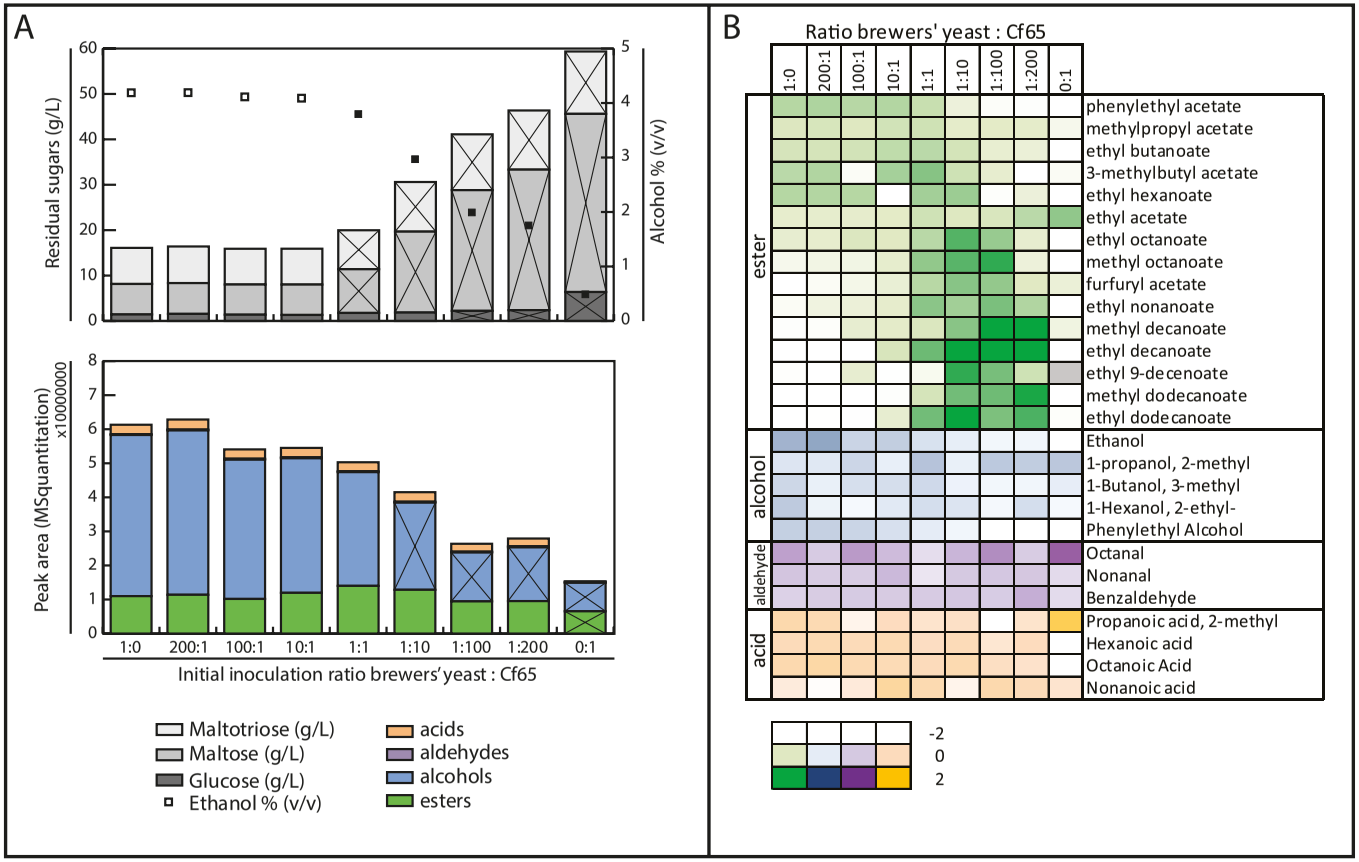


Figure 4.3: Dose response effect. Box A, top graph: residual sugars (glucose (dark grey), maltose (mid grey) and maltotriose (light grey)) and ethanol % (v/v, open squares) in the final product. Box A, bottom graph: Sum of VOCs per group of compounds (esters (green), alcohols (blue), aldehydes (purple) and acids (orange)). Significant difference to initial inoculum (only brewers' yeast (ratio 1:0)) is indicated by black cross in the bar (residual sugars and VOCs) and black closed squares (ethanol) ($p < 0.05$). Box B: A colour gradient is used to indicate the intensity of the compound among the different inoculation ratios (0= median, -3 = below median and 3=above median); (log2(compound/median compound all samples)). The compounds are grouped as esters (green), alcohols (blue), aldehydes (purple) and acids (orange). Grey = not detected.

Brewers' yeast is inhibited due to low oxygen availability

Cf65 efficiently grows in the presence of oxygen displaying higher growth rates than brewers' yeast, but it cannot grow anaerobically. In aerobic conditions brewers' yeast grows on sugars in a fermentative mode (Crabtree-effect) and it can grow anaerobically but requires traces of molecular oxygen to synthesize ergosterol and unsaturated fatty acids (UFAs). Supplementation of wort with ET80 (ergosterol + Tween80) ensures that brewers' yeast grows in the complete absence of molecular oxygen (Longley, et al., 1978). To test whether growth of brewers' yeast in co-culture with Cf65 is inhibited by the lack of molecular oxygen, brewers' yeast was cultivated in a bioreactor (batch culture) together with Cf65 (inoculation ratio 1:100, with 10^4 and 10^6 CFU/ml respectively) in wort with and without the supplementation of ET80.

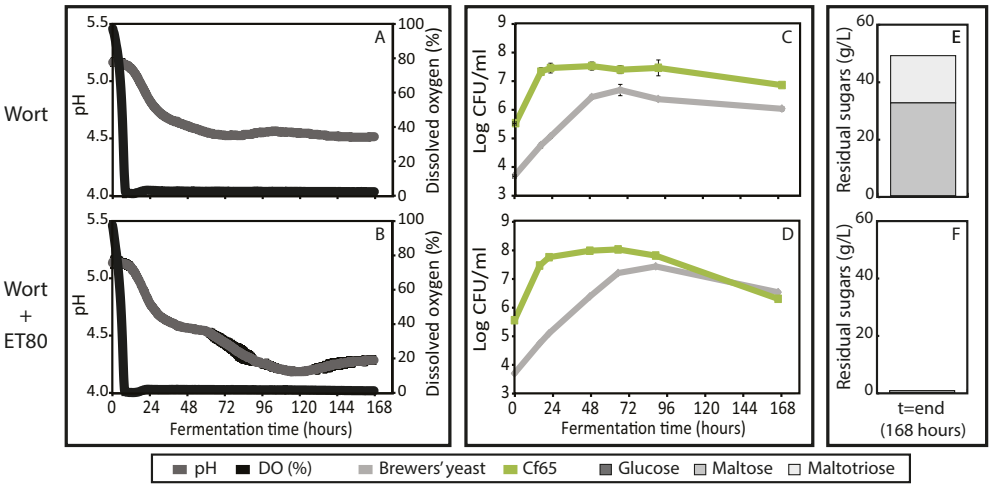


Figure 4.4: Effect of ET80 supplementation on yeast growth and performance. Wort, either with (B, D and E) or without (A, C and E) ET80, was initially inoculated with brewers' yeast + Cf65 (ratio 1:100). Dissolved oxygen (black, A and B), pH (dark grey, A and B) and colony forming units of brewers' yeast (grey, C and D) and Cf65 (green, C and D) were followed over time (average ± standard deviation, n=4). The residual sugars (glucose (dark grey), maltose (mid grey) and maltotriose (light grey)) were measured at the end of fermentation (E and F).

The dissolved oxygen decreases in both fermentations from 100% to approximately 0 % within 8 hours (Figure 4.4A and 4.4B). During the batch cultivation, the pH drops from 5.2 to 4.5 in 58 hours for both fermentations. Beyond 58 hours, the pH of the culture supplemented with ET80 reaches 4.2 in 115 hours while the non-supplemented culture remained at pH 4.5 (Figure 4.4A and B). In line with these results we found the plate counts of brewers' yeast to increase to 7.4 ± 0.04 log CFU/ml in the culture supplemented with ET80 and to 6.7 ± 0.2 log CFU/ml in the non-supplemented wort (Figure 4.4C and D). Moreover, only trace amounts of residual sugar were detected after 7 days of fermentation in the culture supplemented with ET80 while significantly more residual sugar was found in the non-supplemented culture

(Figure 4.4E and F). These results align with the conclusion that brewers' yeast is inhibited by oxygen depletion in a co-culture with Cf65 unless ET80 is added. The observation that no residual sugars are left in the culture supplemented with ET80 confirms that there are no other inhibiting factors for brewers' yeast (Figure 4.4F and Suppl. Figure 4.1).

Discussion

For wort fermentation, usually a mono-culture of an in-house brewers' yeast is used. This is either *Saccharomyces cerevisiae* (ale-type yeast) or a natural hybrid of *S. cerevisiae* and *S. eubayanus* (lager-type yeast) (Hebly, et al., 2015). Many attempts to screen for or develop strains with diverse characteristics have been made, however the range of biodiversity is limited when strains are sourced from a single genus (Gallone, et al., 2016; Saerens, et al., 2010; Snoek, et al., 2015; Steensels, et al., 2012; Steensels, et al., 2014b). In order to increase the range of diversity a number of research groups have recently investigated the use of non-*Saccharomyces* yeast (so called non-conventional yeast) (Gamero, et al., 2016; Johnson, 2013; Jolly, et al., 2014; Steensels, et al., 2015; Steensels and Verstrepen, 2014). They all conclude that non-conventional yeast harbour interesting traits for industrial fermentations, however, an approach for their application in wort fermentation remains to be explored. Here, we investigated the use of (non-)conventional yeast in mono-cultivation and co-cultivation with brewers' yeast (*S. cerevisiae)*. These approaches potentially offer the opportunity to add new product characteristics to fermented beverages.

We first monitored the diversity in aroma production and growth characteristics among 49 wild yeast isolates, belonging to the species *S. cerevisiae*, *C. fabianii* and *P. kudriavzevii* in wort. Both *C. fabianii* and *P. kudriavzevii* are not able to grow in the absence of oxygen and therefore can only grow at early stages of the fermentation. Hence, they mainly utilized glucose, where *S. cerevisiae* strains utilized – as expected - glucose and maltose. The potential benefit of using *C. fabianii* and *P. kudriavzevii* is their ability to produce relatively high levels of esters in comparison to *S. cerevisiae* (Table 4.1) which is in line with findings by Nyanga, et al. (2013). Additionally, *C. fabianii* and *P. kudriavzevii* produce not only relatively more esters, but also the balance of the different esters is compared to *S. cerevisiae* (Figure 4.1).

In the brewing industry it is custom to use pure cultures consisting of a single strain to ferment wort, since breweries tend to recycle their yeast for a few batches. However, in various other fermentation industries (i.e. fermented dairy, wine) it is common practice to add multiple strains to obtain a consistent, more complex aroma bouquet which can be fine-tuned by varying the dose of each strain (Andorrà, et al., 2012; Ciani, et al., 2010; El Soda, et al., 2000; Spus, et al., 2017). Recently, Saerens and Swiegers (2014) patented the use of co-cultivation of *Pichia kluyverii* and brewers' yeast to produce a beer with enhanced flavour. However, no data on aroma and ethanol formation in the co-cultivation conditions were supplied neither is the synergy between the two yeast species described.

In our study co-cultivation was performed with one representative strain per species selected based on the performance as mono-culture; Sc131, Cf65 and Pk129. These strains were co-cultivated with brewers' yeast at a ratio of 1:1. Due to the poor fermentation

performance of Cf65 and Pk129 in mono-cultures, brewers' yeast was expected to dominate the fermentation, since it can perform best under the applied conditions. Interestingly, we found significantly more residual sugars in the co-cultures (maltotriose for all co-cultures and maltose for the co-culture with Cf65 and Pk129). This observation aligns with the detected reduced ethanol production. Interestingly, the relative abundance of volatile esters in the co-cultivation of Cf65 with brewers' yeast is significantly higher (Table 4.2) compared to what is found in the brewers' yeast mono-culture. Therefore, we chose to further explore the fermentation process by varying the inoculum ratios of Cf65 over brewers' yeast.

Interestingly, a clear dose response relationship was observed. A higher initial dose of Cf65 resulted in more residual sugars, lower ethanol % (v/v), a decrease in volatile alcohols and an increase of relative abundance of volatile esters. This relationship shows that it is possible to steer the end-product composition by varying the ratios of Cf65 and brewers' yeast. Esters are the one the most important aroma compounds determining the flavour, due to their low odour-threshold. Among the ester, the most important compounds are ethyl acetate (solvent-like aroma), 3-methylbutyl acetate (banana aroma), methylpropyl acetate (fruity, sweet aroma), phenylethyl acetate (rose, apple, honey aroma), ethyl hexanoate (apple, aniseed aroma) and ethyl octanoate (sour apple aroma) and are either positively or negatively correlated with a higher initial inoculation dose of Cf65 (Figure 4.3B)(Michel, et al., 2016). If the concentration of these compounds is too high it is perceived as an off-flavour, therefore the balance between all aroma compounds is of utmost importance.

Additionally, the complexity of the VOCs increases with a higher dose of Cf65 (Shannon diversity index, Table 4.2). Interestingly, no dose response relationship was found for the volatile aldehydes and acids. These findings indicate that the sensorial properties can be changed by varying the initial inoculum ratios of Cf65 and brewers' yeast. However, the VOCs profile does not provide information on the sensorial impact. Therefore it is recommended for future studies to evaluate product characteristics using a sensorial panel.

The observed dose response relationship is the result of competition between Cf65 and brewers' yeast. We found no evidence for the production of compounds by Cf65 which inhibit brewers' yeast (Suppl. Figure 4.1). Further investigation revealed that fast oxygen depletion by Cf65 inhibits the performance of brewers' yeast. Brewers' yeast needs oxygen to synthetize ergosterol (12 moles oxygen per mole ergosterol (Rosenfeld and Beauvoit, 2003)) when insufficient amounts are stored intracellularly. Supplementation of wort with ET80 overrules this effect, and resulted in complete fermentation of all sugars by brewers' yeast in the co-culture. This also indicates that there are no other factors inhibiting the performance of brewers' yeast in co-culture with Cf65.

It needs to be stressed that the conditions of pre-culturing could also affect the outcome of the competition. In our experimental design brewers' yeast was pre-grown in static cultures and it is conceivable that pre-growth in aerated cultures results in higher intracellular ergosterol

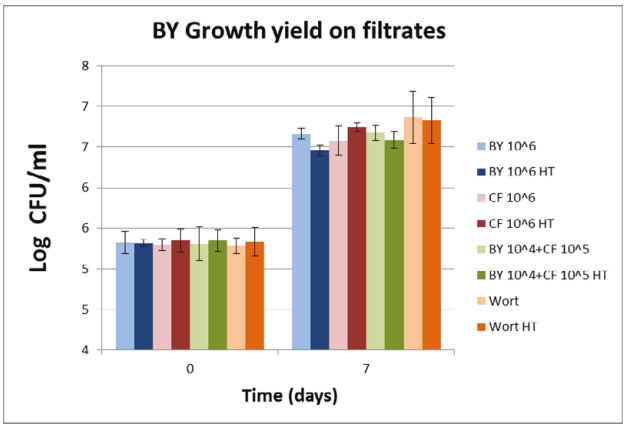
levels that may enable completion of the fermentation in the presence of Cf65. Obviously, this is one of the parameters that can be included in future co-culture experiments.

Understanding the underlying mechanism of the dose response relationship of Cf65 in co-culture with brewers' yeast makes it now possible to apply the same principle for other (aerobic) non-conventional yeast selected after screening for specific features including novel (combinations of) aroma compounds, such as Pk129 identified in the current study. Additionally, the dose response relation makes it possible to model the performance of both yeasts to optimize the optimal ratio Cf65 over brewers' yeast by prediction of the final product characteristics (such as volatile esters an ethanol content). This approach can therefore now be used for product innovation to enhance the aroma bouquet of the final product beyond the capacities of brewers' yeast alone.

Acknowledgements

This research was financially supported by the Graduate School VLAG (Wageningen University & Research, Wageningen, The Netherlands) and HEINEKEN Supply Chain B.V. (Zoeterwoude, The Netherlands). The authors would like to acknowledge Jan-Maarten Geertman and Niels Kuijpers from HEINEKEN for sharing their knowledge and Wageningen University & Research MSc thesis students for support of the experimental work; Saskia Zweers, Ralph van der Ploeg, Maria Alarcon Cabezas, Ivana Mik and Niccolo Ferretti.

Supplementary materials



Supplementary figure 4.1: Brewers' yeast growth yields on various filtrates to test if Cf65 produces inhibitory compounds against brewers' yeast. Three pre-cultures on wort were grown for 7 days at 20 °C (static with water lock); 1) brewers' yeast, 2) Cf65 and 3) brewers' yeast + Cf65 (ratio 1:10). The precultures were filtrated and half of it was heat treated for 25 minutes at 55 °C (to test for heat labile inhibitory compounds). All filtrates were inoculated with the same amount of brewers' yeast (t=0) and incubated for 7 days at 20 °C static with waterlock. After 7 days the CFU/ml was determined (t=7), but no inhibition was observed in any of the variants.

References

Andorrà, I., Berradre, M., Mas, A., Esteve-Zarzoso, B. and Guillamón, J. M. (2012). Effect of mixed culture fermentations on yeast populations and aroma profile. *LWT - Food Science and Technology* **49,** 8-13.

Basso, R. F., Alcarde, A. R. and Portugal, C. B. (2016). Could non-*Saccharomyces* yeasts contribute on innovative brewing fermentations? *Food Research International* **86,** 112-20.

Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J. C., Gerds, M. L., Hammes, W. P., Harnett, J., Huys, G., Laulund, S., Ouwehand, A., Powell, I. B., Prajapati, J. B., Seto, Y., Ter Schure, E., Van Boven, A., Vankerckhoven, V., Zgoda, A., Tuijtelaars, S. and Hansen, E. B. (2012). Food fermentations: Microorganisms with technological beneficial use. *Int J Food Microbiol* **154,** 87-97.

Ciani, M., Comitini, F., Mannazzu, I. and Domizio, P. (2010). Controlled mixed culture fermentation: a new perspective on the use of non-*Saccharomyces* yeasts in winemaking. *FEMS Yeast Res* **10,** 123-33.

Contreras, A., Hidalgo, C., Schmidt, S., Henschke, P. A., Curtin, C. and Varela, C. (2015). The application of non-*Saccharomyces* yeast in fermentations with limited aeration as a strategy for the production of wine with reduced alcohol content. *Int J Food Microbiol* **205,** 7-15.

Del Monaco, S. M., Barda, N. B., Rubio, N. C. and Caballero, A. C. (2014). Selection and characterization of a Patagonian *Pichia kudriavzevii* for wine deacidification. *J Appl Microbiol* **117,** 451–64.

El Soda, M., Madkor, S. A. and Tong, P. S. (2000). Adjunct cultures: Recent developments and potential significance to the cheese industry. *Journal of Dairy Science* **83,** 609-19.

Erten, H. and Tanguler, H. (2010). Influence of *Williopsis saturnus* yeasts in combination with *Saccharomyces cerevisiae* on wine fermentation. *Lett Appl Microbiol* **50,** 474-9.

Gallone, B., Steensels, J., Prahl, T., Soriaga, L., Saels, V., Herrera-Malaver, B., Merlevede, A., Roncoroni, M., Voordeckers, K., Miraglia, L., Teiling, C., Steffy, B., Taylor, M., Schwartz, A., Richardson, T., White, C., Baele, G., Maere, S. and Verstrepen, K. J. (2016). Domestication and divergence of *Saccharomyces cerevisiae* beer yeasts. *Cell* **166,** 1397-410.

Gamero, A., Quintilla, R., Groenewald, M., Alkema, W., Boekhout, T. and Hazelwood, L. (2016). High-throughput screening of a large collection of non-conventional yeasts reveals their potential for aroma formation in food fermentation. *Food Microbiology* **60,** 147-59.

Hebly, M., Brickwedde, A., Bolat, I., Driessen, M. R., de Hulster, E. A., van den Broek, M., Pronk, J. T., Geertman, J. M., Daran, J. M. and Daran-Lapujade, P. (2015). *S. cerevisiae* x *S. eubayanus* interspecific hybrid, the best of both worlds and beyond. *FEMS Yeast Res* **15,** 1-14.

Johnson, E. A. (2013). Biotechnology of non-*Saccharomyces* yeasts - the ascomycetes. *Appl Microbiol Biotechnol* **97,** 503-17.

Jolly, N. P., Varela, C. and Pretorius, I. S. (2014). Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Res* **14,** 215-37.

Kaneda, H., Kimura, T., Kano, Y., Koshino, S., Osawa, T. and Kawakishi, S. (1991). Role of fermentation conditions on flavour stability of Beer. *Journal of Fermentation and Bioengineering* **72,** 26-30.

Kim, D. H., Hong, Y. A. and Park, H. D. (2008). Co-fermentation of grape must by *Issatchenkia orientalis* and *Saccharomyces cerevisiae* reduces the malic acid content in wine. *Biotechnol Lett* **30,** 1633-8.

Landaud, S., Latrille, E. and Corrieu, G. (2001). Top pressure and temperature control the fusel alcohol/ester ratio through yeast growth in beer fermentation. *J. Inst. Brew.* **107,** 107-17.

Lei, H., Zhao, H., Yu, Z. and Zhao, M. (2012). Effects of wort gravity and nitrogen level on fermentation performance of Brewer's yeast and the formation of flavor Volatiles. *Appl Biochem Biotechnol* **166,** 1562-74.

Longley, R. P., Dennis, R. R., Heyer, M. S. and Wren, J. J. (1978). Selective *Saccharomyces* media containing ergosterol and tween 80. *J. Inst. Brew.* **84,** 341-5.

Michel, M., Meier-Dörnberg, T., Jacob, F., Methner, F.-J., Wagner, R. S. and Hutzler, M. (2016). Review: Pure non-*Saccharomyces* starter cultures for beer fermentation with a focus on secondary metabolites and practical applications. *Journal of the Institute of Brewing* **122,** 569-87.

Nyanga, L. K., Nout, M. J., Gadaga, T. H., Theelen, B., Boekhout, T. and Zwietering, M. H. (2007). Yeasts and lactic acid bacteria microbiota from masau (*Ziziphus mauritiana*) fruits and their fermented fruit pulp in Zimbabwe. *Int J Food Microbiol* **120,** 159-66.

Nyanga, L. K., Nout, M. J., Smid, E. J., Boekhout, T. and Zwietering, M. H. (2013). Fermentation characteristics of yeasts isolated from traditionally fermented masau (*Ziziphus mauritiana*) fruits. *Int J Food Microbiol* **166,** 426-32.

Quiros, M., Rojas, V., Gonzalez, R. and Morales, P. (2014). Selection of non-*Saccharomyces* yeast strains for reducing alcohol levels in wine by sugar respiration. *Int J Food Microbiol* **181,** 85-91.

Rosenfeld, E. and Beauvoit, B. (2003). Role of the non-respiratory pathways in the utilization of molecular oxygen by *Saccharomyces cerevisiae*. *Yeast* **20,** 1115-44.

Saerens, S. M., Delvaux, F., Verstrepen, K. J., Van Dijck, P., Thevelein, J. M. and Delvaux, F. R. (2008). Parameters affecting ethyl ester production by *Saccharomyces cerevisiae* during fermentation. *Appl Environ Microbiol* **74,** 454-61.

Saerens, S. M., Duong, C. T. and Nevoigt, E. (2010). Genetic improvement of brewer's yeast: current state, perspectives and limits. *Appl Microbiol Biotechnol* **86,** 1195-212.

Saerens, S. M. and Swiegers, H. (2014). Enhancement of beer flavor by a combination of *Pichia* yeast and different hop varieties: City, pp. 27.

Snoek, T., Picca Nicolino, M., Van den Bremt, S., Mertens, S., Saels, V., Verplaetse, A., Steensels, J. and Verstrepen, K. J. (2015). Large-scale robot-assisted genome shuffling yields industrial *Saccharomyces cerevisiae* yeasts with increased ethanol tolerance. *Biotechnol Biofuels* **8,** 1-19.

Spus, M., Liu, H., Wels, M., Abee, T. and Smid, E. J. (2017). Isolation and characterization of *Lactobacillus helveticus* DSM 20075 variants with improved autolytic capacity. *Int J Food Microbiol* **241,** 173-80.

Steensels, J., Daenen, L., Malcorps, P., Derdelinckx, G., Verachtert, H. and Verstrepen, K. J. (2015). *Brettanomyces* yeasts--From spoilage organisms to valuable contributors to industrial fermentations. *Int J Food Microbiol* **206,** 24-38.

Steensels, J., Meersman, E., Snoek, T., Saels, V. and Verstrepen, K. J. (2014a). Large-scale selection and breeding to generate industrial yeasts with superior aroma production. *Appl Environ Microbiol* **80,** 6965-75.

Steensels, J., Snoek, T., Meersman, E., Picca Nicolino, M., Aslankoohi, E., Christiaens, J. F., Gemayel, R., Meert, W., New, A. M., Pougach, K., Saels, V., van der Zande, E., Voordeckers, K. and Verstrepen, K. J. (2012). Selecting and generating superior yeasts for the brewing industry. *Cerevisia* **37,** 63-67.

Steensels, J., Snoek, T., Meersman, E., Picca Nicolino, M., Voordeckers, K. and Verstrepen, K. J. (2014b). Improving industrial yeast strains: exploiting natural and artificial diversity. *FEMS Microbiol Rev* **38,** 947-95.

Steensels, J. and Verstrepen, K. J. (2014). Taming wild yeast: potential of conventional and nonconventional yeasts in industrial fermentations. *Annu Rev Microbiol* **68,** 61-80.

Zhang, C.-Y., Liu, Y.-L., Qi, Y.-N., Zhang, J.-W., Dai, L.-H., Lin, X. and Xiao, D.-G. (2013). Increased esters and decreased higher alcohols production by engineered brewer's yeast strains. *European Food Research and Technology* **236,** 1009-14.

Chapter 5

Dynamic modelling of brewers' yeast and *Cyberlindnera fabianii* co-culture behaviour for steering fermentation performance

Irma M. H. van Rijswijck[1], Oscar van Mastrigt[1], Gerco Pijffers[1], Judith C. M. Wolkers – Rooijackers[1], Tjakko Abee[1], Marcel H. Zwietering[1] and Eddy J. Smid[1]

Manuscript in preparation

Affiliations:

1. Laboratory of Food Microbiology, Wageningen University & Research, P.O. Box 17, 6700 AA Wageningen, The Netherlands

Abstract

Co-cultivation of brewers' yeast (*Saccharomyces cerevisiae*) with *Cyberlindnera fabianii* makes it possible to steer aroma and alcohol levels by changing the inoculation ratio of the two yeasts. A dynamic model was developed based on mono-culture performance of brewers' yeast and *C. fabianii* in controlled bioreactors with aerated wort as media, describing growth rate, carbohydrate utilization, ethanol production, maintenance, oxygen consumption and ergosterol biosynthesis/use for cell membrane synthesis (the last one only for brewers' yeast). The parameters were estimated by fitting models to experimental data of both mono-cultivations. To predict the fermentation outcome of brewers' yeast and *C. fabianii* in co-cultivation, the two models were combined and the same parameter settings were used. The co-cultivation model was experimentally validated for the inoculum ratios 1:10 and 1:100 brewers' yeast over *C. fabianii*. The use of predictive modelling supported the hypothesis that performance of brewers' yeast in co-cultivation is inhibited by oxygen depletion which is required for the biosynthesis of ergosterol. This dynamic modelling approach and the parameters involved may also be used to predict the performance of brewers' yeast in the co-cultivation with other yeast species and to give guidance to optimize the fermentation outcome.

Introduction

Spontaneously fermented foods generally have a complex aroma bouquet that is perceived as authentic and valuable compared to the same raw materials fermented with simple defined starter cultures. However, in order to reduce batch to batch variation and also as result of industrial innovations more use is made of defined starter cultures composed of only one or a limited number of selected well performing strains, providing consistent end-products with specific, but generally less complex aroma bouquets. To overcome this drawback of defined starter cultures, the cheese industry developed the so-called "adjunct cultures", which are lactic acid bacteria that do not contribute to the acidification of the milk, but produce specific desired aroma compounds (El Soda, et al., 2000; Spus, et al., 2017). Recently, the wine industry also implemented this approach and developed multiple strain starter cultures to produce wines with consistent quality but also a complex aroma bouquet (Andorrà, et al., 2012; Barbosa, et al., 2015; Ciani, et al., 2010; Erten and Tanguler, 2010; Kim, et al., 2008; Medina, et al., 2012; Renault, et al., 2015; Sadoudi, et al., 2012; Tristezza, et al., 2016). So far, the beer industry has focussed on optimization of the performance of single strain starter cultures (including hybrids and non-*Saccharomyces* yeast) (Michel, et al., 2016; Steensels, et al., 2012).

Recently, van Rijswijck, et al. (2017) showed that co-cultivation of brewers' yeast (*Saccharomyces cerevisiae*) with *Cyberlindnera fabianii* resulted in a beer with a more complex aroma bouquet. Interestingly, inoculation ratios with a higher dose of *C. fabianii* over brewers' yeast revealed specific esters to be formed in higher levels compared to mono-cultivations of the two yeasts, whereas ethanol concentrations were lower, indicating inhibition of brewers' yeast by *C. fabianii*. This pointed to metabolic interactions between the two yeasts and it was hypothesized that oxygen depletion by *C. fabianii* limits the growth performance of brewers' yeast (Smid and Lacroix, 2013; van Rijswijck, et al., 2017).

Molecular oxygen is required for several pathways in brewers' yeast, such as biosynthesis of sterols and fatty acids (Daum, et al., 1998; David and Kirsop, 1972; Snoek and Steensma, 2007). For the production of 1 mole ergosterol under aerobic conditions, brewers' yeast needs 12 moles oxygen. Excess produced ergosterol is stored intracellularly and can be used for membrane synthesis during growth under anaerobic conditions in the absence of added ergosterol (0.113 mmoles of stored ergosterol is needed to produce 1 Cmol biomass) (Klug and Daum, 2014; Rosenfeld, et al., 2003; Snoek and Steensma, 2007; Vanegas, et al., 2012). Supplementation of the wort with ergosterol (sterol) and tween80 (medium chain fatty acids) eliminates oxygen requirements for brewers' yeast thus supporting growth in anaerobic conditions (Longley, et al., 1978; van Rijswijck, et al., 2017).

Here we developed a dynamic model to describe the interactions between brewers' yeast and *C. fabianii* in co-cultivation and to predict the fermentation outcome. The model is based

on mono-culture performance of both yeasts and considers oxygen consumption, substrate utilization, ethanol production, growth and maintenance. Additionally, for brewers' yeast, ergosterol biosynthesis and use for cell membrane synthesis are accounted for. Finally, the aroma profiles of both mono-cultivations and co-cultivations are discussed.

Materials and Methods

Yeast strains

Two yeast species were used in this study: *Cyberlindnera fabianii* 65 isolated from the microbiota of fermented masau fruits (Nyanga, et al., 2007) and brewers' yeast (*Saccharomyces cerevisiae*) from Lallemand danstar called "Nottingham". Both strains were stored at -80 °C in 15 % (v/v) glycerol.

Media

For viable counts of mono-cultures and total viable counts of the co-cultures, Malt Extract Agar (MEA) plates (Oxoid, prepared according manufactures instructions) were used. In co-cultures, the viable counts per species were distinguished using selective plates.

Brewers' yeast was distinguished using MEA supplemented with ergosterol and Tween 80 (0.01 volume of 2.5 g/l ergosterol (SIGMA-Aldrich Co., St. Louis, USA) dissolved in a solution of 50 % (v/v) ethanol (VWR Chemicals, Amsterdam, The Netherlands) and 50 % (v/v) Tween 80 (Merck, KGaA, Darmstadt, Germany)). Plates were incubated anaerobically at 30 °C for 48 hours.

C. fabianii was distinguished using Yeast Nitrogen Base (YNB)/sorbitol agar plates (1x YNB w/o amino acids and ammonium (Becton, Dickinson and Company Sparks, USA), 45.4 mM $(NH_4)_2SO_4$ (Merck, KGaA, Darmstadt, Germany), 20 g/l sorbitol (Merck, KGaA, Darmstadt, Germany), 20 g/l agar (Oxoid Limited, Basingstoke, UK) incubated aerobically at 30 °C for 48 hours.

Appropriate dilutions of the cultures were made in Peptone Physiological Salt (PPS) tubes (Tritium Microbiology BV, Eindhoven, the Netherlands).

Wort preparation

Pils wort extract (Brewferm®, Beverlo, Belgium) was heated in a water bath at 55 °C for 1 hour to liquefy the extract. The extract was diluted with approximately 12 litre of 55 °C water until 12 °Brix was reached (measured using a refractometer). The wort was sterilized at 105 °C for 15 minutes and stored at 4 °C until use.

Propagation

The stock of strains was streaked onto MEA and incubated for 24 hours at 30 °C. A single colony was picked and inoculated in 10 ml sterile wort and incubated at 30 °C, 200 RPM for 24 hours. 1 % of the overnight culture was inoculated in an Erlenmeyer flask containing 100 ml sterile wort and incubated static for 3 days at 30 °C.

Fermentation

Wort fermentations were performed in 500 mL Infors bioreactors (Multifors, Infors HT, Bottmingen, Switzerland). Temperature, pH (not regulated) and dissolved oxygen (DO) was continuously monitored with internal probes. The bioreactors were continuously stirred at 100 rpm and before inoculation the wort was flushed with sterile air (1 hour) for complete saturation with oxygen (at 20 °C and 21 % oxygen in air). At this point, the DO probe was set at 100 %. The air inlet was closed, just before inoculation. The sample point of the bioreactor was 115 mm from the top. The volume at the start of the fermentations was 450 ml for the liquid phase and 200 ml for the gas phase.

The wort was inoculated with brewers' yeast and/or *C. fabianii*. Before inoculation the cells were counted using a Bürker-Türk counting chamber (Cell vision technologies, Heerhugowaard, The Netherlands) and the appropriate volume was centrifuged for 5 minutes at 10.620 x g to obtain the desired pitching rate. Samples were taken at appropriate time points. One part of the sample was immediately used for viable cell count, another 2 x 2 ml was transferred and stored at -20 °C in GC-MS vials and the left was separately frozen at -20 °C until analysis of the residual sugars and ethanol.

Residual sugars and ethanol analysis

High Performance Liquid Chromatography (HPLC) was performed to quantify ethanol, glucose, maltose and maltotriose on an Ultimate 3000 HPLC (Dionex) equipped with an RI-101 refractive index detector (Shodex, Kawasaki, Japan), an auto sampler and an ion-exclusion Aminex HPX – 87H column (7.8 × 300 mm) with a guard column (Bio-Rad, Hercules, CA). As mobile phase, 5 mM H_2SO_4 was used at a flow rate of 0.6 mL/minute and the column was kept at 40 °C. Total run time was 30 minutes. The injection volume was 10 µl. Samples were deproteinated with 0.5 volume Carrez A (0.1 M potassium ferrocyanide trihydrate) and 0.5 volume Carrez B (0.2 M zinc sulfate heptahydrate) and 2 times diluted with MilliQ water. Diluted samples and standards (4-20 mM for glucose, 8-40 mM for maltose and 2-10 mM for maltotriose; 0.72-3.6 % (v/v) for ethanol) were injected onto the column.

Volatile organic compound analysis

Immediately after taking the sample, 2 ml was transferred to a GC-MS vial and closed with the appropriate caps. Samples were kept frozen (20 °C) until analysis. The following method was used to determine the volatile organic compounds present in the sample using headspace solid phase micro extraction gas chromatography mass spectrometry (HS-SPME GC-MS) analysis. Samples were defrosted and incubated for 5 minutes at 60 °C, followed by extraction for 20 minutes at 60 °C using a SPME fiber (Car/DVB/PDMS, Supelco). The compounds were desorbed from the fiber for 10 minutes on a Stabilwax®- DA-Crossband®-Carbowax®-polyethylene-glycol column (30 m length, 0.25 mmID, 0.5 µm df). The gas

chromatograph settings were: PTV Split-less mode (5 minutes) at 250 °C. Helium was used as carrier gas at a constant flow of 1.5 ml/min. The GC oven temperature, initially at 40 °C for 2 minutes, raised to 240 °C (10 °C/min) and kept at 240 °C for 5 minutes(Gamero, et al., 2013). Total run time was 28 minutes. Mass spectral data was collected over a range of m/z 33-250 in full scan mode with 3.0030 scans/seconds. Peaks were annotated using Chromeleon® 7.2. The ICIS algorithm was used for peak integration and the NIST main library to match the mass spectral profiles with the profiles of NIST. Peak areas were calculated using the MS quantitation peak (highest m/z peak per compound).

Statistical analysis

Statistical analysis on the data was performed using the student t-test ($p < 0.05$).

Modelling

Dynamic models were built based on experimental data of brewers' yeast and *C. fabianii* in mono-cultivation, which were combined to confirm and predict the behaviour of both yeast species in co-cultivation. The model for *C. fabianii* is a simplified version of the model for brewers' yeast. Therefore, we first explain the model for brewers' yeast in mono-cultivation. The time interval used was 0.2 hour.

The cultivation of brewers' yeast consists of 5 different growth phases: i) a lag phase, ii) an aerobic growth phase, iii) an anaerobic growth phase, iv) a stationary phase, and v) a death phase (Figure 5.1).

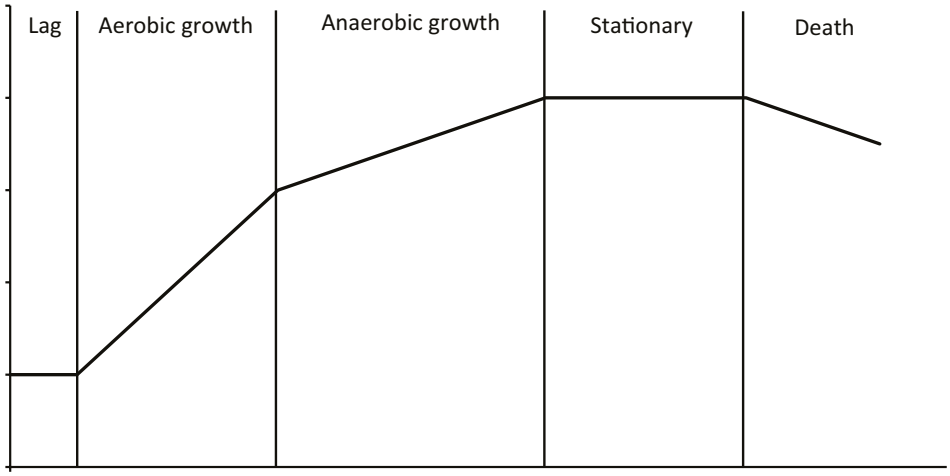


Figure 5.1: Yeast growth phases considered in the model.

The following assumptions were made:

i) Lag phase:
   a. Growth rate = 0 h^{-1}
   b. Consumption of internal carbohydrates (glycogen and/or trehalose)
   c. Consumption of oxygen to generate energy for synthesis of enzymes necessary for growth (implied to be maximum ())

ii) Aerobic growth phase:
   a. Oxygen concentration > 0
   b. Growth rate depends on oxygen concentration (Monod kinetics)
   c. Oxygen consumption depends on the amount of biomass formed
   d. Carbohydrate consumption depends on the amount of biomass formed
   e. Ergosterol is produced (1 mol ergosterol per 12 moles oxygen)

iii) Anaerobic growth phase:
   a. Oxygen concentration = 0
   b. Ergosterol > 0
   c. Growth rate depends on substrate consumption rates, biomass yield and maintenance
   d. Ergosterol depletion depends on the amount of biomass formed.

iv) Stationary phase:
   a. Oxygen concentration = 0
   b. Ergosterol depletion depends on the ergosterol consumption rate
   c. Ergosterol is not yet depleted for more than 24 hours

v) Death phase:
   a. Oxygen concentration = 0
   b. Substrate concentration = 0 and/or ergosterol is depleted for more than 24 hours
   c. Cell number decreases

All the above assumptions are made for brewers' yeast. For *C. fabianii* ii(e),iii(b), iii(d), iv(b), iv(c) and v are not assumed. Glucose, maltose and maltotriose are considered as substrates for brewers' yeast, whereas only glucose is considered as substrate for *C. fabianii*.

The above assumptions were combined in dynamic models using the equations given in Table 5.1 including the equations for growth rate, sugar consumption rate and differential equations for biomass, oxygen, ergosterol and sugars.

The models for growth, sugar and oxygen consumption were fitted using the experimental data of the mono-cultivations. The fraction of oxygen used for ergosterol production (F_E) and death rate (K_d) of brewers' yeast could only be fitted by the co-cultivations, since ergosterol was not limiting in the mono-cultivation of brewers' yeast. The parameters were fitted first by hand, then using the solver add-in in Microsoft excel 2010 by minimizing the sum squared error of the following experimental data (LogN (experimental data - the fitted value), residual sugars and oxygen ((experimental data - the fitted data)/minimum (experimental and fitted data)).

Results

Brewers' yeast mono-cultivation on wort

The carbohydrate composition of wort consists of maltose (150 mmol/L), glucose (58 mmol/L) and maltotriose (40 mmol/L). Before fermentation, the wort and headspace are saturated with oxygen by flushing with air (21 % oxygen). Bioreactors were used to monitor growth, sugar utilization, Dissolved Oxygen (DO %), ethanol formation and Volatile Organic Compounds (VOCs) profiles. The wort was pitched with 10^5 Colony Forming Units (CFU)/mL brewers' yeast and fermented for 7 days at ambient temperature (20 °C). Under these circumstances, brewers' yeast reached 10^8 CFU/mL and was able to ferment all carbohydrates in wort to carbon dioxide, ethanol (5.2 % v/v) and biomass (Figure 5.2). As a result of catabolite repression glucose is utilized first, followed by maltose and maltotriose, respectively (Gancedo, 1998; Phillips, 1954). At the start of the fermentation, the DO was set at 100 % and reached 0 % in 15.5 hours. Under anaerobic conditions, brewers' yeast needs ergosterol for cell membrane synthesis (Rosenfeld and Beauvoit, 2003; Snoek and Steensma, 2007). Under the circumstances used here, sufficient oxygen is present at the start of the fermentation to produce and store enough ergosterol to support growth (biomass production) throughout the experiment and consume all fermentable sugars under anaerobic conditions.

A dynamic model was built as described and fitted to the experimental data. The parameters used to describe aerobic growth of brewers' yeast are maximum growth rate (μmax) and affinity for oxygen (K_O), and includes the variable oxygen concentration (C_O) (Equation 1a, Table 5.1). Anaerobic growth of brewers' yeast is described using the yield of ATP per Cmol substrate ($Y_{ATP/S}$), the maintenance factor (m_{ATP}) and yield of ATP per Cmol biomass ($Y_{ATP/X}$) as parameters and substrate consumption rates (q_G (glucose), q_M (maltose) and q_R (maltotriose)) as variables (Table 5.1, Equation 1b). The growth of brewers' yeast was fitted to the experimental data (LogN (CFU/ml)) using equation 4 (Table 5.1). The model for oxygen consumption includes the maximum oxygen consumption rate and biomass produce during the lag phase and variables growth rate (μ) and biomass produced (C_X), and the amount of oxygen needed to produce the biomass ($Y_{O/X}$) as parameter (Equation 2a and b, Table 5.1). The fraction of oxygen used for ergosterol production (F_E) was fitted using the growth rate (μ), biomass (C_X), yield of oxygen consumed per Cmol biomass ($Y_{O/X}$) yield of ergosterol per mole oxygen ($Y_{E/O}$) (Equation 3b, Table 5.1).

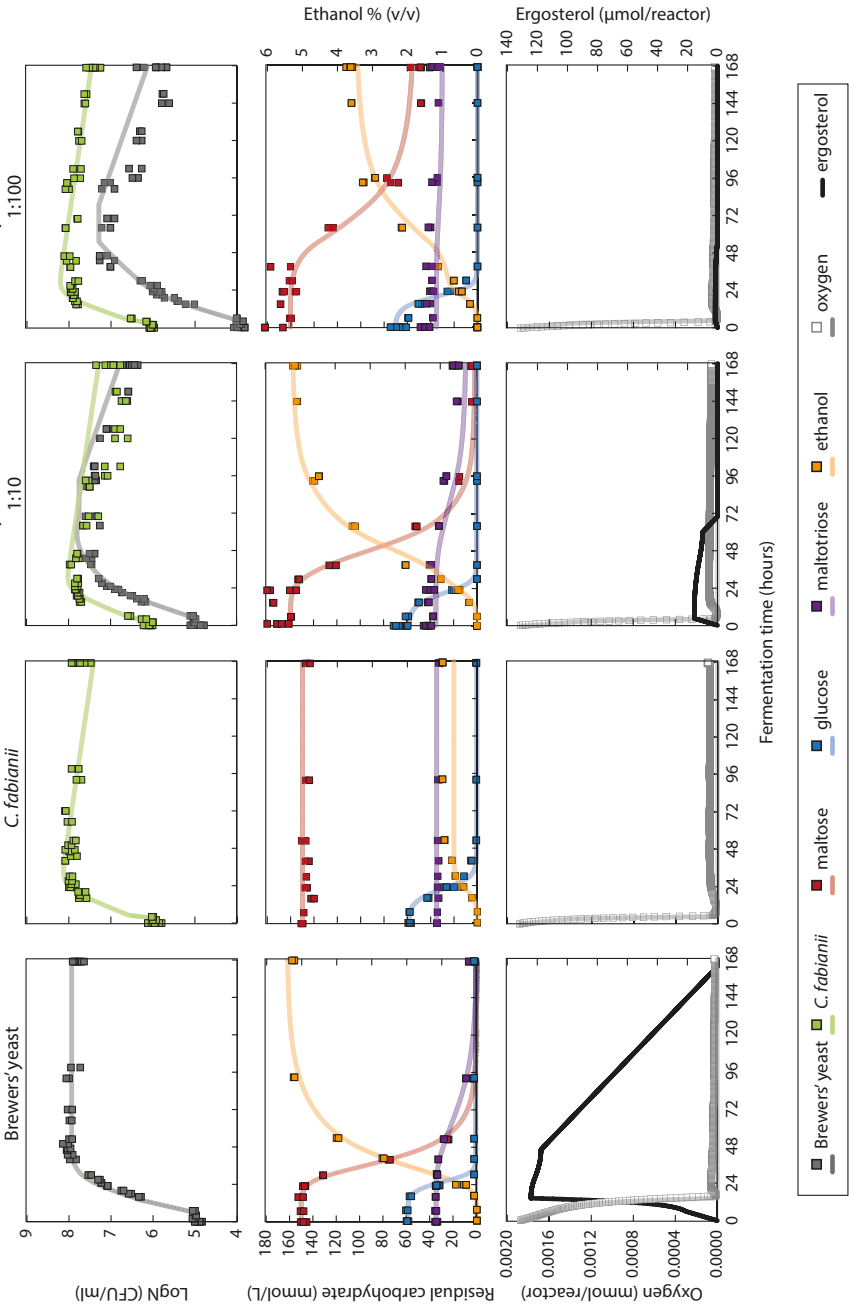


Figure 5.2: Visualization of experimental data (dots) and model (line) of mono- and co-cultivations of brewers' yeast and *C. fabianii*. On the top, the amount of viable cells is expressed in log (CFU/ml) over time (grey (brewers' yeast) and green (*C. fabianii*) dots and lines). In the middle the residual sugars (red (maltose), blue (glucose) and purple (maltotriose) dots and lines) and ethanol percentage (orange dots and line) are shown over time. The bottom graphs show the oxygen (grey dots and line) over time and model for ergosterol production and consumption by brewers' yeast (black line).

The carbohydrate concentrations can be described using the biomass (C_X) and consumption rate for each substrate (Equation 5, Table 5.1). This consumption rate is described by the carbohydrate concentration (C_G for glucose, C_M for maltose and C_R for maltotriose), the affinity constant ($K_{m,G}$ for glucose, $K_{m,M}$ for maltose and $K_{m,R}$ for maltotriose) and the maximal consumption rate for the appropriate carbohydrate (q_G^{max} for glucose, q_M^{max} for maltose and q_R^{max} for maltotriose). For maltose, an inhibition factor for glucose is included in the model and for maltotriose, inhibition factors for both glucose and maltose are included (catabolite repression) (Equation 7 and 8, Table 5.1). The use of intracellularly stored ergosterol is described by the growth rate (μ), biomass (C_X) and the moles ergosterol needed to produce the biomass ($Y_{E/X}$) (Equation 3c, Table 5.1). When the cells reach stationary phase, ergosterol is used for cell membrane synthesis and maintenance for membrane integrity which is expressed in a constant ergosterol consumption rate (R_E) (Equation 3d, Table 5.1) (Klug and Daum, 2014). Once ergosterol is depleted, the specific death rate (K_d) results in a decline in cell numbers (Equation 1e, Table 5.1). Under these circumstances ergosterol is not depleted after 7 days of fermentation, thus no decline in cell numbers is predicted, which is in line with the experimental data (Figure 5.2). All parameter settings are fitted to the experimental data of brewers' yeast in mono-cultivation or taken from literature (Table 5.2).

C. fabianii mono-cultivation on wort

Identical wort composition and bioreactor settings were used for *C. fabianii* as for brewers' yeast mono-cultivation. The wort was pitched with 10^6 CFU/ml *C. fabianii* and fermented for 7 days at ambient temperature (20 °C). *C. fabianii* grows up to ~10^8 CFU/ml, quickly consumes the oxygen (in 5.5 hours), can only ferment the carbohydrate glucose and therefore only 0.67 % (v/v) alcohol was produced (Figure 5.2).

The model to describe the behaviour of *C. fabianii* in mono-cultivation on wort is a simplified version of the model of brewers' yeast, since it can only grow and ferment glucose and utilize maltose when oxygen is available. No ergosterol production, storage and depletion is taken into account. The parameters and variables used to describe the growth rate for *C. fabianii* are; maximum growth rate (μ), concentration of oxygen (C_O), affinity for oxygen (K_O), glucose consumption rate (q_G), yield ATP per Cmol substrate ($Y_{ATP/S}$), yield ATP per biomass ($Y_{ATP/X}$) and the maintenance factor (m_{ATP}) (Equation 1 b and c, Table 5.1). Growth (LogN) of *C. fabianii* was described using equation 11 (Table 5.1). The oxygen consumption during the lag phase described by the maximum oxygen consumption rate (q_O^{max}) and biomass (C_X) (Equation 10a, Table 5.1). In the aerobic growth phase, the oxygen consumption is described using the growth rate (μ), biomass (C_X) and the amount of oxygen needed for biomass production ($Y_{O/X}$) (Equation 10b, Table 5.1).

Table 5.1: Equations used to describe the model of brewers' yeast and *C. fabianii*

Brewers' yeast	(a) Lag	(b) Aerobic growth	(c) Anaerobic growth	(d) Stationary	(e) Death
(1) μ	0	$\mu_{max} * \frac{C_O}{C_O + K_O}$	$\frac{(q_G + q_M + q_R) * Y_{ATP/S} - m_{ATP}}{Y_{ATP/X}}$	0	$-K_d$
(2) $\frac{dC_O}{dt}$	$-q_O^{max} * C_X$	$-\mu * C_X * Y_{O/X}$	0	0	0
(3) $\frac{dC_E}{dt}$	0	$\frac{\mu * C_X * Y_{O/X} * F_E}{Y_{O/E}}$	$-\mu * C_X * Y_{E/X}$	$-R_E$	0
(4) $\frac{dC_N}{dt}$	$\mu * C_N$ and $C_X = C_N * Y_{X/N}$				
(5) $\frac{dC_i}{dt}$ with i=G, M or R	$-q_i * C_X$				
(6) q_G	$q_G^{max} * \frac{C_G}{C_G + K_{m,G}}$				
(7) q_M	$q_M^{max} * \frac{C_M}{C_M + K_{m,M}} * \left(\frac{1}{1 + \frac{C_G}{K_{i,G}}}\right)$				
(8) q_R	$q_R^{max} * \frac{C_R}{C_R + K_{m,R}} * \left(\frac{1}{1 + \frac{C_G}{K_{i,G}}}\right) * \left(\frac{1}{1 + \frac{C_M}{K_{i,M}}}\right)$				

C. fabianii	(a) Lag	(b) Aerobic growth	(c) Anaerobic growth	(d) Stationary	(e) Death
(9) μ	0	$\mu_{max} * \frac{C_O}{C_O + K_O}$	$\frac{q_G * Y_{ATP/S} - m_{ATP}}{Y_{ATP/X}}$	0	0
(10) $\frac{dC_O}{dt}$	$-q_O^{max} * C_X$	$-\mu * C_X * Y_{O/X}$	0	0	0
(11) $\frac{dC_X}{dt}$	$\mu * C_X$ and $C_X = C_N * Y_{X/N}$				
(12) $\frac{dC_G}{dt}$	$-q_G * C_X$				
(13) q_G	$q_G^{max} * \frac{C_G}{C_G + K_{m,G}}$				

The substrate consumption rate is fitted using the maximum consumption rate (), substrate concentration (C_G for glucose) and the affinity constant (K_m) (Equation 13, Table 5.1). Since *C. fabianii* cannot ferment maltose and maltotriose, those concentrations remain constant and do not affect the growth rate. Once glucose is depleted, cells use ATP for maintenance of the cell and is fitted by the factor m_{ATP}, resulting in a decline in cell numbers which is in line with the experimental data. The ethanol production is calculated by the substrate consumption (2 moles of ethanol per mole glucose). All parameter settings are fitted to the experimental data of *C. fabianii* in mono-cultivation or taken from literature (Table 5.2).

Model of brewers' yeast and *C. fabianii* co-culture on wort

The models for mono-cultivations of brewers' yeast and *C. fabianii* were combined to predict the outcome of two inoculation ratios; 1:10 and 1:100 brewers' yeast over *C. fabianii* using the same conditions as set for the mono-cultivations (n=4) and samples were taken during the fermentation (Figure 5.2). All dynamics of cell growth, ethanol production and substrate and oxygen consumption were surprisingly well predicted by using the model for both inoculation ratios. However, the fraction of oxygen used for ergosterol production (F_E) and the death rate (K_d) were fitted to the experimental data of the co-cultivations, since it was not possible to fit these parameters to the experimental data of brewers' yeast in mono-cultivation.

The model predicts maltose and glucose depletion for the ratio 1:10 within 7 days of fermentation and for the ratio 1:100 only glucose depletion is predicted within this timeframe. The amount of ethanol produced depends on the amount of carbohydrates consumed, therefore lower amounts of alcohol were predicted in the ratios 1:10 and 1:100 compared to brewers' yeast in mono-cultivation. Most oxygen is quickly consumed by *C. fabianii* and only a limited amount of oxygen is consumed by brewers' yeast. Thereby, insufficient levels of ergosterol are produced by brewers' yeast resulting in growth inhibition and limited fermentation providing a final product with lower alcohol levels.

Using this model, the outcome of 7 days of fermentation inoculated with decreasing ratios of brewers' yeast over *C. fabianii* was predicted (Figure 5.3). It is clear that starting from a ratio of 1:10 brewers' yeast and *C. fabianii* resulted in significant inhibition of brewers' yeast performance resulting in higher levels of residual sugars and lower alcohol levels. Next to these product characteristics, the aroma formation is of major importance for the final product characteristics.

Aroma formation

HS-SPME GC-MS was used to determine the impact of co-cultivations with inoculum ratio 1:10 and 1:100 brewers' yeast to *C. fabianii* on aroma formation compared to mono-cultivations. Significant differences in volatile organic compounds (VOCs) profiles were observed (Figure

5.4). The sum of peak areas is clearly higher for both co-cultivations compared to both mono-cultivations (Figure 5.4, pie charts). Especially, the marked compounds represented by ethyl 9-hexanodecanoate, ethyl decanoate, ethyl dodecanoate, ethyl hexanoate, ethyl pentadecanoate, propyl decanoate, propyl octanoate, 2-methylpropyl hexanoate, 3-methylbutyl octanoate, 3-methylbutyl pentadecanoate and decanoic acid (indicated with ←) are found in higher levels in co-cultivation compared to the mono-cultivations (Figure 5.4). Further information on the secondary metabolite production and cross-interactions is needed in order to model and predict the aroma formation in co-cultivations.

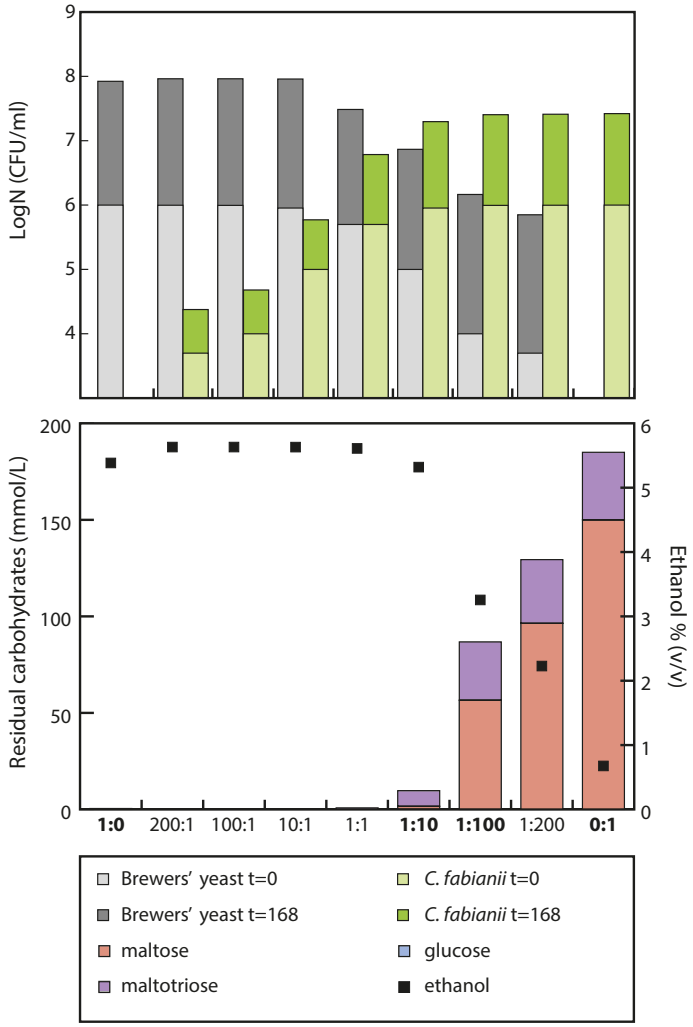


Figure 5.3: Predicted outcome after 7 days of fermentation with various inoculum ratios of brewers' yeast (grey) over *C. fabianii* (green). The graph on the top displays the inoculum (light grey/green) and the predicted final number of cells after 7 days of fermentation (dark grey/green). The graph on the bottom displays the predicted ethanol % (v/v) (black square) and residual sugars (maltose (red), maltotriose (purple) and glucose (blue)) after 7 days of fermentation. The ratios 1:0, 1:10, 1:100 and 0:1 are experimentally validated (Figure 5.2).

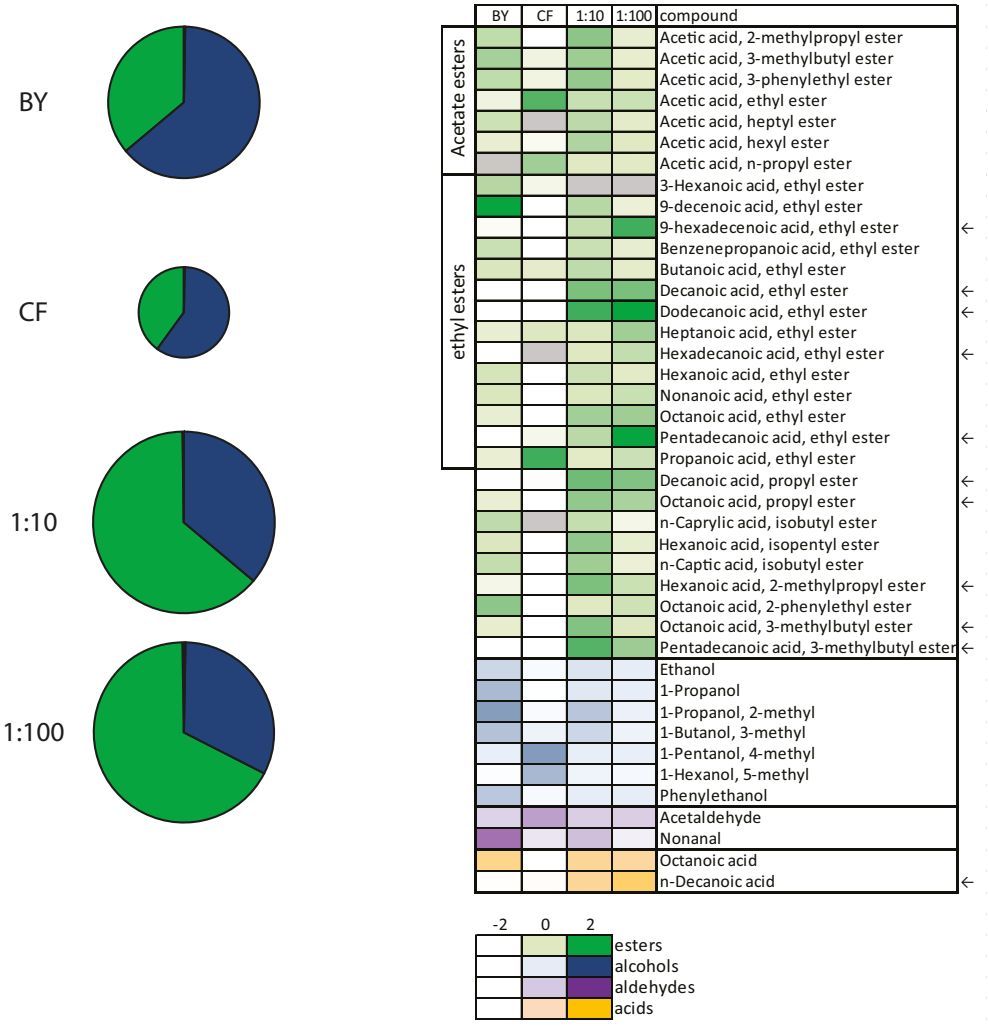


Figure 5.4: Volatile organic compounds of brewers' yeast (BY) and *C. fabianii* (CF) in mono-cultivation and in co-cultivation in ratio 1:10 (1:10) and 1:100 (1:100). The pie charts on the left represent the total sum of all peak areas where the compound groups are indicated by colour (esters (green), alcohols (blue), aldehydes (purple) and acids (orange)). On the right the peak areas are normalized per compound (log2(compound/median compound all samples) to indicate differences in intensity between the variants. A colour gradient is used to indicate the intensity of the compound (0= median, -2 = below median and 2=above median);). The compounds are grouped as esters (green), alcohols (blue), aldehydes (purple) and acids (orange). Grey means not detected. Arrows indicate compounds found in higher levels in co-cultivation compared to the mono-cultivations.

Table 5.2: parameters used in the models

symbol	Explanation	BY	Cf	Reference
$Y_{O/X}$	Mol oxygen needed per Cmol biomass (mol/Cmol)	1	4	Fitted
$Y_{G/X}$	Mol glucose needed per Cmol biomass (mol/Cmol)	0.74	0.74	Fitted
$Y_{E/X}$	mole ergosterol needed per Cmol biomass (mol/Cmol)	0.000113	/	(27)
$Y_{X/N}$	Cmol biomass per yeast cell (Cmol/cell)	1.71E-12	4.57E-13	Determined, (Dry weight/MW_x)
MW_X	Molecular weight of biomass (g/Cmol) using $CH_{1.748}N_{0.148}O_{0.596}P_{0.09}S_{0.0019}M_{0.018}$	26.4	26.4	(31)
$Y_{O/E}$	Mole oxygen needed per mol ergosterol (mol/mol)	0.083	/	(16)
$Y_{ATP/S(resp)}$	Yield ATP per Cmol substrate (ATP/Cmol) (respiration). 16 ATP from 6 Cmole substrate.	2.67	2.67	(32)
$Y_{ATP/S(ferm)}$	Yield ATP per Cmol substrate (ATP/Cmol) (fermentation). 2 ATP from 6 Cmol substrate.	0.33	0.33	Assumed
$Y_{ATP/X}$	ATP needed per Cmol biomass (ATP/Cmol)	1.97	2.29	Calculated ($Y_{ATP/S(resp)}$ / $Y_{X/S}$)
$Y_{X/S}$	Cmol biomass per Cmol substrate (Cmol/Cmol)	1.35	1.16	Fitted
$Y_{EtOH/G}$ $Y_{EtOH/M}$ $Y_{EtOH/R}$	Mol ethanol per mol substrate (mol/mol)	2 4 6	2 4 6	
t_{lag}	Time of lag phase (hours)	6.29	3.98	Fitted
m_{ATP}	Mol ATP needed per Cmol biomass per hour for maintenance (mol $Cmol^{-1}$ h^{-1})	0.124	0.029	Fitted
$K_{m,O}$	Concentration of oxygen where μ_{resp} is half the μ_{max} (mol/L)	0.0004	0.0001	Fitted
$K_{m,G}$	Concentration of glucose where q_G is half the (mol/L)	1.5	1	Fitted
$K_{m,M}$	Concentration of maltose where q_M is half the (mol/L)	15	/	Fitted
$K_{m,R}$	Concentration of maltotriose where q_R is half the (mol/L)	1	/	Fitted
$K_{i,G}$	Inhibition factor by glucose (mol/L)	0.6	/	Fitted
$K_{i,M}$	Inhibition factor by maltose (mol/L)	0.3	/	Fitted
q_G^{max}	Maximum specific consumption rate of glucose (Cmol $Cmol^{-1}$ h^{-1})	7	6	Fitted
q_M^{max}	Maximum specific consumption rate of maltose (Cmol $Cmol^{-1}$ h^{-1})	8	/	Fitted
q_R^{max}	Maximum specific consumption rate of maltotriose (Cmol $Cmol^{-1}$ h^{-1})	0.3	/	Fitted
q_O^{max}	Specific consumption rate of oxygen during the lagphase (mol $Cmol^{-1}$ h^{-1})	0.727	1.066	Fitted
R_E	Rate of ergosterol degradation when no growth and oxygen is present (mol h^{-1} L^{-1}))	$2*10^{-7}$	/	Fitted
μ_{max}	Maximum specific growth rate (h^{-1})	0.9	1.0	Fitted
K_d	Specific death rate when ergosterol is depleted for more than 24 hours (h^{-1})	0.03	/	Fitted
F_E	Fraction of oxygen used for ergosterol production	0.8	/	Fitted
V_l	Liquid volume in the bioreactor (L)	0.45	0.45	Measured

Discussion

The use of dynamic modelling to predict the outcome of a fermentation process and to describe the interaction between multiple organisms strengthens hypotheses made based on experimental data. In this study, a model was developed based on yeast performance in mono-cultivations taking growth, carbohydrate utilization, maintenance, oxygen consumption and ergosterol production/depletion (only for brewers' yeast) into account (Table 5.1). Parameters were fitted to the experimental data or obtained from literature as indicated in Table 5.2. Finally, mono-culture models of both brewers' yeast and *C. fabianii* were combined and the fraction of oxygen needed for ergosterol production and death rate were fitted to the experimental data to describe the interactions in co-cultivation.

It is well known that brewers' yeast is able to ferment all wort carbohydrates (glucose, maltose and maltotriose). As a result of catabolite repression, first glucose, then maltose and finally maltotriose is utilized, which is also confirmed by the experimental data displayed in Figure 5.2. The oxygen consumption was fitted based on the known amount of oxygen in the bioreactor at t=0 and the approximate time of oxygen depletion, measured using a dissolved oxygen probe. The yield of ergosterol per mole oxygen was based on literature (0.083 mole ergosterol per mole oxygen (Snoek and Steensma, 2007)), whereas the fraction of oxygen consumed used for ergosterol production was fitted based on the experimental data. Once oxygen was depleted, the use of ergosterol was based on the yield of biomass per mole ergosterol ($1.13*10^{-4}$ mole ergosterol per Cmol biomass) as described by Rosenfeld and Beauvoit (2003). It was assumed that brewers' yeast uses ergosterol for maintenance of the cell membrane functionality after carbohydrate depletion (Daum, et al., 1998). The amount of oxygen present in the wort at the start of fermentation supports sufficient ergosterol production for anaerobic growth by brewers' yeast in mono-cultivation, therefore experimental data of the two co-cultivations were needed to fit the fraction of oxygen used for ergosterol production, since it was hypothesised that ergosterol was limiting as a result of competition for oxygen with *C. fabianii* (Smid and Lacroix, 2013). For brewers' yeast in co-cultivation, a decline in cell numbers was observed 24 hours after ergosterol depletion, possibly caused by insufficient maintenance of the cell membrane due to ergosterol depletion (Daum, et al., 1998). This has been incorporated in the model by introducing the specific death rate (K_d) (Maier, 2009), that signifies the decline in cell numbers starting 24 hours after depletion of ergosterol.

Using the model described in this paper, it is now possible to predict the outcome of various other brewers' yeast and *C. fabianii* inoculation ratios such as used in van Rijswijck, et al. (2017) (Figure 5.3). Indeed, a similar trend was observed, but slightly different yields of ethanol and residual sugars were obtained. This is conceivably caused by a different experimental set-up, where van Rijswijck, et al. (2017) used culture flasks (with different volumes of liquid (V_l) and headspace (V_g)) whereas the model is based on data obtained from

controlled bioreactors used in the current study (see materials and methods). The amount of oxygen at the start of fermentation is an important determinant of the fermentation outcome of the co-cultivation. The model describes the competition for oxygen, where *C. fabianii* has an advantage over brewers' yeast due to a higher specific oxygen consumption rate (1.066 and 0.727 h^{-1}, respectively). Reduced oxygen consumption by brewers' yeast will result in less ergosterol production and therefore an inhibition of fermentation performance (biomass production and maintenance). The model can be adapted for the use of other yeast species and for different fermentation and headspace volumes (oxygen availability) to predict and optimize the fermentation outcome (provided that no other than the described inhibitory microbial interactions occur).

The pre-culture conditions used consist of two steps; the first pre-culture incubation is performed aerobically for 1 day, where the second pre-culture incubation is performed static for 3 days. During the static incubation brewers' yeast will conceivably consume all ergosterol produced in the first pre-cultivation. In the model it is assumed that the cells used for inoculation do not have intracellularly stored ergosterol (at t=0, C_E=0). If both first and second pre-culture incubations are performed aerobically it is conceivable that sufficient intracellularly stored ergosterol is available to complete the wort fermentation without the need for oxygen. Since this parameter is an important determinant in the fermentation outcome, the intracellular ergosterol concentration at the start of fermentation (t=0 C_E>0) can be included in the model.

Obviously, modelling of production of secondary metabolites in mono- cultivation and in co-cultivation is challenging and more information is needed about the enzymatic/ chemical reactions especially concerning metabolic interactions during co-cultivation. In the current study we investigated the volatile organic compounds produced in mono- and co-cultivations (Figure 5.4). The sum of the peak areas of all esters produced by brewers' yeast and *C. fabianii* in mono-cultivation is smaller than the sum of peak area of all esters found in the tested co-cultivations (brewers' yeast + *C. fabianii* = 1.9 * 10^7, ratio 1:10 = 3.4 * 10^7 and ratio 1:100 = 3.6 * 10^7). This indicates that in co-cultivation additional reactions take place by which esters are formed at higher levels (Figure 5.4, marked with ←). Interestingly, among those compounds no acetate esters are found, but mainly medium chain fatty acid (MCFA) esters such as esters from decanoic acid and octanoic acid. These MCFAs can act as inhibitory compounds due to intracellular acidification, resulting in a sluggish fermentation (Legras, et al., 2010; Viegas, et al., 1989). It is suggested that esterification of these acids is a way for detoxification i.e. maintenance of intracellular pH homeostasis (Legras, et al., 2010). Obviously, more insight is required in the extra- and intracellular enzymatic and chemical reactions in such co-cultivations to enable adequate prediction of the fermentation outcome concerning the production of secondary metabolites.

In conclusion, our dynamic modelling approach predicts co-culture fermentation performance of different doses of brewers' yeast over *C. fabianii* resulting in lower alcohol beer with a more complex aroma bouquet in the co-cultures with a high *C. fabianii* inoculum. The use of predictive modelling delivered support for our hypothesis on the interaction between brewers' yeast and *C. fabianii* in co-cultivation and shows that brewers' yeast fermentation performance in mono- and co-cultivation can be predicted based on the availability of oxygen, carbohydrates and intracellularly stored ergosterol. According the model, the addition of a fast oxygen consumer will result in less oxygen consumption by brewers' yeast, thus less ergosterol production and an inhibition of the fermentation performance. At the same time, *C. fabianii* contributes positively to the aroma formation, which masks the unfermented wort flavour. Notably, the described approach can also be used to predict the fermentation outcome of co-cultivations using other selected yeasts. Additionally, the outcome can be fine-tuned by changing the amounts and doses of the inoculation, the amount of oxygen present at the start of fermentation or by adding ergosterol or its precursors to the wort. Our dynamic modelling approach may further support the implementation of co-cultivations as a novel approach for fermented product innovations.

Acknowledgements

This research was financially supported by the Graduate School VLAG (Wageningen University & Research, The Netherlands) and HEINEKEN Supply Chain B.V. (Zoeterwoude, The Netherlands). The authors would like to acknowledge Jan-Maarten Geertman and Niels Kuijpers from HEINEKEN for sharing their knowledge.

Abbreviations

N = yeast cell (number)
X = biomass (Cmol)
G = glucose (mol)
M = maltose (mol)
R = maltotriose (mol)
O = oxygen (mol)
E = ergosterol (mol)
F = fraction
t = time (hours)
q = specific consumption rate (h^{-1})
m = maintenance (mol Cmol-1 h-1)
Y = yield
MW = molecular weight (g/mol)
Km = half-velocity constant (mol/L)
Ki = inhibition constant (mol/L)
ATP = adenosinetriphosphate
Resp = respiration
Ferm = fermentation
R = rate of degradation (h^{-1})
EtOH = ethanol (mol)

Supplementary materials

Model mono-cultures

$\mu_{(resp)}$ (h^{-1}) (BY/ CF)

- IF: $t < t_{lag}$
  - TRUE: 0
  - FALSE:$(C_{O(mol/L)} * \mu_{max})/ (C_{O(mol/L)} + K_{i,O})$

$\mu_{(ferm)}$ (h^{-1}) (BY)

- IF: t < 24h
  - TRUE: IF $C_E <= 0$
    - TRUE: 0
    - FALSE:IF $C_{O(mol/reactor)} > 0$
      - TRUE:0
      - FALSE: $((q_G + q_M + q_R) * Y_{ATP/S} – m_{ATP} / Y_{ATP/x}$
  - FALSE: IF $C_E <= 0$
    - TRUE:IF $C_{E(t=t-24h)} <=0$
      - TRUE: 0
      - FALSE: $-K_d$
    - FALSE: IF $C_{O(mol/reactor)} > 0$
      - TRUE:0
      - FALSE: $MAX((((q_G + q_M + q_R)* Y_{ATP/S} – m_{ATP} / Y_{ATP/x}),0)$

$\mu_{(ferm)}$ (h^{-1}) (CF)

- IF $C_{O(mol/reactor)} > 0$
  - TRUE: 0
  - FALSE: $(q_G * Y_{ATP/S} – m_{ATP}) / Y_{ATP/X}$

N (cells/ml) (BY/CF)

- IF $C_{O(mol/reactor)} > 0$
  - TRUE: $N_{(t=t-\Delta t)} * \exp(\mu_{(resp,\ t=t-\Delta t)} * \Delta t)$
  - FALSE: $N_{(t=t-\Delta t)} * \exp(\mu_{(ferm,\ t=t-\Delta t)} * \Delta t)$

LogN (log(cells/ml)) (BY/CF)

- $Log_{10}(N)$

C_x (mol/reactor) (BY/CF)

- $N * (V_l * 1000) * Y_{X/N}$

$C_{O(consumed)}$ (mol/reactor) (BY/CF)

- IF $t < t_{lag}$
  - TRUE: $q_O^{max} * C_{X(mol/reactor,\ t=0)} * t$
  - FALSE: IF $logN_{(t=t)} < logN_{(t=t-\Delta t)}$
    - TRUE: $C_{O(consumed,\ mol/reactor,\ t=t-\Delta t)}$
    - FALSE: $t_{lag} * q_O^{max} * C_{X(mol/reactor,\ t=0)} + (C_{X(mol/reactor,\ t=t)} – C_{X(mol/reactor,\ t=0)})*Y_{O/X}$

C_O (mol/reactor) (BY/CF)

- IF $C_{O(mol/reactor,\ t=0)} - C_{O(consumed,\ mol/reactor,\ t=t)} > 0$
  - TRUE: $C_{O(mol/reactor,\ t=0)} - C_{O(consumed,\ mol/reactor,\ t=t)}$
  - FALSE: 0

C_O (mol/L) (BY/CF)

- IF ($C_{O(mol/reactor)}$/450*1000) > Max dO2 in water
  - TRUE: Max dO2 in water
  - FALSE: $C_{O(mol/reactor)}$/450*1000

$C_{O(mol/reactor)}$ > 0 (oxygen for respiration, mol/reactor) (BY/CF)

- IF $C_{O(mol/reactor)} > 0$
  - TRUE
  - FALSE

C_G (mol/reactor) (BY/CF)

- IF $C_{O(mol/reactor)} > 0$
  - TRUE: $C_{G(mol/reactor,\ t=0)} - ((C_{O(consumed,\ mol/reactor,\ t=t)} / Y_{O/X}) * C_{G/X})$
  - FALSE: $C_{G(mol/reactor,\ t=t-\Delta t)} - \Delta t * C_{X(mol/reactor)} * q_{G(t=t-\Delta t)}$

C_G (mol/L) (BY/CF)

- $C_{G(mol/reactor)} / 450 * 1000$

q_G (BY/CF)

- IF $C_{O(mol/reactor)} > 0$
  - TRUE: 0
  - FALSE: $(C_{G(mol/L)} / (C_{G(mol/L)} + K_{m,G})) * q_G^{max}$

C_M (mol/reactor) (BY)

- IF $C_{O(mol/reactor)} > 0$
  - TRUE: $C_{M(mol/reactor,\ t=t-\Delta t)}$
  - FALSE: $C_{M(mol/reactor,\ t=t-\Delta t)} - \Delta t * C_{X(mol/reactor)} * q_{M(t=t-\Delta t)}$

q_M (BY)

- IF $C_{O(mol/reactor)} > 0$
  - TRUE: 0
  - FALSE: $(C_{M(mol/L)} / (C_{M(mol/L)} + K_{m,M})) * (1 / (1 + (C_{G(mol/L)} / K_{i,G}))) * q_M^{max}$

C_R (mol/reactor) (BY)

- IF $C_{O(mol/reactor)} > 0$
  - TRUE: $C_{R(mol/reactor,\ t=t-\Delta t)}$
  - FALSE: $C_{R(mol/reactor,\ t=t-\Delta t)} - \Delta t * C_{X(mol/reactor)} * q_{R(t=t-\Delta t)}$

q_R (BY)

- IF $C_{O(mol/reactor)} > 0$
  - TRUE: 0
  - FALSE: $(C_{R(mol/L)} / (C_{R(mol/L)} + K_{m,R})) * (1 / (1 + (C_{G(mol/L)} / K_{i,G}))) * (1 / (1 + (C_{M(mol/L)} / K_{i,M}))) * q_R^{max}$

C_E (mol/reactor) (BY)

- IF (($C_{O(mol/reactor)}$ > 0) = FALSE) AND ($\mu_{(ferm)}$ <= 0)
  - TRUE: $C_{E(mol/reactor,\ t=t-\Delta t)} - R_E$
  - FALSE: IF $C_{O(mol/reactor)}$ > 0
    - TRUE: $C_{O(consumption)} * F_E * Y_{O/E}$
    - FALSE: IF ((MAX($C_{X(mol/reactor,\ IF\ (CO(mol/reactor) > 0))} - (C_{X(mol/reactor,\ t=t)} - C_{X(mol/reactor,\ t=0)}) * Y_{E/X}$ > 0
      - TRUE: ((MAX($C_{X(mol/reactor,\ IF\ (CO(mol/reactor) > 0))} - (C_{X(mol/reactor,\ t=t)} - C_{X(mol/reactor,\ t=0)}) * Y_{E/X}$)
      - FALSE: 0

C_{EtOH} (mmol/L) (BY/CF)

- IF $C_{O(mol/reactor)}$ > 0
  - TRUE: 0
  - FALSE: (($C_{G(t=0)} - C_{G(t=t)}$) * 2) + (($C_{M(t=0)} - C_{M(t=t)}$)*4) + (($C_{R(t=0)} - C_{R(t=t)}$)*6)

Ethanol % (v/v) (BY/CF)

- ((($C_{EtOH(mmol/L)}$ / 1000) * 46.07) / (0.789 / 1)) /10

Model co-culture

$\mu_{(resp)}$ (h^{-1}) (BY)

- IF: t < $t_{lag(BY)}$
  - TRUE: 0
  - FALSE:($C_{O(mol/L)} * \mu_{max(BY)}$)/ ($C_{O(mol/L)} + K_{i,O(BY)}$)

$\mu_{(resp)}$ (h^{-1}) (CF)

- t < $t_{lag(CF)}$
  - TRUE: 0
  - FALSE:($C_{O(mol/L)} * \mu_{max(CF)}$)/ ($C_{O(mol/L)} + K_{i,O(CF)}$)

$\mu_{(ferm)}$ (h^{-1}) (BY)

- IF: t < 24h
  - TRUE: IF C_E <= 0
    - TRUE: 0
    - FALSE:IF $C_{O(mol/reactor)}$ > 0
      - TRUE:0
      - FALSE: (($q_{G(BY)} + q_{M(BY)} + q_{R(BY)}$) * $Y_{ATP/S(BY)} - m_{ATP(BY)} / Y_{ATP/x(BY)}$
  - FALSE: IF C_E <= 0
    - TRUE:IF $C_{E(t=t-24h)}$ <=0
      - TRUE: 0
      - FALSE: $-K_d$
    - FALSE: IF $C_{O(mol/reactor)}$ > 0

- TRUE:0
- FALSE: MAX((((q$_{G(BY)}$ + q$_{M(BY)}$ + q$_{R(BY)}$)* Y$_{ATP/S(BY)}$ – m$_{ATP(BY)}$ / Y$_{ATP/x(BY)}$),0)

$\mu_{(ferm)}$ (h^{-1}) (CF)

- IF $C_{O(mol/reactor)} > 0$
  - TRUE: 0
  - FALSE: $(q_{G(CF)} * Y_{ATP/S(CF)} – m_{ATP(CF)}) / Y_{ATP/X(CF)}$

N (cells/ml) (BY)

- IF $C_{O(mol/reactor)} > 0$
  - TRUE: $N_{(BY, t=t-\Delta t)} * \exp(\mu_{(resp\ (BY),\ t=t-\Delta t)} * \Delta t)$
  - FALSE: $N_{(BY, t=t-\Delta t)} * \exp(\mu_{(ferm\ (BY),\ t=t-\Delta t)} * \Delta t)$

N (cells/ml) (CF)

- IF $C_{O(mol/reactor)} > 0$
  - TRUE: $N_{(CF, t=t-\Delta t)} * \exp(\mu_{(resp\ (CF),\ t=t-\Delta t)} * \Delta t)$
  - FALSE: $N_{(CF, t=t-\Delta t)} * \exp(\mu_{(ferm\ (CF),\ t=t-\Delta t)} * \Delta t)$

C_x (mol/reactor) (BY)

- $N_{(BY)} * (V_l * 1000) * Y_{X/N(BY)}$

C_x (mol/reactor) (CF)

- $N_{(CF)} * (V_l * 1000) * Y_{X/N(CF)}$

$C_{O(consumed)}$ (mol/reactor) (BY)

- IF $t < t_{lag(BY)}$
  - TRUE: $q_O{}^{max}{}_{(BY)} * C_{X(BY,\ mol/reactor,\ t=0)} * t$
  - FALSE: IF $\log N_{(BY, t=t)} < \log N_{(BY, t=t-\Delta t)}$
    - TRUE: $C_{O(consumed,\ BY,\ mol/reactor,\ t=t-\Delta t)}$
    - FALSE: $t_{lag(BY)} * q_O{}^{max}{}_{(BY)} * C_{X(BY,\ mol/reactor,\ t=0)} + (C_{X(BY,\ mol/reactor,\ t=t)} – C_{X(BY,\ mol/reactor,\ t=0)}) * Y_{O/X(BY)}$

$C_{O(consumed)}$ (mol/reactor) (CF)

- IF $t < t_{lag(CF)}$
  - TRUE: $q_O{}^{max}{}_{(CF)} * C_{X(CF,\ mol/reactor,\ t=0)} * t$
  - FALSE: IF $\log N_{(CF, t=t)} < \log N_{(CF, t=t-\Delta t)}$
    - TRUE: $C_{O(consumed,\ CF,\ mol/reactor,\ t=t-\Delta t)}$
    - FALSE: $t_{lag(CF)} * q_O{}^{max}{}_{(CF)} * C_{X(CF,\ mol/reactor,\ t=0)} + (C_{X(CF,\ mol/reactor,\ t=t)} – C_{X(CF,\ mol/reactor,\ t=0)}) * Y_{O/X(CF)}$

C_O (mol/reactor) (total)

- IF $C_{O(mol/reactor,\ t=0)} – C_{O(consumed,\ BY,\ t=t)} - C_{O(consumed,\ CF,\ t=t)} > 0$
  - TRUE: $C_{O(mol/reactor,\ t=0)} – C_{O(consumed,\ BY,\ t=t)} - C_{O(consumed,\ CF,\ t=t)}$
  - FALSE: 0

C_O (mol/L) (total)

- IF $(C_{O(mol/reactor)}/450*1000) >$ Max dO2 in water

- TRUE: Max dO2 in water
- FALSE: $C_{O(mol/reactor)}$/450*1000

$C_{O(mol/reactor)}$ > 0 (oxygen for respiration, mol/reactor) (total)

- IF $C_{O(mol/reactor)} > 0$
  - TRUE
  - FALSE

q_G (BY)

- IF $C_{O(mol/reactor)} > 0$
  - TRUE: 0
  - FALSE: $(C_{G(mol/L)} / (C_{G(mol/L)} + K_{m,G(BY)})) * q_G^{max}{}_{(BY)}$

q_G (CF)

- IF $C_{O(mol/reactor)} > 0$
  - TRUE: 0
  - FALSE: $(C_{G(mol/L)} / (C_{G(mol/L)} + K_{m,G(CF)})) * q_G^{max}{}_{(CF)}$

q_M (BY)

- IF $C_{O(mol/reactor)} > 0$
  - TRUE: 0
  - FALSE: $(C_{M(mol/L)} / (C_{M(mol/L)} + K_{m,M(BY)})) * (1 / (1 + (C_{G(mol/L)} / K_{i,G(BY)}))) * q_M^{max}{}_{(BY)}$

q_R (BY)

- IF $C_{O(mol/reactor)} > 0$
  - TRUE: 0
  - FALSE: $(C_{R(mol/L)} / (C_{R(mol/L)} + K_{m,R(BY)})) * (1 / (1 + (C_{G(mol/L)} / K_{i,G(BY)}))) * (1 / (1 + (C_{M(mol/L)} / K_{i,M(BY)}))) * q_R^{max}{}_{(BY)}$

C_G (mol/reactor) (total)

- IF $C_{O(mol/reactor)} > 0$
  - TRUE: $C_{G(mol/reactor,\ t=0)} - ((C_{O(consumed,\ BY,\ mol/reactor,\ t=t)} / Y_{O/X(BY)}) * Y_{G/X(BY)}) - ((C_{O(consumed,\ CF,\ mol/reactor,\ t=t)} / Y_{O/X(CF)}) * Y_{G/X(CF)})$
  - FALSE: $C_{G(mol/reactor,\ t=t-\Delta t)} - (\Delta t * C_{X(BY,\ mol/reactor)} * q_{G(BY,t=t-\Delta t)}) - (\Delta t * C_{X(CF,\ mol/reactor)} * q_{G(CF,\ t=t-\Delta t)})$

C_M (mol/reactor) (total)

- IF $C_{O(mol/reactor)} > 0$
  - TRUE: $C_{M(mol/reactor,\ t=t-\Delta t)}$
  - FALSE: $C_{M(mol/reactor,\ t=t-\Delta t)} - (\Delta t * C_{X(BY,\ mol/reactor)} * q_{M(BY,\ t=t-\Delta t)})$

C_R (mol/reactor) (total)

- IF $C_{O(mol/reactor)} > 0$
  - TRUE: $C_{R(mol/reactor,\ t=t-\Delta t)}$
  - FALSE: $C_{R(mol/reactor,\ t=t-\Delta t)} - (\Delta t * C_{X(BY,mol/reactor)} * q_{R(BYt=t-\Delta t)})$

C_{EtOH} (mmol/L) (BY/CF)

- IF $C_{O(mol/reactor)} > 0$

- TRUE: 0
- FALSE: $((C_{G(t=0)} - C_{G(t=t)}) * 2) + ((C_{M(t=0)} - C_{M(t=t)})*4) + ((C_{R(t=0)} - C_{R(t=t)})*6)$

Ethanol % (v/v) (BY/CF)

- $(((C_{EtOH(mmol/L)} / 1000) * 46.07) / (0.789 / 1)) /10$

C_E (mol/reactor) (BY)

- IF $((C_{O(mol/reactor)} > 0)$ = FALSE) AND $(\mu_{(ferm, BY)} <= 0)$
  - TRUE: $C_{E(mol/reactor, t=t-\Delta t)} - R_E$
  - FALSE: IF $C_{O(mol/reactor)} > 0$
    - TRUE: $C_{O(consumption, BY)} * F_E * Y_{O/E}$
    - FALSE: IF $((MAX(C_{X(BY, mol/reactor, IF (CO(mol/reactor) > 0))} - (C_{X(BY, mol/reactor, t=t)} - C_{X(BY, mol/reactor, t=0)}) * Y_{E/X} > 0$
      - TRUE: $((MAX(C_{X(BY, mol/reactor, IF (CO(mol/reactor) > 0))} - (C_{X(BY, mol/reactor, t=t)} - C_{X(BY, mol/reactor, t=0)}) * Y_{E/X})$
      - FALSE: 0

References

Andorrà, I., Berradre, M., Mas, A., Esteve-Zarzoso, B. and Guillamón, J. M. (2012). Effect of mixed culture fermentations on yeast populations and aroma profile. *LWT - Food Science and Technology* **49,** 8-13.

Barbosa, C., Mendes-Faia, A., Lage, P., Mira, N. P. and Mendes-Ferreira, A. (2015). Genomic expression program of *Saccharomyces cerevisiae* along a mixed-culture wine fermentation with *Hanseniaspora guilliermondii*. *Microb Cell Fact* **14,** 1-17.

Ciani, M., Comitini, F., Mannazzu, I. and Domizio, P. (2010). Controlled mixed culture fermentation: a new perspective on the use of non-*Saccharomyces* yeasts in winemaking. *FEMS Yeast Res* **10,** 123-33.

Daum, G., Lees, N. D., Bard, M. and Dickson, R. (1998). Biochemistry, cell biology and molecular biology of lipids of *Saccharomyces cerevisiae*. *Yeast* **14,** 1471-510.

David, M. H. and Kirsop, B. H. (1972). Yeast growth in relation to the dissolved oxygen and sterol content of wort. *J. Inst. Brew.* **79,** 20-5.

El Soda, M., Madkor, S. A. and Tong, P. S. (2000). Adjunct cultures: Recent developments and potential significance to the cheese industry. *Journal of Dairy Science* **83,** 609-19.

Erten, H. and Tanguler, H. (2010). Influence of *Williopsis saturnus* yeasts in combination with *Saccharomyces cerevisiae* on wine fermentation. *Lett Appl Microbiol* **50,** 474-9.

Gamero, A., Wesselink, W. and de Jong, C. (2013). Comparison of the sensitivity of different aroma extraction techniques in combination with gas chromatography-mass spectrometry to detect minor aroma compounds in wine. *J Chromatogr A* **1272,** 1-7.

Gancedo, J. M. (1998). Yeast carbon catabolite repression. *Microbiol Mol Biol Rev* **62,** 334-61.

Kim, D. H., Hong, Y. A. and Park, H. D. (2008). Co-fermentation of grape must by *Issatchenkia orientalis* and *Saccharomyces cerevisiae* reduces the malic acid content in wine. *Biotechnol Lett* **30,** 1633-8.

Klug, L. and Daum, G. (2014). Yeast lipid metabolism at a glance. *FEMS Yeast Res* **14,** 369-88.

Lange, H. C. and Heijnen, J. J. (2001). Statistical reconciliation of the elemental molecular biomass composition of *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering* **75,** 334-44.

Legras, J. L., Erny, C., Le Jeune, C., Lollier, M., Adolphe, Y., Demuyter, C., Delobel, P., Blondin, B. and Karst, F. (2010). Activation of two different resistance mechanisms in *Saccharomyces cerevisiae* upon exposure to octanoic and decanoic Acids. *Applied and Environmental Microbiology* **76,** 7526-35.

Longley, R. P., Dennis, R. R., Heyer, M. S. and Wren, J. J. (1978). Selective *Saccharomyces* media containing ergosterol and tween 80. *J. Inst. Brew.* **84,** 341-5.

Maier, R. M. (2009). Chapter 3 - Bacterial Growth, *Environmental Microbiology (Second Edition)*, Academic Press: San Diego, pp. 37-54.

Medina, K., Boido, E., Dellacassa, E. and Carrau, F. (2012). Growth of non-*Saccharomyces* yeasts affects nutrient availability for *Saccharomyces cerevisiae* during wine fermentation. *Int J Food Microbiol* **157,** 245-50.

Michel, M., Meier-Dörnberg, T., Jacob, F., Methner, F.-J., Wagner, R. S. and Hutzler, M. (2016). Review: Pure non-*Saccharomyces* starter cultures for beer fermentation with a focus on secondary metabolites and practical applications. *Journal of the Institute of Brewing* **122,** 569-87.

Nyanga, L. K., Nout, M. J., Gadaga, T. H., Theelen, B., Boekhout, T. and Zwietering, M. H. (2007). Yeasts and lactic acid bacteria microbiota from masau (*Ziziphus mauritiana*) fruits and their fermented fruit pulp in Zimbabwe. *Int J Food Microbiol* **120,** 159-66.

Phillips, A. W. (1954). Utilization by yeasts of the carbohydrates of wort. *Journal of the institute of brewing* **61,** 122-6.

Renault, P., Coulon, J., de Revel, G., Barbe, J. C. and Bely, M. (2015). Increase of fruity aroma during mixed *T. delbrueckii*/*S. cerevisiae* wine fermentation is linked to specific esters enhancement. *Int J Food Microbiol* **207,** 40-8.

Rosenfeld, E. and Beauvoit, B. (2003). Role of the non-respiratory pathways in the utilization of molecular oxygen by *Saccharomyces cerevisiae*. *Yeast* **20,** 1115-44.

Rosenfeld, E., Beauvoit, B., Blondin, B. and Salmon, J. M. (2003). Oxygen consumption by anaerobic *Saccharomyces cerevisiae* under enological conditions: Effect on fermentation kinetics. *Applied and Environmental Microbiology* **69,** 113-21.

Sadoudi, M., Tourdot-Marechal, R., Rousseaux, S., Steyer, D., Gallardo-Chacon, J. J., Ballester, J., Vichi, S., Guerin-Schneider, R., Caixach, J. and Alexandre, H. (2012). Yeast-yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by single or co-culture of non-*Saccharomyces* and *Saccharomyces* yeasts. *Food Microbiol* **32,** 243-53.

Smid, E. J. and Lacroix, C. (2013). Microbe-microbe interactions in mixed culture food fermentations. *Curr Opin Biotechnol* **24,** 148-54.

Snoek, I. S. and Steensma, Y. H. (2007). Factors involved in anaerobic growth of *Saccharomyces cerevisiae*. *Yeast* **24,** 1-10.

Spus, M., Liu, H., Wels, M., Abee, T. and Smid, E. J. (2017). Isolation and characterization of *Lactobacillus helveticus* DSM 20075 variants with improved autolytic capacity. *Int J Food Microbiol* **241,** 173-80.

Steensels, J., Snoek, T., Meersman, E., Picca Nicolino, M., Aslankoohi, E., Christiaens, J. F., Gemayel, R., Meert, W., New, A. M., Pougach, K., Saels, V., van der Zande, E., Voordeckers, K. and Verstrepen, K. J. (2012). Selecting and generating superior yeasts for the brewing industry. *Cerevisia* **37,** 63-7.

Tristezza, M., Tufariello, M., Capozzi, V., Spano, G., Mita, G. and Grieco, F. (2016). The oenological potential of *Hanseniaspora uvarum* in simultaneous and sequential co-fermentation with *Saccharomyces cerevisiae* for industrial wine production. *Frontiers in Microbiology* **7,** 1-14.

van Rijswijck, I. M. H., Wolkers-Rooijackers, J. C. M., Abee, T. and Smid, E. J. (2017). Performance of non-conventional yeasts in co-culture with Brewers' yeast for steering ethanol and aroma production. *(Accepted) Microbial Biotechnology*.

Vanegas, J. M., Contreras, M. F., Faller, R. and Longo, M. L. (2012). Role of unsaturated lipid and ergosterol in ethanol tolerance of model yeast biomembranes. *Biophys J* **102,** 507-16.

Verduyn, C., Stouthamer, A. H., Scheffers, W. A. and van Dijken, J. P. (1991). A theoretical evaluation of growth yields of yeasts. *Antonie van Leeuwenhoek* **59,** 49-63.

Viegas, C. A., Rosa, M. F., Sá-Correia, I. and Novais, J. M. (1989). Inhibition of yeast growth by octanoic and decanoic acids produced during ethanolic fermentation. *Applied and Environmental Microbiology* **55,** 21-8.

Chapter 6

General discussion

Before the industrial revolution, fermentations occurred naturally by the microbiota present on the raw materials, air microbiota or from a previous batch (back-slopping) resulting in an authentic product which varied from batch to batch. Consumer demands changed towards a consistent product quality. Therefore, the industry started to sterilize the raw materials, to induce the fermentation with a pure culture and controlled parameters to obtain consistent quality. The drawback is that such controlled procedures potentially deliver fermented products with a less complex aroma bouquet and a less authentic character. To overcome this, the dairy industry developed "mixed starter cultures" (multiple strain starter cultures) and "adjunct cultures" (strains with a desired trait (i.e. aroma production) and low fermentation performance) (El Soda, et al., 2000; Spus, et al., 2017). The wine industry recently explored the use of non-conventional yeast to yield a wine with a more complex aroma bouquet (Ciani, et al., 2010; Del Monaco, et al., 2014; Jolly, et al., 2014; Pretorius, 2000). Uncontrolled fermentations, such as the Belgian Lambic fermentation, which is induced by the air microbiota and microbiota present on the raw materials, involve the activities of a wide range of microorganisms including yeast and bacteria (Spitaels, et al., 2014). However, for controlled fermentations, the traditional beer industry is still hesitating to use multiple strain starter cultures. Nevertheless, there is an increasing interest in the use of non-conventional yeast species for beer production and product innovation (Canonico, et al., 2016; Crauwels, et al., 2015; De Francesco, et al., 2015; Gamero, et al., 2016; Michel, et al., 2016a; Michel, et al., 2016b; Saerens and Swiegers, 2014; Saerens and Swiegers, 2016; Schifferdecker, et al., 2014; Steensels, et al., 2015; Steensels and Verstrepen, 2014; Varela, 2016). Some strains have been identified as potential candidates, but unfortunately they were tested only as single strain starter cultures. Therefore strains with poor fermentation characteristics have been neglected, whereas they could still contribute positively to aroma formation in co-cultivation together with brewers' yeast (Chapter 4 and 5).

To evaluate the aroma profiles of various types of beers currently on the market, head space solid phase microextraction gas chromatography mass spectrometry (HS-SPME GC-MS) was used. In Figure 6.1, the sum of all volatile esters are plotted as function of the alcohol content, shows the lack of low alcohol beers which are at the same time high in volatile esters. Nowadays, consumers are more aware of their health and therefore low-alcohol and alcohol-free beers are more trending than ever before. Many attempts have been made to produce a low-alcohol or alcohol-free beer with more fruity flavours, for example by pervaporation (Olmo, et al., 2014). But it is extremely costly and difficult to retain the aroma in such beers. Thus beer innovation is needed to yield beers with a more complex aroma bouquet, high in fruity flavours (esters) and low in alcohol (% v/v). In the scope of this thesis, we will further discuss beer innovation using the natural biodiversity and generation of artificial diversity. Finally, we will emphasise the industrial importance of aroma formation by yeast.

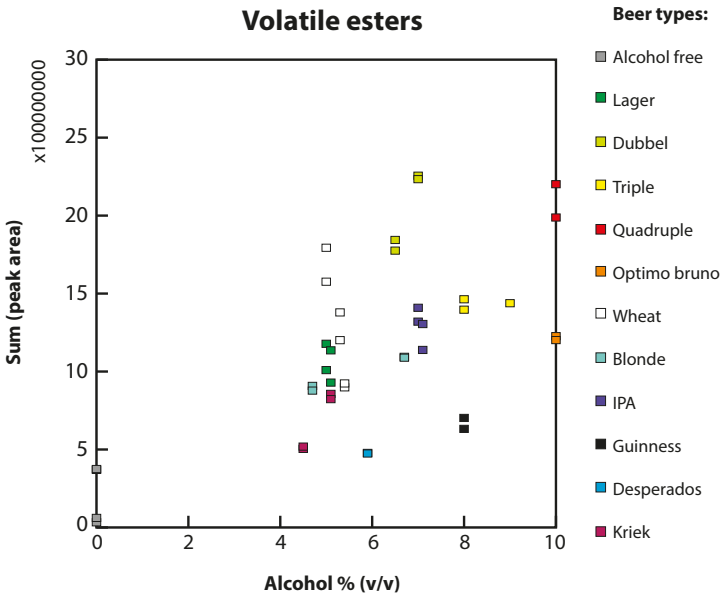


Figure 6.1: Volatile esters of various commercial beers. Sum of peak areas of all esters plotted against the soluble alcohol % (v/v) of the beer.

Exploring natural diversity (non-conventional yeast)

There are about 1500 yeast species currently identified (Kurtzman, et al., 2011) and it is about time to unravel the capacities of these non-conventional yeast species. Some are known for their excellent fermentation performance and others for their desired aroma production. Results discussed in chapter 4 and 5 prove that both yeast types are of use for product innovation. It is demonstrated that the use of co-cultivation of an excellent fermentative yeast (brewers' yeast) with an aroma producing yeast (*Cyberlindnera fabianii*) results in a beer with a more complex aroma bouquet and lower alcohol content (Chapter 4). Additionally, metabolic interactions between the two yeast species were observed, signified by that fact that some esters were at much higher levels following co-cultivation as compared to mono-cultivations. This suggests that esters are formed by reactions between fermentation products of both yeasts, and thereby strengthening the power of co-cultivation (Chapter 4, Figure 4.3). A dynamic model including the crucial parameters which fits both mono-cultivations and co-cultivations (at different inoculum ratios) reveals that the performance of brewers' yeast and *C. fabianii* in co-cultures is governed by competition for oxygen and fermentable substrates and not by direct antagonistic interactions (Chapter 5). Brewers' yeast needs 12 moles of oxygen for the production of 1 mole ergosterol, which is needed for cell membrane synthesis under anaerobic conditions. In co-cultivation, oxygen is

quickly captured by *C. fabianii*, whereby brewers' yeast is only able to produce insufficient amounts of ergosterol to complete the fermentation and therefore lower ethanol levels are found (Figure 6.2). Obviously, less oxygen at the onset of fermentation will also result in incomplete fermentation by brewers' yeast, and only limited aroma compounds will be formed. Discovering and defining these interactions offers interesting opportunities for other co-cultivation approaches with good and poor fermentative yeast strains.

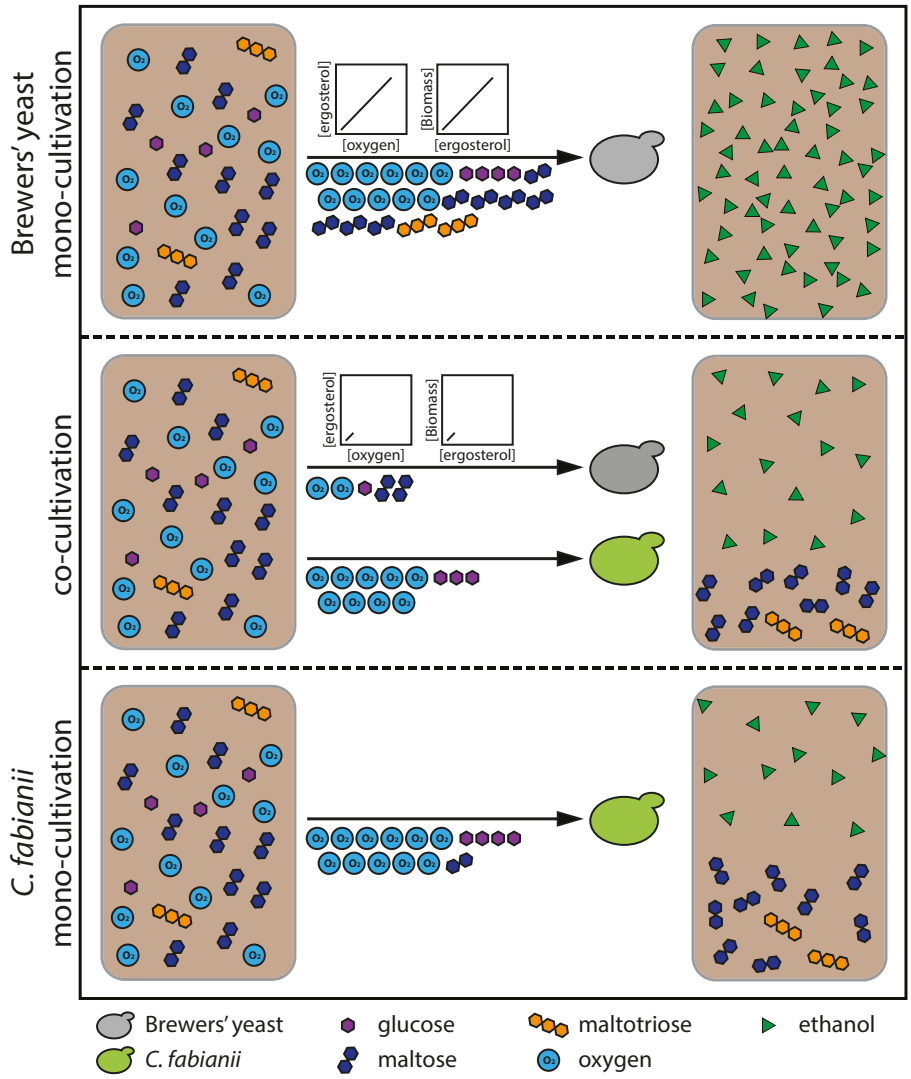


Figure 6.2: Schematic overview of the primary metabolism of brewers' yeast and *C. fabianii* in mono- and co-cultivation on wort. In mono-cultivation sufficient oxygen is available for brewers' yeast to produce ergosterol and to complete fermentation. In co-cultivation, more molecular oxygen is consumed by *C. fabianii* resulting in limited availability of molecular oxygen causing a lack of ergosterol production and reduced fermentation capacity resulting in residual sugars and less ethanol formation.

It should be noted that future application of *C. fabianii* requires standard evaluation of the "Qualified Presumption of Safety (QPS)" and the "Generally Recognized as Safe (GRAS)" status by the European Food Safety Authority or Food and Drug Authority (FDA, United States of America) respectively (EFSA, 2007; EFSA, 2013). Notably, up to now, the requirements to obtain the QPS or GRAS-status for new fermentation starters are unclear. Bourdichon, et al. (2012) have reviewed and updated the inventory of microorganisms used in food fermentations. They used criteria such as; positive contribution of the microorganism to the fermented food and the presence in fermented foods has to be well documented (Bourdichon, et al., 2012). They excluded a microorganism from the list if there is a lack of documentation for any desirable function in the fermentation process, if it is a contaminant and/or does not harbour any relevant metabolic activity or if the species is undesirable in food for scientifically documented reasons (Bourdichon, et al., 2012). Altogether this is ambiguous and by using this approach it is not possible or at least very difficult to introduce new species that have no long history of safe use in fermented foods but are non-pathogenic. Additionally, biodiversity among strains of one species should be considered. It is known that, for instance, the production of undesirable (toxic) biogenic amines is strain dependent and thus should be addressed separately (Spano, et al., 2010).

Requirements of an organism to obtain any label of safe use should be revised and clearly specified. First, two risk levels can be separately evaluated: (i) products containing living microorganisms and (ii) products containing no living microbes. I suggest to subcategorize into these two types of products. The microbes which are used only to produce the product but which are not present or inactive/killed in the final product, should be screened for the production of potentially harmful compounds, such as biogenic amines, methanol, urea and other relevant compounds.

If the microorganism is still present in the end product its survival in the gastrointestinal (GI) tract might be additionally assessed by passages through artificial GI-tract juices and assessment for adhesion to mucus cells. At least there should be a protocol to evaluate if a microbe or its metabolites are of potential risk for the consumer. If not, it can be further explored on laboratory scale and if used in the industry it can be submitted for GRAS or QPS notification. As long as no clear rules are implemented it will limit the opportunities for product innovation using novel non-conventional yeast. These limitations make sure that the industry will consider other ways to obtain strains with altered performance such as the generation of artificial diversity.

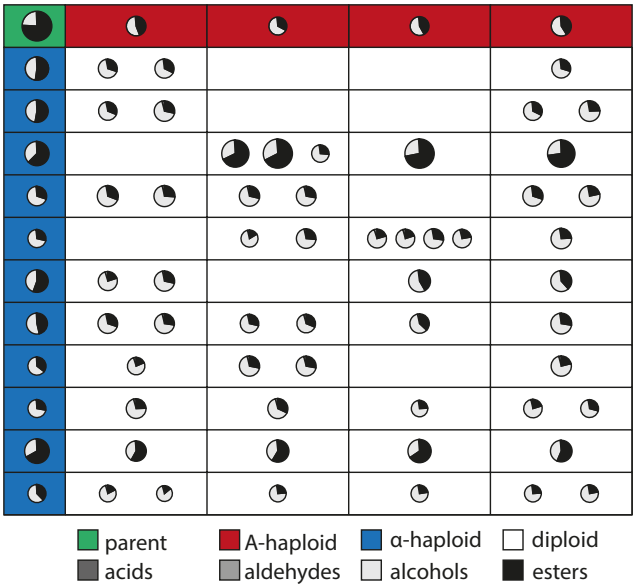


Figure 6.3: Generation of new diploid variants. Haploids (A-mating type (red) and α-mating type (blue)) are obtained by sporulation of the diploid parent (*S. cerevisiae* 131, green). Characterized haploids were mated with opposite mating type and new diploids (white) were obtained (confirmed by PCR). All strains were grown on malt extract broth for 48 hours after which the VOCs profile was measured using HS-SPME GC-MS. The sum of peak area of each compound group (acids, aldehydes, alcohols and esters) are visualised in pie charts (size indicates total amount).

Generation of artificial diversity

The performance of yeast can be altered or optimized by generation of artificial biodiversity. As already described in Chapter 1, various approaches such as, hybridization, mutagenesis and experimental evolution can be used to obtain artificial strain diversity (Figure 1.3). This has been extensively reviewed by Steensels, et al. (2014), and will therefore only be discussed briefly here in the context of beer innovation.

In nature, haploid yeast cells from opposite mating type can fuse (also known as direct mating) to form a diploid cell, this is called sexual hybridization. Of course, this can be steered by selecting haploids harbouring traits of interest. In the agricultural sector this is called selective breeding. However, the phenotypic outcome of the diploid cell is difficult to predict, therefore strong screening criteria are needed (Unpublished data, Figure 6.3). The latter is obviously more straightforward for optimization for growth kinetics than for aroma formation. Various types of sexual hybridization can be used such as (i) direct mating (two haploid cells (α/A)), (ii) rare mating (diploid with haploid cell), (iii) mass mating and (iv) genome shuffling. Often, especially for the generation of interspecific and intergeneric hybrids, the frequencies for sexual hybridization can be extremely low or even zero. In

such cases, it is recommended to use asexual hybridization such as protoplast fusion and cytoduction (Steensels, et al., 2014). Strains obtained via these approaches are in Europe considered as genetically modified (GM) organisms and due to consumers disapproval not used in the food and beverage industry. To circumvent the GM-label, techniques are developed for induced sexual hybridization (Alexander, et al., 2016). Whether such approaches find their way in industrial applications remains to be seen.

Another approach to create artificial diversity is random mutagenesis. This method relies on the exposure of yeasts to specific chemicals or ultraviolet light. However, since the mutations are not targeted, they often result in unexpected and unintended side effects (Sybesma, et al., 2006). High throughput screening methods are needed and good selection criteria to select for the best variant with the desired trait(s).

Higher product yields, faster substrate consumption rates or higher resistance to harsh conditions can be obtained using experimental evolution under selective environmental conditions. Often sequential propagations are applied for many generations to obtain genetically altered yeast cells that show elevated production of the desired metabolite or consume particular substrates at higher rate. One such example is the selection for variants of brewers' yeast based on ethanol tolerance and rapid growth at high osmotic strength resulting in variants that ferment the wort sugars faster (Ekberg, et al., 2013).

In summary, there are various ways to alter the yeast performance, either using non-GM or GM approaches. Implementation of these approaches is obviously affected at various levels. The European Union (EU) and a significant fraction of consumers disapprove GM approaches, although in most cases only targeted mutations have been introduced and therefore the risk of unexpected and unintended side effects is very limited, whereas untargeted mutations by non-GM approaches are likely to result in unexpected, unintended and therefore sometimes unknown side effects (Sybesma, et al., 2006).

Importance of aroma formation

In 2014, around 6500 active breweries were located in Europe, which produced around 383 million hectolitres beer (Europe, 2016). The quality of beer is mainly determined by key elements produced by yeast: (fusel) alcohols, (fusel) aldehydes, esters and vicinal diketones (Pires, et al., 2014). Compounds such as fusel alcohols and esters are perceived as pleasant, whereas vicinal diketones and aldehydes are perceived as unpleasant. The shelf life and quality of beer is mainly determined by the stability of the aroma balance.

The relevance of aroma formation by yeast goes far beyond the importance for beer manufacturing only. It is relevant for all industries involved in the production of (fermented) foods, beverages, flavour and fragrances. Various biotechnological approaches are used to

produce “natural” flavour compounds or aroma blocks. To be able to understand aroma formation by yeast completely, the unravelling of the underlying mechanisms of flavour formation by yeast is of key industrial relevance.

Fusel alcohols, especially tryptophol, tyrosol, isoamyl alcohol and phenylethyl alcohol, are suggested to be involved in quorum sensing (Chen and Fink, 2006; Lorenz, et al., 2000; Zupan, et al., 2013). Under nitrogen limiting conditions *S. cerevisiae* produces more fusel alcohols simultaneously with entry of the stationary phase (liquid media) or invasive growth (solid media) (Chen and Fink, 2006; Zupan, et al., 2013). However, in Chapter 2, no correlation between invasive growth and higher fusel alcohol levels was observed under conditions of nitrogen limited growth.

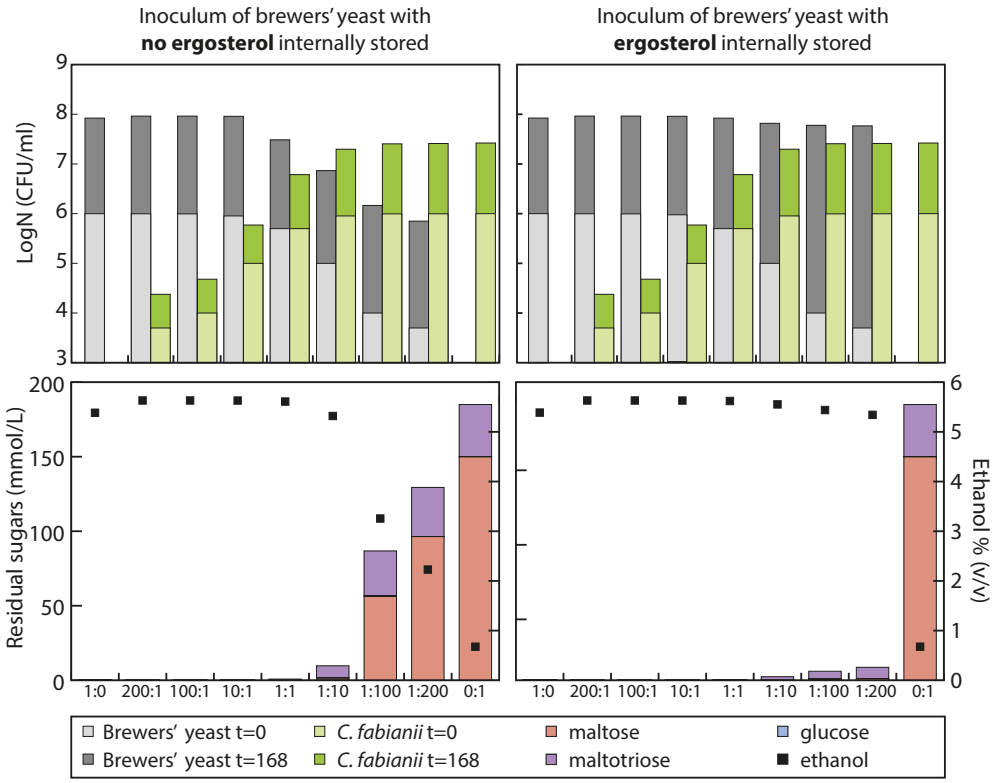


Figure 6.4: The effect of static (left) and aerobic (right) pre-culture conditions on predicted fermentation outcome using various ratios of brewers' yeast over *C. fabianii* as inoculum. The outcome is predicted using the model described in chapter 5, assuming that intracellular ergosterol is stored in aerobic pre-cultured cells, which is not assumed in the static pre-cultured cells. The inoculum and final cell numbers are indicated with light grey (brewers' yeast), light green (*C. fabianii*), dark grey (brewers' yeast) and dark green (*C. fabianii*) bars respectively. Final ethanol levels are displayed with a black square and residual sugar with blue/red/purple bars (glucose, maltose and maltotriose respectively).

Various hypotheses for the biological function of ester production by yeast have been proposed and discussed (Saerens, et al., 2010). First, ester synthesis has been discussed to function as cellular detoxification mechanism to reduce levels of alcohol and/or acids (Nordström, 1964). Secondly, the function of esters as unsaturated fatty acid analogues in the cell membrane to assure optimal membrane fluidity has been proposed (Mason and Dufour, 2000). Thirdly, ester formation is suggested to play a role in the regeneration of CoA (Malcorps and Dufour, 1992). Finally, due to the volatility of esters it has been suggested that yeast produce esters to attract flies and insects to use them as transport vehicle to new sources of nutrients (Christiaens, et al., 2014; Palanca, et al., 2013). None of these hypotheses have been validated yet, but it is also possible that the biological role is a combination of the mentioned factors. Interestingly, to our knowledge, no biological role for ester hydrolysis has been suggested so far.

Conclusions and future perspectives

Work described in this thesis encompasses a broad range of approaches and techniques which has given insight in the distinct aroma formation capacity of *S. cerevisiae* 131, *C. fabianii* 65 and *Pichia kudriavzevii* 129 and their applicability for beer innovation. Where *S. cerevisiae* is the most studied model organism, our research shows that other yeast species such as *C. fabianii* and *P. kudriavzevii* harbour special features that make them interesting candidates to apply in food fermentation processes. It was suggested that fusel alcohols would function as signalling molecules to induce invasive growth in nitrogen limitation. A distinct morphological response for each isolate was observed in nitrogen limited conditions, however, no correlation with fusel alcohol production was found. Unravelling the cell organization of *C. fabianii* and *P. kudriavzevii* grown under nitrogen limitation revealed the power of cell-cell adhesion for penetrative growth and the formation of meta-filaments or pseudo-hyphae to extend the cell surface area. The cells showed clear enhanced substrate penetration by changing growth mode, which may be of relevance for solid-state fermentation processes, such as fruit fermentations. Notably, a significant increase in the production of esters (ethyl acetate, ethyl propanoate, isobutyl acetate and isopentyl acetate) by *C. fabianii* and *P. kudriavzevii* was found under conditions of nitrogen limitation. Further research is needed to reveal the different mechanisms and communication between the cells.

The amount of esters in the final product depends on the levels of the corresponding precursor alcohol(s) and acid(s) steering ester production and hydrolysis of the formed product(s). Ester production depends on the substrate availability (alcohol and ac(et)yl-CoA) and the enzyme activity (alcohol acetyltransferase). Recently, Kruis, et al. (2017) discovered a new elusive alcohol acetyltransferase (EAT) in *Wickerhamomyces anomalus*, *Wickerhamomyces cifferii*, *kluyveromyces marxianus*, *Kluyveromyces lactis*, *Cyberlindnera jadinii*, *C. fabianii*, *Hanseniaspora uvarum*, *Eremothercium cymbalariae* and *S. cerevisiae* responsible for ethyl

acetate production. Proteins of these EAT homologs were produced in *S. cerevisiae* CEN.PK2-1D from the pCUP1 plasmid and analysed using HS-SPME GC-MS and compared. This revealed the product range of the EAT enzymes: ethyl acetate, propyl acetate, isobutyl acetate, isoamyl acetate, phenylethyl acetate and ethyl propanoate (unpublished data, Kruis, van Rijswijck et al.). This experiment has been performed in defined minimal media, whereby a more diverse product range (mainly acetate esters) is expected when cells are grown in complex media such as wort.

Alcohols are produced by alcohol dehydrogenases using NAD(P)H as co-factor. Esters are hydrolysed by esterases. Linking the specific alcohol dehydrogenase and esterase enzyme activities with the corresponding aroma profiles revealed that acetate esterases are of key importance in determining the final levels of acetate esters (Chapter 3). This study revealed very low acetate ester hydrolysing activities for *C. fabianii* which is in line with high ester yields. The assay developed in this study can now be used to screen for strains with low acetate ester hydrolysing activities.

Special features of *C. fabianii* and *P. kudriavzevii*, such as high ester production and distinct aroma formation, makes them interesting candidates for beer innovation. Learning from other industries, we applied these yeast either in mono-cultivation and co-cultivation with brewers' yeast. Co-cultivation revealed most promising findings, showing that it is possible to fine-tune the final product characteristics by varying the ratio of *C. fabianii* over brewers' yeast in the inoculum (Chapter 4, Figure 4.3). Modulation of the parameters responsible for the dose-response relationship revealed that this relationship is a result of inhibition of brewers' yeast due to limited oxygen availability and thus insufficient ergosterol biosynthesis (Chapter 5, Figure 5.2). Ergosterol is necessary for cell membrane synthesis under anaerobic conditions, insufficient amounts of ergosterol prevents brewers' yeast to dominate the co-culture and to finish all fermentable sugars in the wort (Chapter 5, Figure 6.2). *C. fabianii* is able to quickly consume the oxygen present at the early stages of fermentation. Moreover, analysis of the aroma profiles revealed that a synergistic effect results in higher amounts of specific esters in co-cultivations which are not or barely found in both mono-cultivations. Thus, as expected, a more complex aroma bouquet is observed in the co-cultivations compared to the mono-cultivations (Chapter 4 and 5).

It has to be noted, that the pre-culture conditions of brewers' yeast are key for the fermentation outcome of brewers' yeast and *C. fabianii* in co-cultivation. Aerobic pre-cultured cells will have sufficient ergosterol intracellularly stored, whereby brewers' yeast will be able to complete the fermentation in complete anaerobiosis. In chapter 4 and 5 static pre-cultured cells were used, for which we assumed that no ergosterol was intracellularly stored at times of inoculation. Using the model described in Chapter 5, various fermentation parameters (growth, residual sugars and ethanol formation) of both aerobic and static pre-cultured inoculums in various ratios of brewers' yeast over *C. fabianii* is predicted (Figure

6.4). No residual sugars are predicted for various ratios brewers' yeast over *C. fabianii* using aerobic pre-cultured cells (Figure 6.4). However, the effect on the aroma formation cannot be predicted using this model and thus has to be experimentally evaluated.

Further unravelling of the special features of other non-conventional yeast species will undoubtedly lead to more possibilities for beer innovation. Although more research has been published on non-conventional yeast species with good fermentation capacity, we have demonstrated with this study that also the non-conventional yeast species with poor fermentative capacities can be key for beer innovation.

References

Alexander, W. G., Peris, D., Pfannenstiel, B. T., Opulente, D. A., Kuang, M. and Hittinger, C. T. (2016). Efficient engineering of marker-free synthetic allotetraploids of *Saccharomyces*. *Fungal Genet Biol* **89,** 10-7.

Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J. C., Gerds, M. L., Hammes, W. P., Harnett, J., Huys, G., Laulund, S., Ouwehand, A., Powell, I. B., Prajapati, J. B., Seto, Y., Ter Schure, E., Van Boven, A., Vankerckhoven, V., Zgoda, A., Tuijtelaars, S. and Hansen, E. B. (2012). Food fermentations: microorganisms with technological beneficial use. *Int J Food Microbiol* **154,** 87-97.

Canonico, L., Agarbati, A., Comitini, F. and Ciani, M. (2016). *Torulaspora delbrueckii* in the brewing process: A new approach to enhance bioflavour and to reduce ethanol content. *Food Microbiol* **56,** 45-51.

Chen, H. and Fink, G. R. (2006). Feedback control of morphogenesis in fungi by aromatic alcohols. *Genes Dev* **20,** 1150-61.

Christiaens, J. F., Franco, L. M., Cools, T. L., De Meester, L., Michiels, J., Wenseleers, T., Hassan, B. A., Yaksi, E. and Verstrepen, K. J. (2014). The fungal aroma gene ATF1 promotes dispersal of yeast cells through insect vectors. *Cell Rep* **9,** 425-32.

Ciani, M., Comitini, F., Mannazzu, I. and Domizio, P. (2010). Controlled mixed culture fermentation: a new perspective on the use of non-*Saccharomyces* yeasts in winemaking. *FEMS Yeast Res* **10,** 123-33.

Crauwels, S., Steensels, J., Aerts, G., Willems, K. A., Verstrepen, K. J. and Lievens, B. (2015). *Brettanomyces bruxellensis*, essential contributor in spontaneous beer fermentations providing novel opportunities for the Brewing industry. *BrewingScience* **68,** 110-21.

De Francesco, G., Turchetti, B., Sileoni, V., Marconi, O. and Perretti, G. (2015). Screening of new strains of *Saccharomycodes ludwigii* and *Zygosaccharomyces rouxii* to produce low-alcohol beer. *Journal of the Institute of Brewing* **121,** 113-121.

Del Monaco, S. M., Barda, N. B., Rubio, N. C. and Caballero, A. C. (2014). Selection and characterization of a Patagonian *Pichia kudriavzevii* for wine deacidification. *J Appl Microbiol* **117,** 451–64.

EFSA (2007). Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. *The EFSA Journal* **587,** 1-16.

EFSA (2013). Scientific opinion on the maintenanace of the list of QPS biological agent intentionally added to food and feed (2013 update). *EFSA Journal* **11,** 1-106.

Ekberg, J., Rautio, J., Mattinen, L., Vidgren, V., Londesborough, J. and Gibson, B. R. (2013). Adaptive evolution of the lager brewing yeast *Saccharomyces pastorianus* for improved growth under hyperosmotic conditions and its influence on fermentation performance. *FEMS Yeast Research* **13,** 335-49.

El Soda, M., Madkor, S. A. and Tong, P. S. (2000). Adjunct cultures: Recent developments and potential significance to the cheese industry. *Journal of Dairy Science* **83,** 609-19.

Europe, T. B. o. (2016). The contribution made by beer to the European Economy, *EU report*: 2016.

Gamero, A., Quintilla, R., Groenewald, M., Alkema, W., Boekhout, T. and Hazelwood, L. (2016). High-throughput screening of a large collection of non-conventional yeasts reveals their potential for aroma formation in food fermentation. *Food Microbiology* **60,** 147-59.

Jolly, N. P., Varela, C. and Pretorius, I. S. (2014). Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Res* **14,** 215-37.

Kruis, A. J., Levisson, M., Mars, A. E., van der Ploeg, M., Garcés Daza, F., Ellena, V., Kengen, S. W. M., van der Oost, J. and Weusthuis, R. A. (2017). Ethyl acetate production by the elusive alcohol acetyltransferase from yeast. *Metabolic Engineering* **42,** 92-101.

Kurtzman, C. P., Fell, J. W. and Boekhorst, J. (2011). *The Yeasts, a taxonomic study*, Fifth edition edn. Elsevier.

Lorenz, M. C., Cutler, N. S. and Heitman, J. (2000). Characterization of alcohol-induced filamentous growth in *Saccharomyces cerevisiae*. *Mol Biol Cell* **11,** 183-99.

Malcorps, P. and Dufour, J. P. (1992). Short-chain and medium-chain aliphatic-ester synthesis in Saccharomyces cerevisiae. *Eur. J. Biochem* **2010,** 1015-22.

Mason, A. B. and Dufour, J. P. (2000). Alcohol acetyltransferases and the significance of esther synthesis in yeast. *Yeast* **16**, 1287-98.

Michel, M., Kopecka, J., Meier-Dornberg, T., Zarnkow, M., Jacob, F. and Hutzler, M. (2016a). Screening for new brewing yeasts in the non-*Saccharomyces* sector with *Torulaspora delbrueckii* as model. *Yeast* **33,** 129-44.

Michel, M., Meier-Dörnberg, T., Jacob, F., Methner, F.-J., Wagner, R. S. and Hutzler, M. (2016b). Review: Pure non-*Saccharomyces* starter cultures for beer fermentation with a focus on secondary metabolites and practical applications. *Journal of the Institute of Brewing* **122,** 569-87.

Nordström, K. (1964). Formation of esters from acids by Brewers' yeast IV. Effect of higher fatty acids and toxicity of lower fatty acids *Journal of the institute of brewing* **70,** 233-42.

Olmo, Á. d., Blanco, C. A., Palacio, L., Prádanos, P. and Hernández, A. (2014). Pervaporation methodology for improving alcohol-free beer quality through aroma recovery. *Journal of Food Engineering* **133,** 1-8.

Palanca, L., Gaskett, A. C., Günther, C. S., Newcomb, R. D. and Goddard, M. R. (2013). Quantifying Variation in the Ability of Yeasts to Attract *Drosophila melanogaster*. *PLoS One* **8,** e75332.

Pires, E. J., Teixeira, J. A., Branyik, T. and Vicente, A. A. (2014). Yeast: the soul of beer's aroma--a review of flavour-active esters and higher alcohols produced by the brewing yeast. *Appl Microbiol Biotechnol* **98,** 1937-49.

Pretorius, I. S. (2000). Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* **16,** 675-729.

Saerens, S. M., Delvaux, F. R., Verstrepen, K. J. and Thevelein, J. M. (2010). Production and biological function of volatile esters in *Saccharomyces cerevisiae*. *Microb Biotechnol* **3,** 165-77.

Saerens, S. M. and Swiegers, H. (2014). Enhancement of beer flavor by a combination of *Pichia* yeast and different hop varieties: US2014/0234480.

Saerens, S. M. and Swiegers, H. (2016). Production of low-alcohol or alcohol-free beer with *Pichia kluyveri* yeast strains In Chr. Hansen A/S, H. D. (Ed), CHR. Hansen A/S, Hoersholm (DK): US2016/0010042.

Schifferdecker, A. J., Dashko, S., Ishchuk, O. P. and Piskur, J. (2014). The wine and beer yeast *Dekkera bruxellensis*. *Yeast* **31,** 323-32.

Spano, G., Russo, P., Lonvaud-Funel, A., Lucas, P., Alexandre, H., Grandvalet, C., Coton, E., Coton, M., Barnavon, L., Bach, B., Rattray, F., Bunte, A., Magni, C., Ladero, V., Alvarez, M., Fernandez, M., Lopez, P., de Palencia, P. F., Corbi, A., Trip, H. and Lolkema, J. S. (2010). Biogenic amines in fermented foods. *Eur J Clin Nutr* **64 Suppl 3,** 95-100.

Spitaels, F., Wieme, A. D., Janssens, M., Aerts, M., Daniel, H. M., Van Landschoot, A., De Vuyst, L. and Vandamme, P. (2014). The microbial diversity of traditional spontaneously fermented lambic beer. *PLoS One* **9,** e95384.

Spus, M., Liu, H., Wels, M., Abee, T. and Smid, E. J. (2017). Isolation and characterization of *Lactobacillus helveticus* DSM 20075 variants with improved autolytic capacity. *Int J Food Microbiol* **241,** 173-80.

Steensels, J., Daenen, L., Malcorps, P., Derdelinckx, G., Verachtert, H. and Verstrepen, K. J. (2015). *Brettanomyces* yeasts--From spoilage organisms to valuable contributors to industrial fermentations. *Int J Food Microbiol* **206,** 24-38.

Steensels, J., Snoek, T., Meersman, E., Picca Nicolino, M., Voordeckers, K. and Verstrepen, K. J. (2014). Improving industrial yeast strains: exploiting natural and artificial diversity. *FEMS Microbiol Rev* **38,** 947-95.

Steensels, J. and Verstrepen, K. J. (2014). Taming wild yeast: potential of conventional and nonconventional yeasts in industrial fermentations. *Annu Rev Microbiol* **68,** 61-80.

Sybesma, W., Hugenholtz, J., De Vos, W. M. and Smid, E. J. (2006). Safe use of genetically modified lactic acid bacteria in food. Bridging the gap between consumers, green groups, and industry. *Electronic Journal of Biotechnology* **9,** 424-48.

Varela, C. (2016). The impact of non-*Saccharomyces* yeasts in the production of alcoholic beverages. *Appl Microbiol Biotechnol* **100,** 9861-74.

Zupan, J., Avbelj, M., Butinar, B., Kosel, J., Sergan, M. and Raspor, P. (2013). Monitoring of quorum-sensing molecules during minifermentation studies in wine yeast. *J Agric Food Chem***,** 2496-505.

Summary

Yeast are used as workhorses to convert hopped wort into beer. Conventionally, such yeasts belong to the genus *Saccharomyces* and most research on fermentation of wort for the production of beer has focussed on the species *Saccharomyces cerevisiae* and *Saccharomyces pastorianus*. Recently, there is an increasing interest in unravelling features of non-conventional yeast species for beer innovation. In this thesis, features of yeast isolates belonging to the species: *Cyberlindnera fabianii*, *Pichia kudriavzevii* and *S. cerevisiae* (all isolated from fermented masau (*Ziziphus mauritiana*) fruits in Zimbabwe), were studied with focus on aroma production. Additionally, a novel approach was used to apply these yeasts in co-cultivation with brewers' yeast (*S. cerevisiae*) for beer innovation.

The characteristics and quality of the beer are mainly determined by aroma compounds in the final product such as esters, alcohols, aldehydes and acids. Yeast use various metabolic pathways such as glycolysis, the fermentative pathway, the tricarboxylic acid (TCA) cycle and the Ehrlich pathway to produce aroma compounds or the precursors for the synthesis thereof (Chapter 1). Among the aroma compounds, esters are of major importance, especially since they are perceived by the human olfactory system at very low concentrations. In general, esters are desirable compounds in beers due to their fruity flavour. Examples are isoamyl acetate (banana), isobutyl acetate (fruity, sweet), phenylethyl acetate (rose, apple, honey), ethyl acetate (sweet pear), ethyl hexanoate (apple, aniseed) and ethyl octanoate (sour apple). Together with an extensive range of other volatile organic compounds (VOCs) these compounds were previously profiled using headspace solid-phase-micro-extraction gas-chromatography mass-spectrometry (GCMS). Interestingly, comparative profiling of aromas showed that *C. fabianii* produces significantly higher amounts of isoamyl acetate and ethyl acetate compared to *S. cerevisiae*.

It has been suggested in literature that products of the Ehrlich pathway, so called "fusel alcohols", can function as signalling molecules for invasive growth upon nitrogen limitation. This suggested that nutrient limitation could affect growth performance and production of aroma compounds. Therefore, in Chapter 2, the metabolic and morphological response of *S. cerevisiae*, *C. fabianii* and *P. kudriavzevii* was analysed upon nitrogen and/or glucose limitation on semi-solid (agar) media. All three yeasts showed a change in growth mode upon nitrogen and/or glucose limitation. Scanning electron microscopy was used to unravel the cell organisation of *C. fabianii* and *P. kudriavzevii* grown under nitrogen limitation. This revealed the power of cell-cell adhesion for penetrative growth and the formation of meta-filaments or pseudo-hyphae to extend the cell surface area. Such changes in growth mode may be of relevance for solid-state fermentation processes, such as fruit fermentations, by enhanced substrate penetration of yeast. Notably, a significant increase in the production of esters (ethyl acetate, ethyl propanoate, isobutyl acetate and isopentyl acetate) by *C. fabianii* and *P. kudriavzevii* was found under conditions of nitrogen limitation. Understanding the

relationship between nitrogen limitation and ester formation gives more insight into the ability to steer ester formation by nutrient availability in wort fermentations.

The amount and type of esters are important determinants of the final flavour characteristics of the beer. Therefore, the diversity in ester production between the three yeast species was investigated by studying enzymatic reactions involved in the production (synthesis) of esters and their degradation (hydrolysis) and to link this to volatile organic compound profiles (Chapter 3). The amount and type of esters depends on substrate availability and a combination of enzyme activities supporting the synthesis and hydrolysis of the different esters formed. Esters are generally formed by a condensation reaction of an alcohol with ac(et)yl CoA by a so-called alcohol acetyltranferase (AATse). The products formed can generally be subdivided into two groups; acetate esters and medium chain fatty acid (MCFA) esters. Alcohols are formed by reduction of aldehydes by alcohol dehydrogenases (ADH) using either the fermentative or Ehrlich pathway. Comparative analysis of the specific ADHs and acetate ester hydrolysing activity and subsequently linking these data with the distinct aroma profiles of the three yeasts revealed that the acetate ester hydrolysing activity is a key step in determining the final pool size of acetate esters found in the fermentation broths (Chapter 3). Under the experimental conditions, *C. fabianii* showed the lowest acetate ester hydrolysing activity correlating with higher extracellular levels of acetate esters indicating the suitability of this yeast for use in co-cultures with brewers' yeast with the objective to enable the enrichment of acetate esters in the fermentation process (Chapters 4 and 5).

Nowadays, there is large interest of consumers in specialty beers such as beers low in alcohol (health awareness) and/or richer is fruity flavours (specialty beers), and this has significantly stimulated the quest for new methods, practices and yeast strains to produce such beers. In Chapter 4 an innovative approach is described using co-cultivations of brewers' yeast and *C. fabianii* to steer wort fermentation performance. Various ratios of brewers' yeast over *C. fabianii* were inoculated in wort. A dose response relationship was observed, where a higher initial dose of *C. fabianii* leads to lower alcohol production and a more complex aroma bouquet. Interestingly, specific esters, i.e. ethyl acetate (sweet pear), ethyl octanoate (sour apple), ethyl decanoate (waxy, sweet apple), ethyl 9-decenoate (fruity, fatty) and ethyl dodecanoate (fruity, waxy), were found in higher levels in co-cultivation compared to both mono-cultivations indicating metabolic interactions. The reduced ethanol production in the co-culture could be explained by inhibition of brewers' yeast performance by *C. fabianii* in the co-cultivations. Further investigations revealed that this growth inhibition is caused by competition for oxygen between brewers' yeast and *C. fabianii*. Depletion of oxygen caused inhibition of growth of brewers' yeast since it needs oxygen to synthesize ergosterol that is required for cell membrane synthesis under anaerobic conditions (Chapter 4).

The interaction between brewers' yeast and *C. fabianii* in co-cultivation can be described using dynamic modelling (Chapter 5). A dynamic model was developed based on brewers'

yeast and *C. fabianii* in mono-cultivation and fitted to experimental data. The two models were combined and the same parameter settings were used to predict the fermentation outcome of brewers' yeast and *C. fabianii* in co-cultivation. The model was experimentally validated using inoculation ratios of 1:10 and 1:100 brewers' yeast over *C. fabianii*. Additionally, the use of dynamic modelling supported the hypothesis that competition for oxygen between brewers' yeast and *C. fabianii* results in inhibition of brewers' yeast fermentation performance. Interestingly, prediction of aroma formation in co-cultivation, especially that of specific esters, appeared to be more challenging due to metabolic interactions resulting in MCFA-esters contributing to fruity aromas, and this aspect requires further study.

The results and findings obtained in the experimental chapters (Chapter 2-5) are further discussed in Chapter 6. Unravelling features of non-conventional yeast generates novel opportunities for beer innovation. Application of *C. fabianii* in co-cultivation with brewers' yeast in wort fermentation offers a novel approach in product innovation resulting in low alcohol beers with enriched aroma bouquet. Finally, the developed dynamic model may be used to predict fermentation outcomes of brewers' yeast with other non-conventional yeast species.

Acknowledgements

Het werk beschreven in dit proefschrift bevat het bewijs dat twee (verschillende gisten) meer complexiteit kunnen produceren dan één (gist). Alleen, had dit proefschrift dan ook nooit dit niveau bereikt. Graag wil ik hier van de gelegenheid gebruik maken om iedereen te bedanken die, op elk mogelijke manier, zijn/haar bijdrage heeft geleverd aan het bereiken van dit mooie resultaat.

Allereerst wil ik mijn promotoren Eddy en Tjakko heel erg bedanken. Zonder jullie vertrouwen in mij had ik deze kans niet gehad. Eddy, bedankt voor het delen van je kennis en expertise, waardevolle meetings en kritische terugkoppelingen. Ik bewonder je passie voor het vak. Tjakko, jouw explosies van ideeën, maar ook het afbakenen van de kaders van elk hoofdstuk heeft ervoor gezorgd dat de boodschap niet is ondergesneeuwd door ruis. Ook wil ik jullie bedanken voor de snelle en kritische feedback in de laatste fase. Samen waren jullie het perfecte duo dat me heeft geholpen om te groeien op persoonlijk en professioneel vlak. Bedankt voor de 4 fijne jaren en ik hoop dat we nog veel mogen samenwerken in de toekomst.

Graag wil ik Marcel Zwietering bedanken als hoofd van de vakgroep. Bedankt voor het faciliteren van zomer barbecues en labuitjes die een belangrijke bijdrage aan de sfeer op de afdeling leveren. Ook wil ik je enorm bedankt en voor je oog voor detail en kritische terugkoppelingen op Chapter 5. Ik heb met plezier 4 jaar doorgebracht bij FHM.

Ook wil ik graag Graduate School VLAG (Wageningen), HEINEKEN Supply Chain B.V. (Zoeterwoude) en de vakgroep levensmiddelenmicrobiologie (FHM) (Wageningen, The Netherlands) bedanken voor de financiële steun. Daarnaast wil ik, Dr Jan-Maarten Geertman en Dr Niels Kuijpers van HEINEKEN bedanken voor het delen van jullie kennis, enthousiasme en input tijdens onze project meetings. Het gaf telkens weer een boost om verder te gaan.

De GC-MS komt in elk wetenschappelijk hoofdstuk terug. Zonder de hulp van Geert Meijer (PDQ) en Dianne Somhorst (FBR) en uiteindelijk de aanschaf van een eigen GC-MS door FHM was dit waarschijnlijk anders geweest. Geert en Dianne, bedankt voor het delen van jullie kennis, flexibiliteit en hulp in nood (wat nogal eens nodig was). Ook wil ik graag Margaret Bosveld (FCH) bedanken voor alle hulp met betrekking tot de HPLC.

Dr Rob Nout, bedankt en voor het delen van je passie en kennis op het gebied van fermentatie en hulp bij het brouwen. Ik heb ook erg genoten van onze samenwerking in de begeleiding van de Advanced Food Fermentation studenten.

Ik wil graag Dr Jan Dijksterhuis bedankt voor het maken van de mooie Cryo-SEM plaatjes (Chapter 2). Jouw passie en kennis heeft er voor gezorgd dat ook de verborgen informatie uit de foto's werd gehaald.

Genoom sequentie data betekent niks zonder goede BioIT analyse. Daarom wil ik Prof. Dick de Ridder, Martijn Derks en Jetse Jacobi bedanken voor jullie hulp en expertise.

De sfeer op de werkvloer is waarschijnlijk nog belangrijker dan het werk zelf. Daarom wil ik alle collega's van FHM bedanken voor de fijne jaren. Gerda, bedankt voor je hulp bij alle administratieve werkzaamheden en het regelen van alle belangrijke randzaken. Ingrid, bedankt voor het op orde houden van de keuken, studenten en de goede zorgen met betrekking tot de bestellingen en bijhouden van de voorraden. Marcel T., helaas zijn onze leuke experimenten niet in dit proefschrift beschreven, maar desalniettemin bedankt voor het introduceren van de FACS.

Big thanks to my colleague PhD students Oscar, Jeroen, Natalia, Yue, Diah, Maciej, Karin, Hasmik, Alicja, Monica, Evelien, Bernard and James. Thank you for the unforgettable PhD trips, advices and fun at the department. Maciej, thank you for taking care of our lab for the first 2 years. Special thanks to my office buddies Diah, Maciej, Natalia and Alessia. Thanks for the fun, support and introducing me to your cultures. Big hug to you all.

Alex, apparently we worked for 4 years at a similar topic. Too bad we only met in the last few months of my project. However, it definitely gave me a boost through the last stretch of my PhD. I hope that our ongoing collaboration will lead to some interesting results, thanks!

Beide paranimfen hebben een speciale rol gespeeld in de afgelopen 4 jaar. Oscar, het is niet meer dan logisch dat jij aan mijn zijde staat vandaag. We deelden het lab, je stond altijd klaar om te helpen en jouw hulp met het modeleren is van onkenbare waarde. Ik heb ongelofelijk veel van je geleerd, bedankt! Succes met het afronden van je eigen PhD, al ben ik er van overtuigd dat dit helemaal goed komt. Judith, het was vanzelfsprekend dat ook jij naast mij staat vandaag. Vanaf het begin was er meteen een klik. Twee handen op één buik, wat hebben we samen gelachen, plezier gehad en bedankt voor je hulp in het lab en steun in de wat mindere periode. Afscheid ging dan ook met een lach en traan. Zo'n vriendschap blijft, bedankt!

I am very grateful to Prof. Dr Richard van Kranenburg, Prof. Dr Han Wösten, Dr John Morrissey and Dr Lucie Hazelwood for critically reviewing of my thesis. Thank you for accepting the invitation to be part of the PhD committee.

I am glad I got the chance to profit from many extra hands in the lab by supervising a broad diversity of MSc and BSc thesis students. Joshua, Mengyuan, Thomas, Wouter, Ivana, Saskia, Ralph, Line, Liang, Maria, Niccolo and Gerco thank you for your interest in my project. Together with you, it was possible to develop all the methods and results described in this thesis. I learned a lot from your different personalities. Thank you for being part of the team and I wish you all the best in your future career.

Studiegenootjes Ntsiki, Jeroen en Jorg, samen gingen we van het MBO naar het HBO. Samen was het elke dag weer een feest om naar school te gaan. Vrienden door dik en dun. Voor

een master gingen we elk onze eigen weg, Ntsiki koos voor een PhD aan de VU waar Jeroen gezellig bij ons op de vakgroep kwam. Een bijzondere vriendschap, bedankt voor alles.

Mariela, we leerde elkaar kennen bij CSK. In Arnhem, bouwden we een speciale vriendschap op. Je gebruikte je netwerk om mij te helpen aan internationale ervaringen. Dit heeft een grote rol gespeeld in het bereiken van mijn doelen. Ik ben je hier enorm dankbaar voor en ik ben blij dat we nu weer collega's zijn.

I would like to thank all my former colleagues and supervisors (at CSK, NIZO, UC Davis, UCC, Corbion) over the past years. Thanks for sharing your knowledge and I will always use your skills as model. I am lucky I got to know you all. Graag wil ik ook mijn huidige collega's van CSK food enrichment bedanken. Het is fijn om weer terug te zijn en ik ben blij dat ik mij bij CSK verder mag ontwikkelen als onderzoeker.

Natuurlijk kan ik dit dankwoord niet afsluiten zonder mijn (schoon)familie en vrienden enorm te bedanken. Wat ben ik toch een geluksvogel met zulke mooie mensen om mij heen. Inhoudelijk staat dit proefschrift waarschijnlijk ver bij jullie vandaan, maar jullie steun en warmte verdient hier zeker een plekje. De vriendinnen van de middelbare school, iedereen is zijn eigen pad gegaan, maar nog altijd bij elkaar. De loopmaatjes van Orion en in het bijzonder An, Kristel en Kelly bedankt voor de vele mooie (ontspannende) kilometers samen. Bedankt!

Pap, Peter en Jan, jullie zijn een voorbeeld voor iedereen. Ondanks vette pech, laten jullie zien dat geluk en liefde het enige is wat er toe doet, dat je gebruik moet maken van je kunnen en dat opgeven geen optie is als iets niet lukt. Ik had het liever anders gezien, maar het heeft me wel geholpen om alles te relativeren naar wat er echt toe doet. Bedankt!

Pap en mam, bedankt voor de vrijheid die jullie me hebben gegeven. Jullie hebben me altijd gesteund, maar lieten me vrij in keuzes zoals doorstuderen en het uitzoeken van mijn interesses. Dit heeft er voor gezorgd dat ik hier nu sta en de afgelopen 4 jaar met veel plezier aan mijn proefschrift heb gewerkt.

Liefste Ad, ik ben zo blij dat wij samen zijn. Bedankt voor jouw liefde, goede zorgen en humor, maar ook je steun in de mindere periodes. Je zorgt er voor dat ik elke dag met een lach op mijn gezicht naar het werk ga en weer huiswaarts rij. Nergens is het zo fijn als thuis samen met jou. Ik kijk er naar uit om samen nog heel veel avonturen aan te gaan.

About the author

Irma Mathea Hendrina van Rijswijck was born on 21 September 1988 in Venlo (The Netherlands). In 2004, she graduated from secondary school at Bouwens van der Boijecollege in Panningen (The Netherlands). In the same year, she started Laboratory Research at ROC Rijn IJssel in Arnhem (The Netherlands). During her internship at CSK food enrichment, she got her first experiences with fermentation by studying lactic acid bacteria as starter culture for cheese and yoghurt. In 2008, she graduated in three subjects: Microbiology, Life Sciences and Botany. In the same year, she started her Bachelor in Biomedical Laboratory Research at Saxion University of Applied Sciences in Deventer (The Netherlands). She conducted her internship at NIZO food research in Ede (The Netherlands) where she studied the plasmid profiles of various lactic acid bacteria in a complex dairy starter culture. She conducted her BSc Thesis at the Marco Lab of the University of California, Davis (United States of America) where she studied the adaptation of lactic acid bacteria to plant and plant tissue. After her graduation in 2011, she worked for 6 months at CSK food enrichment. Irma got awarded with a VSB fonds scholarship to fund a MSc degree in Food Microbiology at the University College Cork (Ireland), which she started in 2011. She conducted her MSc thesis at the Food Microbiology department where she studied a phage resistance mechanism in lactic acid bacteria. In 2012, she graduated with a First Class Honours Degree and moved back to The Netherlands where she was employed for 5 months at Corbion in Gorinchem. In 2013, she got the opportunity to start her PhD project entitled: ”Co-cultivation of non-conventional yeast with *Saccharomyces cerevisiae* to increase the aroma complexity of fermented beverages” at the Laboratory of Food Microbiology at Wageningen University & Research in Wageningen (The Netherlands). Her work is described in this thesis. Currently, Irma is working as Postdoctoral Researcher at CSK food enrichment in Wageningen (The Netherlands).

List of publications

Erkus, O., de Jager, V. C., Spus, M., van Alen-Boerrigter, I. J., van Rijswijck, I. M. H., Hazelwood, L., Janssen, P. W., van Hijum, S. A., Kleerebezem, M. and Smid, E. J. (2013). Multifactorial diversity sustains microbial community stability. *ISME J* 7, 2126-36.

van Rijswijck, I. M. H., Dijksterhuis, J., Wolkers-Rooijackers, J. C. M., Abee, T. and Smid, E. J. (2015). Nutrient limitation leads to penetrative growth into agar and affects aroma formation in *Pichia fabianii*, *P. kudriavzevii* and *Saccharomyces cerevisiae*. *Yeast* 32, 89-101.

van Rijswijck, I. M. H., Derks, M. F. L., Abee, T., de Ridder, D. and Smid, E. J. (2017a). Genome sequences of *Cyberlindnera fabianii* 65, *Pichia kudriavzevii* 129, and *Saccharomyces cerevisiae* 131, isolated from fermented masau fruits in Zimbabwe. *Genome Announcements* 5, 1-3.

van Rijswijck, I. M. H., Wolkers-Rooijackers, J. C. M., Abee, T. and Smid, E. J. (2017b). Performance of non-conventional yeasts in co-culture with brewers' yeast for steering ethanol and aroma production. *Accepted in Microbial Biotechnology.*

Overview of training activities

Discipline specific activities

Courses

Genetics and physiology of food associated microorganisms	VLAG, Wageningen	2013
BSDL Microbial physiology and fermentation technology	EMBL, TU Delft	2014
Food Fermentation	VLAG, Wageningen	2016

Meetings and conferences

COST Bioflavour meeting, poster presentation	Freising, Germany	2013
COST Bioflavour meeting, oral presentation	Montreux, Switzerland	2014
Kluyvercenter meeting	Delft	2014
KNVM fall meeting, poster presentation	Papendal	2014
SfAM conference, poster presentation	Dublin, Ireland	2015
Belgian brewing conference, poster presentation	Leuven, Belgium	2015
ISSY33, oral presentation, oral presentation	Cork, Ireland	2017

General courses

VLAG PhD week	VLAG, Wageningen	2013
Data management	VLAG, Wageningen	2013
Basic statistics	Biometris, Wageningen	2013
Writing grant proposals	VLAG, Wageningen	2016
Career assessment	VLAG, Wageningen	2016

Optional courses

Preparation of research proposal	WUR, Wageningen	2013
PhD trip 2014 Ireland	FHM, Wageningen	2014
Organizing PhD trip 2014 Ireland	FHM, Wageningen	2014
PhD trip 2017 Italy	FHM, Wageningen	2017
Food Microbiology department seminars	FHM, Wageningen	2013-2017

The research described in this thesis was financially supported by the Graduate School VLAG (Wageningen University & Research, Wageningen, The Netherlands), HEINEKEN Supply Chain B.V. (Zoeterwoude, The Netherlands) and the Laboratory of Food Microbiology (Wageningen University & Research, Wageningen, The Netherlands).

Cover design & layout: Iliana Boshoven-Gkini | www.AgileColor.com
Printed by: Digiforce, Vianen (NL) | www.dfprint.nl