MAKING SENSE OF WASTE:

FERMENTATIVE PRODUCTION OF FLAVOUR AND AROMA COMPOUNDS FROM AGRO-INDUSTRIAL BY-PRODUCTS.

MELODIE A. LINDSAY

A thesis submitted in fulfilment
of the requirements for the degree of
Doctor of Philosophy

School of Biological Sciences

The University of Auckland

2019

School's out forever.

-Alice Cooper

Abstract

The global flavour and fragrance industry is expected to increase in value from $27 billion US to $37 billion US by 2021. This is largely due to consumer preferences for sustainable and ethical natural flavours, and fragrances. This study investigated a biotechnological approach to identify novel and alternative sources of natural flavours and fragrances. This thesis explored the potential fermentation to produce flavour and fragrance compounds from an ethical and abundant substrate – agro-industrial by-products. Nine agro-industrial by-products were investigated and fermented with five food-grade microorganisms. Hundreds of potentially valuable, volatile compounds were produced and identified through a preliminary screen using HS-SPME coupled to GC-MS from 39 fermentations. Several were extracted and quantified including a rose-scented compound − phenylethyl alcohol. This was produced by the yeast *Bretannomyces bruxellensis* on carrot pomace at a yield of 50 mg/kg (w/w) and was selected for further optimisation efforts. Based on two potential microbial pathways to produce phenylethyl alcohol 1) *de novo* from glycolysis and the Shikimate pathway, and 2) bioconversion of phenylalanine in the Ehrlich pathway, five additional yeasts were selected for comparison of production on carrot pomace. An enhanced extraction process allowed yields of 123 mg/kg using *B. bruxellensis*, while extending the fermentation duration resulted in 509 mg/kg (w/w) yield. *Candida* spp. however, were more efficient producers of phenylethyl alcohol, yielding over 1000 mg/kg. Given carrot pomace was deficient in essential nutrients, the food-grade yeast *Candida utilis* was selected for further optimisation trials supplementing carrot pomace with sucrose, yeast-available nitrogen and phosphate. Using factorial design, the key factors affecting phenylethyl alcohol yield were determined. Consequently, high sucrose (10 g/L) gave the highest yield of phenylethyl alcohol, while increasing levels of nitrogen and phosphate were detrimental to production.

In conclusion, *C. utilis* is an efficient producer of phenylethyl alcohol on carrot pomace and after optimisation a yield of 11.14 g/kg (w/w) was achieved. This finding provides a promising method for the *de novo* production of natural phenylethyl alcohol using an abundant and ethical substrate. Future work involves determining optimal sucrose and nitrogen concentrations for fermentation, followed by scale-up efforts to a pilot plant level to examine the commercial feasibility of this novel production method.

Acknowledgements

I would like to express my utmost gratitude to the people and organisations who have supported me throughout this PhD research. Without their contribution, none of this would have been possible.

First, I would like to thank: The Bioresource Processing Alliance, Callaghan Innovation, Onions New Zealand and The University of Auckland for financial support. I would like to thank my supervisors Associate Professor Silas G. Villas- Bôas and Associate Professor David Greenwood for supporting me and allowing me the freedom to pursue both a thesis topic that interested me, and other opportunities along the way throughout this project. Thank you for holding out until the end and seeing me through it all. Thank you also to Ninna Granucci for your support and input that helped get this project off the ground. A heartfelt thank you to Professor Gillian Lewis and Professor Phillip Harris for stepping in at the last minute to see me through to submission. I really appreciate all of your support.

To my colleagues and members of the Metabolomics Lab – past and present, thank you for your support and encouragement throughout this project. I have learned so much and have been privileged to have had the chance to work with all of you. To Elizabeth Mackenzie and Tony Chen, thank you for helping me to set up my initial SPME methods. To Erica Zarate and Saras Green, thank you for helping me to not only conduct my research in your lab, but also for going above and beyond and letting me get hands-on experience with everything GC-MS. I really appreciate your kind words and encouragement throughout this project. To Francesca Casu, Matias Kinzurik and Farhana Pinu, thank you for your support and advice.

To Raphael Bang, words cannot express how grateful I am to have you as a dear friend and colleague. Thank you for your support and encouragement and data processing mastery. Alex Lee and Aqfan Jamaluddin, it has been a tough few years. Thank you for being there for me and always having my back. To all three of you, I couldn't have asked for better to go through this journey with. Alastair, Matt, Pauline and the Obar crew, thank you for everything. You guys are the best.

To Brandon, thank you for being there for me and for all your love and support. To Sam, we did it. Thank you for the years of friendship, support and showing up any time I needed you.

Finally, to my family, I couldn't have done this without your love, support and encouragement. To my Canadian family, I've missed you. Thank you for your love and encouragement. To my parents, thank you for giving me every opportunity to succeed in life and for all your love, advice and support. To my beautiful sisters Ashley and Onalee, and their partners, thank you for everything you have done for me. I really appreciate the love and support you all give.

Contents

CHAPTER I

1 GENERAL INTRODUCTION

1.1 Flavour and aroma compounds and their global significance

Flavour and fragrance ingredients are typically volatile, low molecular weight compounds that confer a characteristic taste and/or odour. As additives, flavour and fragrance ingredients contribute up to 5% of the total cost of many consumer goods and products (*Givaudan Investor presentation*, 2015.; Jani, 2017). Slight subtleties in these additives can be enough to sway consumer choice between almost identical products, and even establish brand loyalty. The global fragrance and flavour industry has an estimated compound annual growth rate of 4-6.4%(Jani, 2017; Market Research Consulting Pvt Ltd, 2018; Sales et al., 2018). This has been explained by increased demand in two key areas: a) bulk additives and b) high-value additives (Jani, 2017). With increasing disposable income, consumer spending on personal care items, ready-to-eat meals, processed foods and beverages has increased the global consumption of bulk flavour and fragrance additives. While increased overall consumption of flavour and fragrance ingredients has seen continuous growth, the value of the industry has skyrocketed in recent years due to the consumer base placing heightened importance on natural, sustainable, organic, ethical, and GMO-free products (Market Research Consulting Pvt Ltd, 2018). This has increased global demand for high-value fragrance and flavour ingredients. In 2017, the global consumption of flavour and fragrance additives exceeded 1.6 million tonnes and the expected impact is to increase the global industry value from $27 billion (USD) in 2016 to $37 billion (USD) by 2021 (*Flavors and Fragrances - Global Market Outlook (2017-2026)*, 2018; Euromonitor International, 2018).

1.2 Flavours and fragrances: Natural vs. synthetic

Natural flavours and fragrances are traditionally sourced from herbs, spices, plants and animals. These are usually in the form of essences, extracts, essential oils, and various other tinctures and preparations. The organoleptic quality and purity as well as the origins of these flavours and fragrances have a large impact on the price a consumer is willing to pay despite

the cost of producing these ingredients being volatile. Global supply is often outstripped by consumer demand, and pricing can be unstable depending on crop harvests, seasonality, adverse weather events, and in some cases – political unrest. In addition, manual labour requirements, energy and purification costs can also increase pricing (Berger, 2007).

Currently, the global supply of many natural fragrances and flavours is insufficient to cater to the volume required per annum e.g. natural vanilla, raspberry and nootkatone. Consequently, the demand for natural flavours and fragrances has created a significant market for synthetic versions of these flavours and fragrances. In response, synthetic alternatives have been developed from cheap and abundant starting materials – often from petrochemical origins, to bridge the gap. While natural flavours and fragrances tend to be complex mixtures of hundreds of compounds that confer a desired sensory profile, their synthetic counterparts are often highly pure formulations containing a single, or small group of compounds that is representative of the overall flavour or fragrance desired (Berger, 2007). This makes the flavour or aroma profile extremely predictable, and provides a reliable year-round supply. For major corporations using large quantities of these ingredients, the cheap cost, and stable supply of synthetic flavour and fragrance ingredients is a significant advantage. However, these often have reduced organoleptic profiles and require product labelling to declare artificial additives. Despite their cheaper price point, manufacturers can be deterred by these labelling constraints, especially when similar products with natural ingredients are available to consumers alongside their product. As a result, there is significant demand for alternative, ethical and non-petrochemical sources of natural flavour and fragrance ingredients.

1.3 Natural flavours and fragrances: Current scope

Increasingly, customers have placed high importance on natural, sustainable, organic, GMO-free and ethical products. As discussed above, natural fragrances and flavours are often

expensive, and in limited supply. Historically, these supply-chain shortcomings have been addressed using synthetic versions of various flavour and fragrance ingredients. However, increasing consumer demand for natural products has created a necessity to investigate alternative sources of natural flavours and fragrances.

1.4 Natural flavours and fragrance definitions

Traditionally, flavours and fragrances were derived exclusively from natural sources. These included various herbs, spices, flowers, oils, resins, extracts and preparations made from plant and animal origins (Berger, 2007). Today, there are two major legal statues that define natural flavours and fragrances which have been set by the FDA and European Union. While they are very similar in defining traditional natural flavours, the guidelines for modern practices of manufacturing natural flavours, including through biotechnological routes are less consistent.

The FDA has set a legal definition for natural flavourings as below:

"The term *natural flavor* or *natural flavoring* means the essential oil, oleoresin, essence or extractive, protein hydrolysate, distillate, or any product of roasting, heating or enzymolysis, which contains the flavoring constituents derived from a spice, fruit or fruit juice, vegetable or vegetable juice, edible yeast, herb, bark, bud, root, leaf or similar plant material, meat, seafood, poultry, eggs, dairy products, or fermentation products thereof, whose significant function in food is flavoring rather than nutritional (U.S. Food and Drug Administration, 2018)."

By contrast, the EU does not have a specific legal definition, rather, a subset of conditions that a natural flavouring additive must meet.

"A 'natural flavouring substance' shall mean a flavouring substance obtained by appropriate physical, enzymatic or microbiological processes from material of vegetable,

animal or microbiological origin either in the raw state or after processing for human consumption by one or more of the traditional food preparation processes listed in Annex II. Natural flavouring substances correspond to substances that are naturally present and have been identified in nature (The European Parliament and the Council of the European Union, 2008)."

A major point of difference is that the FDA regulation encompasses the process by which the natural flavouring is obtained, while the EU regulations are more focused on the final natural product. The biggest implication of this is that flavourings derived from GMOs in the EU can be considered natural, provided they meet the other criteria in the regulations and the final product does not contain any GMO material. By contrast, in the FDA regulations, the same flavourings would not be 'natural' as they were derived from GMOs – a non-natural source.

1.5 Biotechnological production of natural flavours and fragrances

1.5.1 Plant cell culture

Plant cell and tissue culture has an extensive, but relatively unsuccessful history of *de novo* volatiles production. While there have been successes for bioactives, pharmaceuticals and other secondary metabolites, volatile compound production has been challenging (Berger, 2015). The initial draw to producing flavour and fragrance compounds in plant cell lines, tissue culture, calluses or root cell culture is that often, whole plant-derived flavour and aromas are often found in low yields from difficult-to-cultivate plants, and plants native to areas with prohibitive political unrest. The ability to produce such flavour and aroma compounds by culturing plant tissues or cells is appealing in order to provide a stable supply, quality product and consistent pricing. Several authors investigated *Citrus* spp. for the production of citrus oil, or citrus-derived flavour compounds. Across a variety of *Citrus* spp. Reil and Berger found only 5% of the orange-peel yield of essential oil components were produced (1996). Other attempts found that cell-culture produced only a few components of essential oils e.g.

limonene, while others produced no volatiles whatsoever (Gounaris, 2010; Niedz et al., 1997). Cultures of *Fragaria* spp. (strawberry) tissues yielded ethyl butyrate, butyl butyrate and the flavour precursor 1,2-propanediol in low levels, but their production required the supplementation of precursor molecules in the culture media (Hong et al., 1990; Zabetakis and Gramshaw, 1998). Dörnenburg & Knorr showed it was possible to produce vanillin from tissue and cell culture of *Vanilla planifolia*, albeit in very low yield (1996). In addition, the garlic volatile aroma compound alliin was produced using root and shoot callus cultures of *Allium sativa* (Ohsumi et al., 1993). It is notable that most examples of plant cell, tissue and callus culture are dated (pre-2000) − likely because these methods have been relatively unsuccessful for the production of volatile compounds (Gounaris, 2010). Compared to the intact plant, tissue cultures have a much lower yield, or no production of the desired compounds at all while in some cases, different volatiles are produced altogether (Gounaris, 2010). As a result, this line of research has been largely abandoned in favour of more promising biotechnological routes.

1.5.2 Enzyme-assisted flavour and aroma compound production

A long-standing and more robust method for the generation of natural flavours and fragrances is through enzymatic reactions. These are traditionally straightforward, simple, enzyme-mediated bioconversions of a precursor molecule into the compound of interest. Often, the most cost-effective enzyme-assisted methods are single-step reactions with no requirements for co-factors (Cheetham, 2002). Enzymes are typically resistant to many solvents, highly specific and highly selective with regards to the regiospecificity, stereospecificity and chemicospecificity of precursors, and often yield enantiomerically pure products (Nestl et al., 2010). In addition, these reactions usually do not require sterile conditions, are simple to carry out, and are easy to scale-up (Cheetham, 2002; De Carvalho, 2010). Lipases, peptidases, oxioreductases, synthases and glycosidases are some enzyme classes that have a role in biotechnological production of many flavour and aroma compounds (Berger, 2015). One

simple reaction involves the use of glycosidases to release terpene flavour compounds from grape-based precursors (Michlmayr et al., 2012). Another, is the conversion of isoamyl alcohol to the banana flavour isoamyl acetate in hexane, using a carboxylesterase from *Bacillus licheniformis* (Torres et al., 2009). Enzymes can also be used to purify racemic mixtures of flavour and aroma compounds for example, $_{L}$-menthol can be purified from a mixture of $_{DL}$-menthol using lipases (Gatfield et al., 2004). Ruiz-Terán *et al* (2001) demonstrated that the extraction of vanilla flavourants from vanilla pods can be improved with the addition of emulsin – an enzyme active preparation purified from crude extracts of almond meal which is rich in β-glucosidase. While enzyme-assisted production of flavours and aromas can be highly successful, it can be limited by complex reactions requiring multiple enzymes. Benzaldehyde can be produced from amygdalin through a two-step enzymic reaction with β-glucosidase and nitrile lyase (Haisman and Knight, 1967). However, reactions with more steps can quickly become costly and inefficient. Other drawbacks of enzyme mediated reactions are difficulties in recycling enzymes, co-factor requirements, and the cost of isolating and purifying enzymes for use or reuse (Berger, 2015). These challenges remain minor and efforts in the enzyme-assisted production of flavour and aroma compounds have been largely successful. There are constant screening efforts to identify new enzymes to catalyse simple reactions, as well as efforts to improve the activities of known enzymes. Of particular interest, is research into directed evolution to enhance the efficiency of known enzymes and the development of *de novo* enzymes based on existing structures and computer modelling (Bornscheuer et al., 2012; Choi et al., 2015). Industry seeks to increase stability, selectivity, substrate range, productivity and to have less stringent reaction conditions necessary for a given enzyme. However, in this instance it is arguable that for these more complex requirements, a whole-cell system may have more success.

1.5.3 Genetically modified organisms

Genetically modified organisms (GMO) or genetically engineered (GE) microorganisms are made using laboratory techniques that alter their DNA – generally to eliminate or introduce specific genes. They are one of the most promising modern sources for *de novo* synthesis of flavour and aroma compounds and have an unmatched potential to do so from simple carbon sources (Schwab, 2007). Both plants and microorganisms are potential hosts for genetic − or "metabolic engineering", and have huge potential for the generation of flavours and fragrances in three main ways: 1) introduce a new biosynthetic pathway, 2) overproduce existing compounds, and 3) express and purify enzyme biocatalysts (Schwab, 2007). Bacteria such as *Bacillus subtilis* and *Escherichia coli* are good candidates for metabolic engineering as they have a simple physiology and short generation time. They can often secrete a gene product into their growth media which can then be used directly for biocatalysis. However, eukaryotic recombinant proteins are often toxic to bacteria, prone to mis-folding, and bacteria lack the ability to conduct post-translational modifications (Schwab, 2007). As a result, yeast are often used in their place.

Overall, metabolic engineering is attractive for industry as it typically takes less time to develop at a lower cost and can be used to produce specific compounds of interest that already have a known market. One of the best examples of a flavour compound produced in this way is vanillin (Goldsmith et al., 2015). It is the first GMO or 'synthetic biology" food additive and is commercially produced by Evolva (Check Hayden, 2014). *De novo* vanillin production can be initiated in yeasts including *Saccharomyces cerevisiae* and *Schitzosaccharomyces pombe* – both GRAS (generally recognised as safe) microorganisms (Hansen et al., 2009). An example of a GMO that has been used to overexpress a naturally produced compound is *Yarrowia lipolytica* that has been genetically modified and produces over 2 g/L of the rose fragrance phenylethyl alcohol (Celinska et al., 2013). This is highly

competitive with existing producers of the rose-scented compound but while considered a GMO, its commercial acceptance is dubious. A second GM strain of *Y. lipolytica* has been engineered to improve yields of gamma-decalactone – a highly desirable fruity/creamy aroma compound (Groguenin et al., 2004; Waché et al., 2000). *Y. lipolytica* naturally converts ricinoleic acid into gamma-decalactone. However, it also degrades the product. The removal of its lactone-degrading activities lead to a 10-fold increase in the formation of gamma-decalactone (Groguenin et al., 2004; Waché et al., 2000).

An interesting GMO approach to producing valuable terpene compounds involved using *E. coli* and expressing the whole plant terpenoid biosynthetic pathway (Martin et al., 2003; Schwab, 2007). Overproducing terpenes in metabolically engineered plants was too slow a process, and required the addition of expensive precursor molecules. In *E. coli*, one of the biggest issues was low precursor supply which was addressed by incorporating additional genes from *S. cerevisiae* to allow *E. coli* to express the mevalonate isoprenoid pathway (Martin et al., 2003). This conferred the ability to synthesize isoprenoids and thus high levels of the precursor amorphadiene and thus high titres of valuable terpene compounds (Martin et al., 2003).

Regarding consumer acceptance of GMO-derived flavour and aroma additives, in some cases manufacturers are able to avoid strict GMO-labelling constraints if the end product does not contain any of the organism used to manufacture it. However, there are still widespread problems with consumer rejection. In addition, some products are unable to be fully separated from their metabolically engineered makers. While new developments in highly targeted gene-editing such as CRISPR-CAS could revolutionise GM products, pushback from the consumer will ultimately decide the fate of this biotechnological method. As a result, finding “wild-type” microorganisms that do not require metabolic engineering to produce flavour and aroma compounds at a commercially acceptable scale is still highly desirable.

1.5.4 GMO-free microbial production of flavours and fragrances

Today, 50-100 flavours are produced microbially and on a commercial scale (Ralf G. Berger, 2007). These include bulk chemicals such as: citric acid, diacetyl, ethanol, acetic acid, lactic acid and L-glutamic acid, as well as more refined flavourings such as vanillin and 3-hexanol (Berger, 1995; Bruhlmann, Fredi; Blomburg, 2016; Carlson, Ting Liu; Peters, 1997; Duboff, Shirley A.; Kwon, Steven S.; Vadehra, 1992; Mayer, 1958; Muheim, Andreas; Müller, Bruno; Münch, Thomas; Wetli, 1998; Shepard, 1960; Toshinori, Matsui; Yukio, Nishimura; Ichijiro, Ide; Hiroshi, Okada; Iwao, Kameyama; Toshinao, 1962). In addition to manufacturing individual flavour additives through fermentation, microbes and fermentation processes are also used to modify or add flavour to foods and beverages: cheese, wine, beer, kombucha, chocolate, and coffee. These products exemplify the potential of using microorganisms to produce more complex flavour and fragrance ingredients using biotechnological methods. The current scope of biotechnologically produced flavour and fragrance additives in a commercial sense is still in its early stages – especially considering most established processes are for bulk chemical production via fermentation. Most of the commercial methods above utilise microorganisms to facilitate simple conversions from a single precursor or simple substrate to the final product. For example, using Baker's yeast (*Saccharomyces cerevisiae*) to make simple conversions of aldehydes to their corresponding alcohols (Bruhlmann, Fredi; Blomburg, 2016). Or using *Acetobacter* spp. to produce acetic acid from ethyl alcohol (Mayer, 1958).

1.6 Fermentation strategies: Liquid-, submerged-, and solid-state fermentation

There are three main commercial fermentation strategies: liquid-, submerged-, and solid-state fermentation. Liquid state fermentation is defined as a liquid media with no solid particles. Conversely, solid-state fermentation is carried out on solid particles in the absence of free water. Submerged-state fermentation is a variation of liquid-state fermentation where solid particles are suspended in a liquid solution. Each method has various pros and cons and must

be carefully selected to suit the type of microorganism and the substrate used for the fermentation.

1.6.1 Liquid state fermentation

One of the most common fermentation strategies is liquid-state fermentation. This strategy is based on the controlled growth of a microorganism, usually bacteria or yeast with the fermentation proceeding in a liquid medium – that is, with no suspended particulates for example, wine and beer fermentations (Camarasa et al., 2018). On an industrial scale, liquid-state fermentation is one of the longest standing and most common fermentation strategies. This encompasses traditional fermented products such as wine, beer and vinegar as well as industrial production of bulk chemicals such as citric acid, diacetyl and acetic acid (Berovic and Legisa, 2007; Duboff, Shirley A.; Kwon, Steven S.; Vadehra, 1992). As the technology has evolved over hundreds – if not thousands of years, liquid-state fermentation is easily the most advanced industrial fermentation strategy. Robust technologies to monitor and control the progress, and fermentation parameters (pH, temperature, aeration, homogeneity of substrate) often make liquid-state fermentations the preferred fermentation method in industry. There are some drawbacks to liquid-state fermentations, most of which involve cost: 1) high energy cost for heating/cooling/drying large quantities of liquid when required, 2) high water consumption, 3) high cost for infrastructure for transport of liquids, 4) high waste water load, 5) foaming, and 6) some microorganisms – especially filamentous fungi do not thrive or produce desired metabolites in a liquid environment (Pandey and Soccol, 2008; Robinson et al., 2001). Despite these drawbacks, the scalability, automation and control over the fermentation parameters make liquid-state fermentation an industry favourite.

1.6.2 Submerged-state fermentation

Submerged-state fermentation is a variation of liquid-state fermentation where submerged-state fermentation has a higher percentage of insoluble particles suspended in the

liquid media. Unlike liquid-state fermentation, submerged-state fermentation has the added benefit of providing a surface for biofilm formation as well as potentially providing a more complex fermentation substrate (Castilho et al., 2009). This fermentation strategy gained popularity in Western countries in the 1940s with the discovery and mass cultivation of *Penicillium* for the production of the antibiotic penicillin (Pandey, 2003). However, submerged-state fermentation has ancient roots in traditional fermentations, for example sake production in Japan, and pickled vegetables e.g. sauerkraut (Peñas et al., 2017; Viander et al., 2003; Yoshizaki et al., 2012). As with liquid-state fermentation, submerged-state fermentation is highly developed in industrial settings and has robust technology to monitor and control the fermentation processes.

*Note some authors consider a submerged-state fermentation to encompass fungal hyphae growth in a liquid media. For clarity, this thesis classifies this as a liquid-state fermentation based on the initial media being a liquid with no insoluble particles.

1.6.3 Solid-state fermentation

Solid-state fermentation is characterised by the growth of microorganisms on a substrate comprised of solid particles in the absence of free water (Hölker and Lenz, 2005; Pandey, 2003; Robinson et al., 2001). Unlike liquid-state and submerged-state fermentations, solid-state offers a complex growth environment that can closely mimic a more natural environment. (Castilho et al., 2009; Hölker and Lenz, 2005). Most often, it is a controlled fermentation where either bacteria or yeast grow across the surface of the substrate in a biofilm, or in the case of fungi, hyphae can penetrate the substrate (Castilho et al., 2009). Traditional use of solid-state fermentation includes the use of *Aspergillus niger* to produce koji, and *Penicillium roqueforti* to produce blue cheese (Behera and Ray, 2016; Couto and Sanromán, 2006; Soccol et al., 2017). While solid-state fermentations are significantly less developed when it comes to the technology available to monitor and control the fermentation, there are

several biological advantages over liquid- and submerged-state fermentations: 1) lower energy requirement, 2) low water requirement, 3) low initial set-up cost, 4) closely mimics natural environment of many microorganisms, 5) large range of substrates including agro-industrial by-products, and 6) often a relatively higher concentration of the end product (Hölker and Lenz, 2005; Pandey, 2003). However, drawbacks include difficulties with controlling aeration, homogeneity and substrate composition during fermentation, maintaining humidity and moisture content, and monitoring the fermentation. Additionally, the fermentation requires a larger pre-inoculum or seed culture. The infrastructure for solid-state fermentation is not yet developed to the extent of liquid- or submerged-state fermentations so scalability can be an issue. Nevertheless, solid-state fermentation is an actively developing fermentation strategy and has significant potential for the production of secondary metabolites – especially flavour and fragrance ingredients (Robinson et al., 2001).

1.7 Natural flavour and fragrance production using fermentation

Research into fermentative production of natural by-products for the production of flavour and fragrance ingredients is a rapidly growing field. Two major approaches used are: 1) investigating microorganisms and substrates to produce *de novo* or facilitate bioconversions of a substrate into a specific compound of interest or, 2) screening of fermentations to identify potential compounds of interest produced.

1.7.1 Fermentative production of targeted compounds of interest

This first method has been used extensively to convert precursor substrates into a desired fragrance or flavour compounds of interest. Compounds of interest have traditionally been natural compounds that are in short supply, or very expensive e.g. vanillin, raspberry ketone, valencene. There is a plethora of research investigating different microorganisms to facilitate simple conversions of precursors into the desired natural product or screening

microbes for their production of a single compound of interest. For example, methods for the production of natural vanillin are highly sought-after and as such, there is extensive research into converting phenolic precursors such as ferulic acid, cinnamic acid or eugenol using a range of microorganisms including: *Streptomyces halstedii, Haematococcus pluvialis, Escherichia coli, Sporotrichum thermophile, Aspergillus niger*, *Pycnoporus cinnabarinus,* and *Phanerochaete cryosporium* (Brunati et al., 2004; Lesage-Meessen et al., 1996; Motedayen et al., 2013; Sindhwani et al., 2012; Topakas et al., 2003; Torre et al., 2004; Tripathi et al., 2002; Zheng et al., 2007). Another, very expensive natural compound, raspberry ketone has only been produced by one strain of the filamentous fungi – *Nidula niveo-tomentosa* (Boker et al., 2001; Feron et al., 2007). Despite significant interest by the industry, this is the most successful *de novo* fermentation producing raspberry ketone to date. Other successes have only been realised using genetically modified organisms (Beekwilder et al., 2007; Lee et al., 2016). While this potentially complies with the EU regulatory definition of 'natural' it is not accepted by US regulations though this may change in the future. A third highly sought-after compound of interest is (+)-nootkatone. This grapefruit flavoured compound has been a key subject of investigation to potentially produce it via the bioconversion of (+)-valencene from orange oil. Screening attempts have successfully used various microorganisms including: *Mucor* spp., *Chlorella* spp., *Botryosphaeria dothidea*, *Botryodiplodia theobromae, Yarrowia lipolytica, Phanerochaete chrysosporium, Kluyveromyces marxianus, Aspergillus tamarii, Pleutorus sapidus, Pseudomonas putida* and *Bacillus megaterium* to facilitate the conversion with varying success, although none at an industrial scale (Fraatz et al., 2009; Furusawa et al., 2005; Palmerín-Carreño et al., 2015; Sowden et al., 2005). Similarly, other authors have screened libraries of microorganisms for production of specific aroma compounds e.g. phenylethyl alcohol (Chreptowicz et al., 2018; Etschmann et al., 2003). While other similar approaches have screened collections of microorganisms for tolerance to fragrance/flavour precursors e.g.

limonene (Bicas and Pastore, 2007). While screening for a targeted volatile compound has produced some significant results, the second hypothesis generating approach is becoming more widespread.

1.7.2 Untargeted screening for volatile compounds of interest

A second method for identifying potential microbial methods for the production of flavour and fragrance compounds is by screening for potential compounds of interest produced by different microorganisms. For example, the fungus *Lactarius fragilis* or "candy cap" mushroom has a characteristic maple syrup aroma which could be a compound of interest as a "natural" maple syrup flavour (Wood et al., 2012). Wood *et al* identified quabalactone III as the compound conferring a maple syrup aroma which could have potential applications as a natural flavouring (2012). Other authors have analysed the volatile profiles of traditionally fermented foods to identify potential odorants and flavourings that have significance in the overall aroma profile e.g. tempeh, soy sauce, wine and sake (Mei Feng et al., 2007; Styger et al., 2011; Yong and Wood, 1977; Yoshizaki et al., 2012). At its most basic, this method of screening can be considered a hypothesis-generating tool to identify major contributors to the flavour and aroma profile of the fermentation from which one can later determine which compounds may be of commercial significance.

1.7.3 Potential of agro-industrial by-products as a low-cost substrate

Agro-industrial by-products comprise a variety of materials from crop harvest residues and bran, to fruit and vegetable pulps, seeds, stems and skins from the beverage, juicing and "ready-to-eat" processing industries as well as whole, blemished, undersized, oversized and misshapen produce. Until recently, agro-industrial by-products were often disposed of by incineration, composting, reapplied to land and for agricultural purposes or landfill. However, nowadays there are a range of opportunities being explored for alternative uses and value-added products to divert these waste streams or valorise the product before disposal. For example,

extracting valuable compounds such as pectin, fibre, phenolics, antioxidants, as well as for production of bioethanol, citric acid, charcoal and animal feed (Aishvarya et al., 2015; Benítez et al., 2011; Brar et al., 2013; Demyttenaere et al., 2003; Pandey et al., 2000; Vojvodić et al., 2016). These have applications across a wide range of industries including supplements, cosmetics, bakery, petrochemicals and agriculture. Of all the management strategies, one of the most common is for animal feed. However, some agro-industrial by-products are an imbalanced feed and must be carefully blended with other nutrients and minerals in order to become a complete feed product (Granato Villas-Bôas et al., 2002). This can be expensive due to the perishability and abundance of agro-industrial by-products at any given time so often, only a small percentage of the total waste can be utilised in this way. In addition, while some by-products such grain-based residues can be used as animal feed, others are higher in free fermentable sugars which can cause alcoholaemia in ruminants while others like olive cake and grape marc are high in lignin and fibre which can result in low digestibility (Dhillon et al., 2013; Granato Villas-Boâs et al., 2003; Granato Villas-Bôas et al., 2002). These factors, combined with the inherent requirement for animal feed to be extremely cheap in order to remain competitive mean there is scope for further development of high-value products from agro-industrial by-products. Agro-industrial by-products are often high in fermentable sugars, fibre, vitamins and minerals, and often other precursor molecules which support and allow accumulation of desirable flavour compounds (Schrader, 2007). As such, while they are insufficient for animal feed, they can be permissive of microbial growth and fermentation. As a result, fermentation – especially solid-state fermentation presents an opportunity to utilise agro-industrial waste streams for the production of flavour and fragrance ingredients, especially where agro-industrial by-products have no other practical applications (Soares et al., 2000). As agro-industrial by-products are usually food grade substrates, the case for their use in flavour and fragrance additives is strengthened. There is a growing pool of research into

bioconversion of agro-industrial by-products using solid-state or submerged-state fermentations with a variety of different microorganisms. Though none of these have been realised industrially, there are some notable laboratory-scale assemblies for producing several flavour and fragrance ingredients. In this way, it may be possible to add value to some of these agro-industrial waste streams beyond that of animal feed.

1.8 Screening agro-industrial by-products as a substrate for fermentative production of natural flavour and fragrance ingredients

Agro-industrial by-products have been identified as a cheap and abundant substrate with great potential for production of secondary metabolites by a wide range of microorganisms – especially flavour and fragrance ingredients. As they are often high in fermentable sugars, fibre, vitamins and minerals they make good candidates for both submerged- and solid-state fermentations. There is extensive, and diverse research into a wide variety of agro-industrial by-products and fermenting microorganisms especially as the types of waste can vary between countries, regions and even towns.

There are many examples of groups undertaking screening of fermented agro-industrial by-products to identify volatile compounds of interest. Bosse *et al* performed a comprehensive screen using 30 different Basidiomycetes to ferment seven different agro-industrial by-products (2013). They identified 14 volatile compounds of interest including benzaldehyde and several sweet and floral aroma compounds in a submerged-state culture of apple pomace using *Tyromyces chioneus* (Bosse et al., 2013). Christen *et al* performed a screen of volatile compounds produced by *Rhizopus* spp. when fermenting apple pomace as well as cassava bagasse (2000). As with the other groups, they found the overall volatile profiles to be enhanced by the fermentation and were pleasant-smelling. Sugarcane bagasse has been used on multiple occasions with Fadel *et al* discovering a strong coconut aroma was produced in a solid-state fermentation with the filamentous fungi *Trichoderma viride* and Martínez *et al*

found that in combination with sugar beet molasses, *Kluyveromyces marxianus* produces a high percentage of fruity esters (35 % total volatile compounds) (Fadel et al., 2014; Martínez et al., 2017).

While there are many screening experiments where compounds of interest have been identified, there are considerably fewer optimisation experiments where groups have tried to improve production of a given flavour or aroma compound. One highly successful candidate microorganism is *Ceratocystis fimbriata* which has been shown on multiple agro-industrial by-products to produce a pleasant fruity aroma including banana and pineapple aromas. Citrus pulp, cassava bagasse, sugar cane bagasse and coffee husk have all been successfully used to produce the characteristic fruity aromas (Christen et al., 1997; Rossi et al., 2009; Soares et al., 2000). Some have been further optimised by using additional supplementations – including with wheat/soya bran (nitrogen supplementation), glucose or molasses (fermentable sugars supplementation) (Rossi et al., 2009). However, there was no commercial production of these flavour compounds.

More recently, Madrera *et al* (2015) investigated large-scale, solid-state fermentations to produce flavour and aroma compounds. They performed 30 L tank fermentations (30 L) using apple pomace and four yeast strains including *Saccharomyces* spp. and *Hanseniaspora* spp. All four yeasts enhanced the volatile profile of apple pomace with ethyl esters and fatty acid esters effectively (Madrera et al., 2015). Each of the fermentations were considered pleasant-smelling and 132 volatile compounds were identified. However, the most abundant and valuable product they produced on a large-scale was ethanol (Madrera et al., 2015). While not a complex nor valuable natural flavour ingredient, their work highlights the potential for large-scale, solid-state fermentations using agro-industrial waste streams. It is notable that no optimisation trials were reported. As such, a well investigated and optimised fermentation may

have significant potential to be a commercial method for production of microbial-derived flavour and fragrance compounds.

While these works are encouraging, they highlight challenges associated with taking laboratory-scale findings into a larger-scale and industrial setting. As such, we are yet to see any agro-industrial by-product derived flavour and aroma compounds as a mainstay on the commercial market.

1.9 Volatile profiling: Analytical tools for the detection and identification of volatile compounds

1.9.1 Detecting and analysing volatiles: GC-MS

The gold standard and powerhouse of volatile compounds separation, detection and identification is gas chromatography coupled to mass spectrometry (GC-MS). Gas chromatography (GC) is highly valuable for volatile profiling as it enhances the identification power of the coupled mass spectrometer (MS). In its most basic sense, GC comprises a column ranging from 10 m to over 100 m in length with an internal diameter ranging from ~100-500 µm (Johnstone and Herbert, 2002). Different film thickness options and polarities of the stationary phase that line the inside of the column can confer selectivity to various classes of compounds (Johnstone and Herbert, 2002; Villas-Bôas et al., 2005). A carrier gas for example, helium, hydrogen or nitrogen is used to flush analytes through the column which sits inside an oven. This can be programmed with various temperature settings to efficiently and effectively separate volatile compounds prior to their introduction into the MS.

Mass spectrometry measures the mass of electrically charged compounds as a mass to charge ratio (Watson and Sparkman, 2013). A MS comprises four main components: sample introduction system, ion source, mass analyser and detection system. A computer coordinates and facilitates all aspects from sample injection to method programming and the identification of analytes. Once a sample enters the MS, it is ionised using one of a number of ionisation

source options. These can be chemical impact (CI), electrospray ionisation (ESI) or electron impact (EI) ionisation sources, though the most common are ESI which is operated at atmospheric pressure and EI which requires vacuum conditions and is the main GC ionisation source (Johnstone and Herbert, 2002; Watson and Sparkman, 2013). Next, the ions are accelerated and deflected magnetically to separate them in the mass analyser component of the MS. As for ionisation sources, there are several options for mass analysers including quadrupole, quadrupole ion-trap (Q-IT), time-of-flight (Tof), orbitrap, ion mobility spectrometry (IMS), Fourier transform ion cyclotron resonance (FT-ICR) (Villas-Bôas et al., 2005). These all use electrical and magnetic forces to separate ions and differ in their resolution and cost. ToF, FT-ICR and orbitrap have the highest resolution of all the mass analysers (Villas-Bôas et al., 2005). However, this comes at a considerable cost. By contrast, quadrupole have a lower resolution and accuracy but are simpler to operate, robust and much cheaper. Overall, GC-MS is considered the gold standard for analysing volatile compounds with a boiling point of under 300 °C. This is the case for fragrance compounds and most flavour compounds. Therefore, any GC-MS system should be acceptable for the purposes of volatile profiling. However, for sample preparation and introduction to the GC-MS, there are far more options to consider.

1.9.2 Sampling techniques for volatile profiling

Solvent extraction is a relatively selective tool by which subsets of volatile compounds can be extracted into solvents of different polarities. This can be a good tool for targeting known classes of volatile compounds or a specific volatile compound of interest. There are significant additional extraction steps to improve the extraction of specific compounds. However, it is challenging to target a broad range of volatiles. This would require the use of many solvents and multiple extraction steps. While solvent extraction could give a good idea of the main aroma compounds present in a sample, often the most aroma-active compounds

are found in trace amounts (Erbaş and Baydar, 2016). Since these volatile compounds are typically low in concentration, solvent extractions exacerbate the dilute nature of these compounds further. Further drawbacks include that they are notoriously difficult to concentrate as drying steps can easily remove highly volatile and trace compounds. For example, compared to 46 compounds detected in rose petal headspace using headspace solid-phase microextraction (HS-SPME), Erbas and Bayder found just 11-15 volatile components following solvent extraction. However, the main components were represented in the extraction and had a good recovery which was quantifiable. As there are a wide range of solvents, including food-grade options, this can be an ideal method for extracting less-volatile or known flavour and aroma compounds from a sample as an extract product e.g. vanilla extract, as well as for quantification of targeted compounds of interest.

1.9.3 Sorptive techniques

Sorptive techniques such as stir-bar sorptive extractions (SBSE) are a good option for concentrating trace volatiles from a sample. For example, in stir-bar sorptive extractions, a magnetic stir-bar is coated with a thick coating of PDMS (polydimethylsiloxane) and either immersed in liquid sample or suspended in the headspace above it. Volatile compounds typically have an affinity for PDMS or other commercially available stationary phase coatings and are concentrated onto the film. Ideally, an equilibrium is formed between the sample and stir-bar or in the case of headspace extractions, a more complex equilibrium between the sample and the headspace; and the headspace and the stir-bar (Contini and Esti, 2006). In this manner, even trace volatile compounds can be adsorbed onto the stir-bar and analysed (Barba et al., 2017; Caven-Quantrill and Buglass, 2006). The major advantage of this technique is the higher capacity of the stir-bar compared with a 1-2 cm SPME fibre to be discussed in detail further in this chapter (Bicchi et al., 2002). It can also be more reproducible and slightly more reliable for quantitative results compared to HS-SPME due to the larger capacity of the stir-bar

coating (Barba et al., 2017; Ruan et al., 2015). However, for the technique to allow automatic sample loading, a thermal desorption unit would be required and the initial investment of this can be prohibitive compared to SPME which can simply be used in place of a normal syringe in an existing GC-MS set up. In addition, a stir-bar is needed for each sample and these need to be cleaned before reuse for another sample. This can be laborious − especially when there are hundreds of samples to analyse.

Direct thermal desorption is another option for profiling volatiles. In this method, a small amount of sample is directly introduced to a thermal desorption tube and heat applied to increase the volatility of compounds. This technique uses a very small sample, is reproducible and relatively fast. Commercial variants of this include the Gerstel thermal desorption unit and the Chromatoprobe. As with sorbtive techniques, the cost of set-up can be prohibitive as it requires a thermal desorption unit. In addition, the sample is often destroyed so is not recommended for precious samples that require further analyses. Another variant of thermal desorption includes the Snifprobe which is worth mentioning for the purposes of air sampling and headspace sampling in the field. With a Snifprobe, air is physically pumped through a piece of film-coated column in order to highly concentrate trace analytes and must be used in conjunction with a Chromatoprobe or thermal desorption unit. However, it can give up to ten times higher sensitivity than HS-SPME (Poliak et al., 2006).

1.9.4 Distillation

Distillation and steam distillation is a common and traditional method for extracting volatile components – often in the form of essential oils. These can be performed in atmospheric pressure or under vacuum. Steam distillation is used for large scale, industrial extractions to produce essential oils. However, in a laboratory scale it can have some drawbacks as when using a small amount of sample, sensitivity is low, thermolabile analytes can be degraded or hydrolysed and it is a relatively time-consuming process (Zabetakis and

Holden, 1997). Vacuum-assisted distillations can help to prevent degradation of some sensitive compounds however, Erbas and Badar found only 15 components of rose oil could be resolved using a laboratory scale steam-distillation apparatus while 46 could be detected using HS-SPME (2016). As a result, distillation should be reserved for much larger-scale extractions and still has a significant role in industrial extractions for major fragrance and aroma compounds.

1.9.5 Head space solid-phase microextraction

While all the above techniques have their merits, headspace (HS) solid-phase microextraction (SPME) remains by far the most utilised technique for volatile profiling. Advantages include: minimal sample preparation and quantity, relatively high throughput of samples, solvent-free extractions, automatable and often no-sample destruction (Barba et al., 2017). There are many examples of SPME and headspace SPME used to complete volatile profiling on food and beverage samples including: honey, wine, beer, spirits, fruits, berries and juices (Alissandrakis et al., 2007; Cacho et al., 2015; Caldeira et al., 2007; de Lourdes Cardeal et al., 2005; Demyttenaere et al., 2003; Ducki et al., 2008; Gamero et al., 2013; González-Mas et al., 2011; Howard et al., 2005; Li et al., 2008; Pellati et al., 2005; Rodrigues et al., 2008; Silva et al., 2008). However, there are also more unusual samples that HS-SPME has been used for to detect, compare or profile volatile compounds emitted from: textiles, hair, microorganisms, mechanically damaged walnuts, giant pandas and insects (Chen, 2017; Dong et al., 2015; Gherghel, S., Morgan, R. M., Arrebola-Liébanas, J., Romero-González, R., Blackman, C. S., Garrido-Frenich, A., & Parkin, 2018; San Román et al., 2015; Sporkert, F.; Pragst, 2000; Wilson et al., 2018; Zhu et al., 2009). Provided the sample can be sufficiently exposed to a SPME fibre, HS-SPME coupled to GC-MS can be used to capture and analyse the volatiles captured within the fibre. Commercially available fibre compositions can dictate the range of volatile compounds adsorbed while fibres also have a much lower capacity compared to SBSE which can decrease sensitivity (López et al., 2006). A number of sample

treatments including the adsorption duration and temperature, agitation settings as well as sample pre-treatment steps such as salt additions, incubation temperatures and duration can be optimised to suit the type of sample or targeted volatile compounds. Currently, HS-SPME is by far the current gold-standard for volatile profiling of a wide variety of volatile compounds. However, the main drawback is that it is not quantitative. As different volatile compounds have different affinities to SPME fibres, and do not necessarily adsorb onto the fibres in the same ratios as they are present in the sample. Fibre coating saturation can result in competition between analytes and result in a misrepresentative profile of volatiles (Contini and Esti, 2006). For this reason, it is very difficult to use SPME quantitatively – even with the use of internal standards as there is no way to know how the standard interacts or otherwise interferes with the adsorption of other analytes in a mixed sample. In addition, different fibre compositions can be selective for specific groups of compounds. In this instance, some compounds may appear to have a higher abundance than others purely based on their affinity to the chosen fibre. Therefore, for quantitative analyses, other techniques such as those listed above must be used in conjunction with SPME.

1.9.6 GC-olfactometry

GC-Olfactometry is an important tool to mention. It does not identify aromatic compounds but can be modified to split flow from the GC to both the MS as well as to an olfactometry port for a user to physically smell compounds as they are eluted. GC-olfactometry is highly subjective and depends on the user and their perception of a given aroma compound as it is separated from its mixture of other analytes in the GC. That said, GC-olfactometry is good for identifying potential new aromatic compounds – like the onion-aroma 3-Mercapto-2-methylpentan-1-ol (Widder et al., 2000). While new aromas are constantly sought after, it can be difficult to identify a market for these compounds, especially as further safety and toxicology assessments must be made. When dealing with novel aroma compounds or

potentially hazardous volatiles, the risk may not be worthwhile. There are still a wide variety of expensive and sought-after natural compounds that have value in discovering new or alternative methods of manufacture (Check Hayden, 2014).

1.10 Microorganisms for fermentation

1.10.1 Aspergillus niger and Aspergillus oryzae

Aspergillus is a group of spore forming fungi generally considered asexual, although a few sexual forms have been identified. The asexual spores serve as an efficient mechanism for their dissemination and are the primary inoculum of the species. They are ubiquitous in nature and widely distributed, likely because of their ability to colonise a wide range of substrates (Pel et al., 2007; Yong and Wood, 1977). In general, Aspergilli are able to tolerate nutrient limited, and harsh conditions including high salt/osmolarity, pH, low oxygen and variable temperatures (Yong and Wood, 1977). Their specialised metabolism includes a set of solute transporter proteins identified by Pel *et al* that allow the transport and use of a wide variety of substrates while others appear to act as nutrient sensors (2007). They secrete a range of enzymes capable of breaking down polysaccharides and other biopolymers to release sequestered nutrients as free amino acids and sugars (Pel et al., 2007; Yong and Wood, 1977). Their tolerance to a wide range of environments including high salt and osmolarity, low oxygen, pH, and low nutrient requirements allows Aspergilli to use a range of alternative, and often harsh substrates such as agro-industrial by-products (Yong and Wood, 1977). *A. niger* and *A. oryzae* are both members of the Aspergillus genus and both have a history of safe use for industrially food and beverage production. Not only are both used for flavour and aroma production, but both have been scaled for industrial-scale fermentation. *A. niger* is already used for the industrial production of the organic acid, and flavouring ingredient – citric acid (Dhillon

et al., 2011; Shepard, 1960). This has been achieved in both submerged-state and solid-state fermentation of a variety of substrates including agro-industrial by-products (Dhillon, Brar, Verma, & Tyagi, 2011; Shepard, 1960). In addition to citric acid, *A. niger* has also been used for the production of other flavour and aroma compounds – most notably playing a role in the first bioconversion step of ferulic acid to vanillin (Motedayen et al., 2013). *A. oryzae* is used in the production of many fermented soybean products including koji, miso, sake and soy sauce where it is responsible for enhancing and producing pleasant aroma and flavour compounds during fermentation (Zhu & Tramper, 2013). Flavour and fragrance compounds produced include: phenylacetaldehyde, isovaleraldehyde and isobutyraldehyde, as well as various volatile mushroom flavours (Kaminski et al., 1974; Yoshizaki et al., 2012). Overall, both *A. niger* and *A. oryzae* are food-grade, GRAS microorganisms with a low risk of alflatoxin production given appropriate fermentation conditions. Both produce a range of enzymes that can hydrolyse substrates and release nutrients in the form of amino acids and sugars for further fermentation and finally, both have an established history of industrial-scale fermentations (Yong and Wood, 1977). Large fermentation volumes up to 150m^3 are common-place, giving promising scale-up potential for any successful fermentative production of flavour or fragrance ingredients produced by these species (Berovic and Legisa, 2007).

1.10.2 Penicillium camemberti

Penicillium camemberti is an Ascomycete fungi first described as a cheese-associated fungus by Thom (Thom, 1906). *P. camemberti* is responsible for conferring the main characteristic flavours and aromas as well as the texture of the soft cheeses brie and camembert. It plays a superior role in fermented, dry sausage products compared to other Penicillium spp. – specifically in flavour and aroma contribution (Joginder Singr and Dincro, 1994). *P. camemberti* is also responsible for formation of methyl ketones, and other desirable volatile compounds conferring mushroom (1-octen-3-ol) and/or green notes (Sunesen and Stahnke,

2003). Karahadian *et al* noted that these compounds were produced not only on dairy and meat products, but also on sugar-based media in a laboratory setting (Karahadian et al., 1985). The use and history of *P. camemberti* to add flavour and aroma to food is extensive and well documented; thus it has GRAS status, and can be safely consumed by humans. Husson *et al* suggest the production of key odourants as found in camembert and brie cheeses by *P. camemberti* occur specifically during secondary metabolism (Husson et al., 2002). The lipolytic and proteolytic enzymes produced by the fungus may play a role in the production of volatile aroma compounds and cheese maturation (Husson et al., 2002). However, in addition, these enzymes may allow *P. camemberti* to utilise some agro-industrial by-products as a substrate for growth and production of potentially industrially relevant flavour and aroma compounds (Boratyński et al., 2018).

1.10.3 Pycnoporus cinnabarinus

The basidiomycete *Pycnoporus cinnabarinus* is a white rot, saprophytic fungi also known as the "orange mushroom" – the cinnabar polypore. It is a robust fungus that is not sensitive to pH, temperature and produces biomass rapidly (Lomascolo et al., 2011). *P. cinnabarinus* gained popularity with its biotechnological potential in the 1990's, especially due to the large range of enzymes it can produce. Specifically, *P. cinabarinus* is an overproducer of the lignolytic enzyme laccase. This enzymes breaks down lignin in a non-specific manner, making it the perfect candidate for the transformation of agro-industrial by-products which are characteristically high in lignin (Lomascolo et al., 2011). In addition, it is classified as both a food-grade and cosmetic-grade microorganism with the potential for use in a wide range of products for human treatment and consumption. There are several cases of valuable aroma compounds being produced by *P. cinnabarinus*, though it is most well-known for its role in vanillin production using a laccase-free mutant in a two-step fermentation with *A. niger* (Lesage-Meessen et al., 1996; Anne Lomascolo et al., 1999). Other valuable aroma compounds

include benzaldehyde – a key component of bitter almond and cherry flavour, as well as the *de novo* production of methylanthranilate − a key component of orange blossom fragrance that is not produced naturally in any easily recoverable concentrations (Gross et al., 1990; A. Lomascolo et al., 1999). A food-grade status combined with the promising production of valuable aroma compounds – especially on agro-industrial waste streams makes *P. cinnabarinus* an ideal candidate for the production of flavour and aroma compounds from agro-industrial by-products.

1.10.4 Brettanomyces bruxellensis

Brettanomyces bruxellensis is a yeast first isolated as a wine spoilage organism and was the first patented microorganism in the 1920's for its role in beer fermentation (Renouf et al., 2007; Schifferdecker et al., 2014; Snowdon et al., 2006; Wedral et al., 2010). It is a Crabtree positive, ethanol tolerant, facultative anaerobe and well adapted to nutrient limited conditions (Hellborg and Piškur, 2009). *B. bruxellensis* produces the characteristic "mousy" or "metallic" aromas conferred by the compounds 2-ethyltetrahydropyridine and 2-acetyltetrahydropyridine which in wine is considered an off-flavour (Renouf et al., 2007; Snowdon et al., 2006). However, the same compounds are key to giving Lambic-style, Belgian beers their characteristic flavour and aroma profiles (Steensels et al., 2015a). In fact, *B. Brettanomyces* plays a distinct role in the flavour and aroma profiles of several other fermented foods and beverages including sourdough bread, kombucha tea and olives (Steensels et al., 2015a). As it has a long history of human consumption in these products, it has obtained GRAS (generally recognised as safe) status making it an ideal candidate microorganism for the production of flavour and aroma compounds. In addition to its potential for flavour production, *B. bruxellensis* is well-adapted to grow in a range of harsh environments as encountered in industrial wine fermentations such as: low pH, high osmotic stress, high ethanol, high sulfites, high acidity and nitrogen-limited conditions (De Barros Pita et al., 2013; Schifferdecker et al.,

2014; Smith and Divol, 2016). In addition, *B. bruxellensis* can utilise a range of substrates such as glucose, fructose, maltose, mannose, ethanol, acetic acid, glycerol as well as lignocellulosic and wood-based substrates (Smith and Divol, 2016). Unlike the typical industrial workhorse − *Saccharomyces cerevisias*, *B. bruxellensis* is able to proliferate and generate high quantities of biomass in nitrogen limited environments (Smith and Divol, 2016; Uscanga et al., 2000). *B. bruxellensis* has a genome enriched in nitrogen and lipid transporter and metabolism genes compared to *S.* cerevisae which may confer this competitive advantage (Woolfit et al., 2007). This could play a role in the fact that *B. bruxellensis* can utilise nitrate as a sole nitrogen source – again unlike *S. cerevisiae* (De Barros Pita et al., 2011). Another potential feature that has given *B. bruxellensis* a potential competitive advantage for utilising diverse substrates and outcompeting yeasts such as *S. cerevisiae* is its high energy efficiency in suboptimal environments (Blomqvist et al., 2010). The maintenance of specific genes including the mitochondrial gene encoding respiratory complex I – NADH dehydrogenase allows the yeast to simultaneously transport excess pyruvate and accumulated acetic acid towards the tricarboxylic acid (TCA) cycle which can allow it to continue growth in nitrogen-limited, low oxygen and low sugar conditions by using energy and amino acids from the TCA cycle (Procházka et al., 2010; Woolfit et al., 2007). This and/or other upregulated respiration-related genes could allow the yeast to continue growth by switching towards respiration to provide energy which could be key to allowing *B. bruxellensis* to maintain a more energy efficient metabolism in suboptimal conditions to outcompete *S. cerevisiae* in industrial fermentations (Blomqvist et al., 2010; Smith and Divol, 2016). The ability of *B. bruxellensis* to survive and proliferate in such nutrient limited conditions and on a wide range of substrates, combined with its potential for flavour and aroma compound generation makes it a well-suited for the fermentation of various agro-industrial by-products which share many of these common features.

Aim and Objectives

Given the clear demand for natural fragrance and flavour ingredients, and the promising potential of using fermentation to access otherwise hard to obtain flavour and aroma compounds, the overall aim of this thesis was to screen, identify and optimise the fermentative production of a flavour/fragrance compound of interest for future scale-up and production in a commercial setting.

1. Ferment a selection of food industry waste products with different fungi and non-*Saccharomyces* yeast.
2. Screen fermented substrates to identify valuable, volatile chemicals with commercial potential.
3. Optimise production of one valuable volatile chemical with commercial potential.

*Authors note: this thesis has been written in a papers-for-publication format. As a result, some sections may be repetitive.

Chapter II

2 SCREENING SOLID-STATE FERMENTATIONS OF AGRO-INDUSTRIAL BY-PRODUCTS TO IDENTIFY NEW NATURAL SOURCES OF INDUSTRIALLY RELEVANT FLAVOUR AND AROMA COMPOUNDS

Melodie A. Lindsay [a], Ninna Granucci [b], David R. Greenwood [a] and Silas G. Villas-Bôas [a,b]

[a] School of Biological Sciences, University of Auckland, 3A Symonds Street, Auckland 1142, New Zealand.

[b] Greenspot Technologies, Toulouse White Biotechnology, 3 rue Ariane, Ramonville-Saint Agne 31520, France.

2.1 Introduction

A high consumer demand for natural flavour and aroma compounds has increased the pressure on traditional, natural sources of many natural additives (Berger, 2007). Natural flavours and fragrances are traditionally sourced from herbs, spices, plants and animals — usually in the form of essences, extracts and oils. The organoleptic properties of such flavours and fragrances tend to be highly complex and difficult to reproduce realistically by synthetic means. As a result, commercial demand for such products often outstrips global supply and prices can be volatile. Partially addressing this problem are synthetic additives consisting of highly pure formulations of single contributing flavour compounds. These synthetic additives are manufactured from cheap and abundant starting materials — often of petrochemical origin (Berger, 2007). Such additives do not qualify as natural products and labelling requirements for these can deter many manufacturers. Recently, heightened public awareness has concentrated efforts on producing sustainable, environmentally friendly and renewable products as well the preference for additives that can be labelled as natural products (Micallef, 2014). This has created a strong case for the search of alternative sources of natural fragrances and flavours.

For centuries, microorganisms have been used to produce and enhance flavours in food and beverage products. Cheese, wine, beer and chocolate are just a few examples of fermented products that have undergone significant changes in their sensory profiles after fermentation (Button and Dutton, 2012; Goddard, 2016). In recent times, there has been a resurgence of fermentation to produce consumer products (Hugenholtz, 2013). There are also many studies investigating various fungi and yeasts and their potential to produce aroma compounds including: *Rhizopus* spp., *Trichoderma* spp., *Ceratocystis* spp., *Saccharomyces* spp., *Hanseniaspora* spp. (Christen et al., 2000, 1997; Hanem et al., 2015; Madrera et al., 2015; Mantzouridou et al., 2015; Rossi et al., 2009; Soares et al., 2000; Zheng et al., 2007). Therefore,

there appears ample opportunity to investigate microorganisms − especially filamentous fungi, for the production of natural flavours and fragrances for the food and cosmetics industries.

To remain competitive, the production of alternative natural fragrances and flavours must be sustainable and reliable. Agro-industrial waste products − especially those produced from the food and beverage industry, are high in: fermentable sugars, fibre, proteins, vitamins and minerals (Kaur et al., 2019). Despite this, they are often disposed of through incineration, composting, landfill or as cheap stock feed. As such, these agro-industrial waste products are an environmentally responsible and ethical biomass with which to produce flavour and fragrance compounds, while there is also opportunity to add value to these by-products. As there are millions of tonnes of different varieties of agro-industrial waste produced annually all over the world, this could be a very competitive feedstock to produce natural flavour and fragrance additives.

Solid state fermentation (SSF) is a promising method to produce natural flavours and fragrances as it offers a complex growth medium that closely mimics the natural environment. SSF is characterised by a fermentation carried out on solid particles, in the absence of free water (Castilho et al., 2009). Most often, this is a controlled fermentation where either bacteria or yeast grow on the surface of the solid particles in a biofilm, or in the case of fungi, hyphae penetrate the solid particles (Castilho et al., 2009). Traditional SSFs include the use of *Aspergillus niger* to produce koji, *Penicillium roqueforti* to produce blue cheese and other traditional brewed foods such as soy sauce and vinegars (Behera and Ray, 2016; Couto and Sanromán, 2006; Soccol et al., 2017). In such cases, microorganisms tend to produce more complex metabolites — including volatile chemicals that contribute to aroma and flavour (Soccol et al., 2017). SSF has successfully produced many different aroma compounds on a range of substrates including coffee husk, cassava bagasse, apple pomace, wheat bran, citrus pulp and sugarcane bagasse (Christen et al., 1997; Hanem et al., 2015; Rossi et al., 2009; Soares

et al., 2000). This forms an encouraging basis to investigate the potential of a range of agro-industrial by-products for use in SSF to produce flavour and aroma chemicals.

In the present study, the volatilomes of 36 fermentations using four different GRAS (generally recognised as safe) filamentous fungi and nine food grade agro-industrial by-products were screened using headspace-solid-phase microextraction coupled to gas chromatography-mass spectrometry (HS-SPME-GC/MS). This enabled us to identify potential valuable aroma chemicals that are produced naturally and from a sustainable, alternative source.

2.2 Materials and Methods

2.2.1 Microorganisms

Four GRAS status fungi were obtained from an inhouse culture collection of microbes purchased from Landcare New Zealand (ICMP) culture collection or isolated from nature: *Aspergillus niger* ICMP 17511, *Aspergillus oryzae* ICMP 1281, *Penicillium camenbertii* sp. and *Pycnorporus cinnabarinus* SVB-F118. These were reactivated and maintained on sabouraud dextrose agar (SDA).

2.2.2 Substrates

Agro-industrial by-products were sourced from various New Zealand producers: Frucor Beverages Limited, Simply Squeezed Limited, RD2 International Limited, Onions NZ, Kiwifruitz, and Pernod Ricard New Zealand. By-products included: apple pomace; orange pomace; carrot pomace; onion pulp; kiwifruit skins; grape marc (red and white); spent brewer's grain; and olive cake and were used without supplementation for solid state fermentations. All substrates were stored at -20°C in vacuum sealed freezer bags until use in fermentations.

2.2.3 Solid-state fermentations

Prior to fermentation, each substrate was thawed and sterilised by autoclaving at 121°C. *Aspergillus niger, Aspergillus oryzae* and *Penicillium camenbertii* were sporulated on SDA. *Pycnorporus cinnabarinus* was propagated on PDA and a mycelium preinoculum cultivated on sterilised rice. 50 g of sterile wet substrate at its original moisture content was inoculated with 3 mL spore suspension (10^7 spores/mL in 0.9% saline solution) or 5 g rice mycelium preinoculum and incubated at 25°C (*P. cinnabarinus and P. camenbertii*) or 28°C (*A. niger* and *A. oryzae*) in the dark. Five replicates for each substrate/fungus combination and corresponding sterile, negative controls were analysed in parallel. After 10 days or >90% visual colonisation was reached, samples were stored at -20°C in glass vials and flushed with nitrogen pending volatilome analysis.

2.2.4 Volatilome analysis

2.2.4.1 Headspace solid-phase microextraction sample preparation and extraction conditions

Samples were prepared in daily batches. For each run, stored fermentations were thawed at 4°C. 2 g samples were weighed into 20 mL amber SPME headspace vials and immediately fitted with a silicone/PTFE septum cap. All analyses were carried out using a 1 cm 50/30 µm DVB/CAR/PDMS (Supelco) fibre. Preincubation was carried out for 10 min at 60°C and was immediately followed by 10 min extraction. 60°C was selected for the incubation temperature as this gave the richest profile of volatiles compared to a 30°C incubation temperature. Desorption in the GC was performed for 1 min at 250°C operating in splitless mode. Following desorption, the fibre was held for a further 5 min at 250°C to clean the fibre in preparation for the next sample.

2.2.4.2 Gas chromatography-mass spectrometry conditions

Volatile compounds entrapped by the fibre were analysed by GC-MS by desorbing into a Shimadzu QP2010 Plus GC-MS system via a CTC analytics Combi PAL autosampler. GC-MS parameters are shown in ***Table 1***.

Table 1. GC-MS parameters for head-space volatiles analysis

General settings		GC-capillary column		Inlet	
Ionization voltage:	70 eV	**Thickness:**	0.25 µm	**Sampling time:**	1 min
Ion source temperature:	200 ˚C	**Diameter:**	0.25 mm	**Injection mode:**	Splitless
Detector voltage:	0.70-0.15 kV	**Length:**	30.0 m	**Injection temperature:**	250 ˚C
Interface temperature:	250 ˚C	**Flow:**	1.10 mL/min	**Flow:**	1 mL/min
Quadrupole temperature:	200 ˚C	**Carrier gas:**	Helium		
Ionization mode:	EI	**Type:**	Rtx®- 5Sil MS		
Acquisition mode:	Scan		(Restek)		
m/z range:	33-400				

GC oven temperature conditions are listed in ***Table 2*** *below.*

Table 2. GC oven temperature conditions for head-space volatiles analysis

Rate °C/min	Temperature °C	Hold Time (min)
-	35.0	0.00
10.00	80.0	0.00
2.00	160.0	0.00
10.00	260.0	0.00

2.2.4.3 GC-MS volatiles identification and data analysis

The volatile compounds detected by GC-MS were analysed and provisionally identified using a suite of compound libraries, software and inhouse R packages. First, a subset of the NIST2014 library was created using Enhanced Chemstation (Agilent). Then, AMDIS (Automated Mass Spectral Deconvolution and Identification system) was used to deconvolute and assign an identity to each peak detected. Automated peak integration was performed using an in-house R package 'MassOmics' and manual corrections were made to remove false identifications and adjust retention time consistency. Compounds that increased in abundance or were produced *de novo* with a match factor of over 90% to NIST2014 library spectra were considered for further characterisation experiments.

2.2.4.4 GC-MS quantification of volatiles extraction

Compounds of interest were identified in several fermentations. These fermentations were repeated and extracted to confirm concentration and identity of the compounds. Given the complex head-space profiles of the fermented samples, extractions were required to reduce the number of volatile compounds in the samples and to allow quantification using standard curves made using the extraction solvent (see **section 1.9.5**). For each extraction, 2 g of homogenised sample was weighed into a Kimax test tube and 1 mL of distilled water, 5 µL of internal standard (10 mM 12-bromo-dodecanol) and ~100 mg sodium chloride was added. 1 mL of tetrahydrofuran was then aliquoted into each, capped and vigorously mixed for 2 min using a vortex mixer. Samples were sonicated for 30 min before centrifuging at 4000 rpm for 10 min. The organic phase was aspirated into GC-MS vials and kept at 4 °C in a cooling tray pending GC-MS analysis.

Gas chromatography-mass spectrometry conditions

Extracted volatiles were quantified using a GC-7890 gas chromatograph (Agilent Technologies, Santa Clara, CA) coupled to a MSD-5975 mass spectrometer (Agilent Technologies, Santa Clara, CA). GC-MS parameters are listed in ***Table 3***.

Table 3. GC-MS parameters for quantification of extracted volatile compounds

General settings		**GC-capillary column**		**Inlet**	
Ionization voltage:	70 eV	**Thickness:**	0.25 µm	**Injection volume:**	1µL
Ion source temperature:	230 ˚C	**Diameter:**	0.25 mm	**Injection mode:**	Splitless
Detector voltage:	0.70-0.15 kV	**Length:**	30.0 m	**Injection temperature:**	200 ˚C
Interface temperature:	250 ˚C	**Flow:**	1.10 mL/min	**Flow:**	1 mL/min
Quadrupole temperature:	200 ˚C	**Carrier gas:**	Helium		
Ionization mode:	EI	**Type:**	Rtx®- 5Sil MS (Restek)		
Acquisition mode:	Scan				
m/z range:	38-550				

GC oven temperature conditions are listed in ***Table 4*** below.

Table 4. GC oven temperature conditions for quantification of extracted volatiles

Rate °C/min	**Temperature °C**	**Hold Time (min)**
-	35.0	0.00
10.00	80.0	0.00
2.00	160.0	0.00
10.00	260.0	0.00

2.2.4.5 GC-MS volatiles identity confirmation and quantification

Volatile compounds of interest were confirmed by comparing both retention time and ion fragmentation patterns with pure chemical standards. A calibration curve was built for each compound of interest using pure chemical standards obtained from Sigma Aldrich and analysed

in the same run as the extracted samples. Each compound was quantified using normalised peak height and comparing to the calibration curve.

2.3 Results

2.3.1 Solid state fermentation performance

Fermentations were visually assessed daily to determine the fermentation end point. All substrates were >90% surface colonised by mycelia 10 days post inoculation with the exception of five fermentations: *A. niger*, on red grape marc; *P. camenbertii*, on olive cake and red grape marc; and *P. cinnabarinus*, on spent brewer's grain and red grape marc.

2.3.2 Volatile profiling

Hundreds of volatile compounds were detected and provisionally identified using the NIST2014 spectral library. All substrates produced volatile compounds following fermentation and numbers are reported in **Figure 1**.

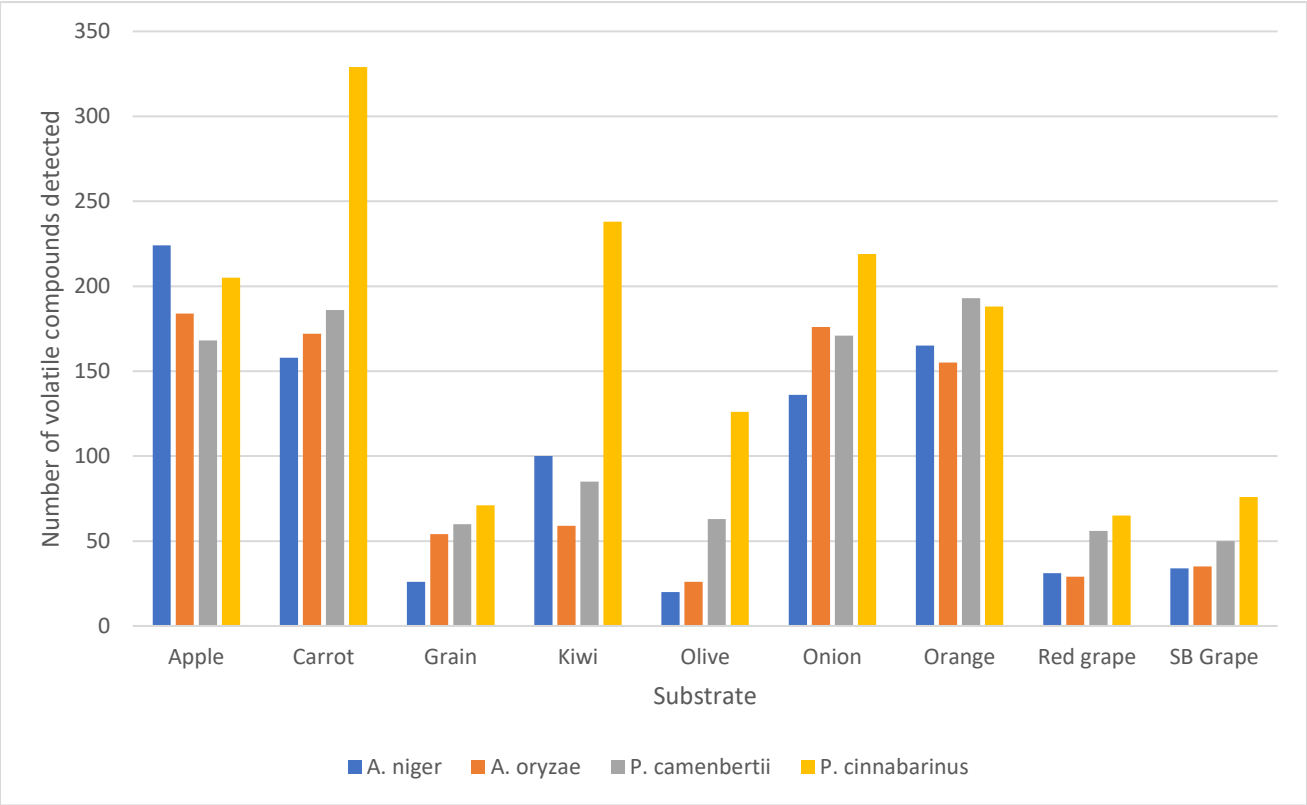


Figure 1. Absolute number of volatile compounds that increased in abundance in all replicates following fermentation with A*spergillus niger, Aspergillus oryzae Penicillium camenberti,* or *Pycnoporus cinnabarinus* on each of nine substrates when compared to an unfermented negative control. Apple=apple pomace, Carrot=carrot pomace, Grain=spent brewer's grain, Kiwi=kiwifruit skins, Olive=olive cake, Onion=onion pulp, Orange=orange pomace, Red grape=red grape marc and SB grape=Sauvignon blanc grape marc. Number of replicates N=5.

The greatest number of compounds based on substrates was detected from: apple (168-224 compounds), followed by carrot (158-329 compounds), onion (136-219 compounds) and orange (155-193 compounds). Considerably fewer compounds were detected in red grape marc (29-65 compounds), olive cake (20-126 compounds), and white grape marc fermentations (34-76 compounds) regardless of the fungi used to ferment them. Among the four fungi used for fermentation, *P. cinnabarinus* yielded the highest number of volatile compounds (1517 compounds) while both *A. oryzae* and *A. niger* yielded less with 890 and 894 compounds respectively. Fermentations using *P. camenberti* allowed the detection of 1023 compounds.

Over 50 compounds of interest were identified across the fermented substrates including flavour and fragrance compounds with characters such as: fruity, floral, spicy, green,

minty and sweet. Only compounds with a match factor of over 90% to the NIST2014 spectral library and those that increased after fermentation when compared to the unfermented control were investigated for organoleptic qualities. A summary of the top fragrance and flavour compounds produced from each fermentation are presented in **Table 5** along with their organoleptic qualities.

Table 5. Organoleptic qualities of potentially industrially-relevant volatile compounds produced during fermentation of nine fruit and vegetable by-products using four filamentous fungi.

Compound	Match %	Reference ion	Substrate	Microorganism	Fold change	Descriptor
1-Butanol	92	56	Apple	*AN*	2.89	Fusel oily-banana
1-Butanol, 2-methyl-	94	57	Onion	*AN*	2.16	Black truffle
	92	57	Onion	*PC1*	191.25	
	92	57	Onion	*PC2*	5.55	
	95	57	Orange	*PC1*	27.80	
1-Heptanol	94	56	BSG	*AO*	60.39	Pleasant, cosmetic
	94	56	BSG	*AN*	10.34	
1-Hepten-3-ol	93	57	Onion	*PC1*	164.70	Oily, green, metallic
1-Hexanol	97	56	BSG	*AO*	7.05	Fresh cut grass

1-Octen-3-ol	90	57	BSG	*PC1*	23.56	Mushroom alcohol
	90	57	BSG	*PC2*	18.89	
	98	57	KFS	*AO*	30.95	
	98	57	KFS	*AN*	29.20	
	92	57	KFS	*PC2*	67.82	
	92	57	KFS	*PC1*	32.63	
	96	57	Onion	*AN*	87.19	
	96	57	Onion	*AO*	37.67	
	95	57	Onion	*PC1*	3118.06	
	95	57	Onion	*PC2*	1554.88	
2-Heptanone, 6-methyl-	95	43	BSG	*AO*	53.97	Camphorous
2-Pentanone	90	43	Apple	*PC1*	7.75	Fruity
3-Octanol	96	59	Carrot	*AO*	2.45	Mushroom, herbal, citrus
	97	59	Carrot	*PC2*	162.88	
	93	59	BSG	*AN*	108.94	

	90	59	BSG	*PC2*	260.96	
	90	59	BSG	*PC1*	99.87	
	90	59	KFS	*PC2*	83.29	
	90	59	KFS	*PC1*	5.07	
	98	59	Onion	*AN*	423.07	
	98	59	Onion	*AO*	705.07	
	94	59	Onion	*PC1*	42.86	
	94	59	Onion	*PC2*	182.12	
	98	59	RG	*AN*	48.19	
	98	59	RG	*AO*	215.10	
	97	59	SBG	*AN*	49.70	
	97	59	SBG	*AO*	102.32	
3-Octanone	98	43	BSG	*AN*	26.24	Herbal, lavender, nectarine
	97	43	BSG	*PC1*	220.63	
	97	43	BSG	*PC2*	20.03	
	98	43	KFS	*AO*	9.35	

	98	43	KFS	*AN*	3.97	
	98	43	KFS	*PC2*	14.35	
	98	43	KFS	*PC1*	4.98	
	99	43	Onion	*AN*	223.92	
	99	43	Onion	*AO*	193.50	
	95	43	Onion	*PC1*	219.54	
	95	43	Onion	*PC2*	85.24	
	96	43	RG	*AO*	57.53	
	96	43	RG	*AN*	30.26	
	98	43	SBG	*AN*	20.34	
	98	43	SBG	*AO*	24.93	
4-Decenoic acid,	95	74	Apple	*PC2*	111.69	Tropical, fishy
methyl ester	95	74	Carrot	*PC2*	353.43	
4-Octenoic acid,	92	74	Carrot	*PC2*	206.55	Fresh pineapple
methyl ester, (Z)-						

	98	43	Apple	*AN*	8.32	Ethereal, sweet,
	98	43	Apple	*AO*	4.75	fruity
Acetic acid,	99	43	Carrot	*PC2*	260.58	
methyl ester	98	43	Carrot	*PC2*	194.72	
	100	43	Orange	*AN*	65.08	
	100	43	Orange	*AO*	14.34	
α.-Cubebene	99	161	Apple	*PC2*	60.99	Herbal
Amyl isovalerate	98	70	Apple	*AN*	70.9	Fruity
	99	108	BSG	*PC1*	7.68	Aniseed
	99	108	BSG	*PC2*	786.14	
Anisole	96	108	Orange	*AN*	21.45	
	97	108	Orange	*PC2*	350.88	
	100	77	BSG	*PC2*	2.32	Cherry, almond
Benzaldehyde	100	106	Orange	*AN*	2.46	
Benzene, 1,2-	91	138	Orange	*AN*	91.78	Insect attractant
dimethoxy-						

Benzene, 1,4-dimethoxy-	100	123	Onion	*PC1*	163.45	Intense sweet, floral
Benzene, 4-ethenyl-1,2-dimethoxy-	96 100	164 164	Orange Orange	*AN* *PC1*	149.35 244.99	Green, floral, smoky
Benzoic acid	99 98 94	105 105 105	Carrot KFS Onion	*PC2* *PC2* *PC2*	272.48 29.44 137.72	Balsamic
Benzoic acid, methyl ester	92 92 98	105 105 105	Orange Orange Orange	*PC1* *PC2* *AN*	16.29 33.35 27.39	Feijoa, ylang ylang, wintergreen
Benzyl alcohol	97 97	79 79	BSG BSG	*PC1* *PC2*	31.32 17.09	Precursor and solvent
β-Pinene	96	93	KFS	*AN*	1.33	Herbal, pine
β-Myrcene	96 96	41 41	Apple Apple	*AO* *AN*	2.03 1.38	Clove-like

	97	93	KFS	*PC1*	2.11	
Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)-	98	93	BSG	*PC1*	7.73	Spicy, black pepper
Butanal, 2-methyl-	96	41	BSG	*PC1*	14.69	Musty, chocolate
	96	41	BSG	*PC2*	13.20	
Butanal, 3-methyl-	96	44	Carrot	*PC2*	60.67	Peach, malty, fatty, chocolate, peach
	98	44	KFS	*PC2*	32.69	
	98	44	KFS	*PC1*	4.28	
	99	44	Onion	*AO*	15.15	
	99	44	Onion	*AN*	8.33	
	94	44	BSG	*AN*	10.76	
	94	44	BSG	*AO*	9.77	
	99	55	Onion	*AO*	306.77	
	99	55	Onion	*AN*	53.09	

Butanoic acid, 3-methyl-, ethyl ester	99	88	Apple	*AN*	210.00	Fruity
Cinnemaldehyde, (E)-	100	131	RG	*AO*	40.00	Cinnamon
Cyclopentanone	92 92	55 55	KFS KFS	*AO* *AN*	36.69 16.60	Minty
Decanal	98	57	Olive	*PC2*	5.39	Citrus
D-Limonene	100	68	Carrot	*AN*	5.68	Citrus
Ethyl tiglate	93	83	RG	*AN*	128.94	Tutti frutti, green olive
Heptanoic acid, methyl ester	94	74	Carrot	*PC2*	105.89	Fruity, green, waxy
Hexanal	93 97 96	44 44 44	BSG KFS Olive	*AO* *PC2* *PC2*	3.00 3.97 9.53	Fresh cut grass

Hexanoic acid, methyl ester	96	74	Apple	*PC2*	133.85	Pineapple, fatty
	94	74	Carrot	*PC2*	75.89	
	93	74	Orange	*PC2*	17.40	
Hexyl n-valerate	95	85	Apple	*AN*	1.49	Green, brandy
iso-Amyl tiglate	91	70	Apple	*AN*	21.04	herbal
Limonene oxide, trans-	99	43	Olive	*PC2*	36.57	Minty, citrus
Methyl 2-furoate	99	95	Apple	*PC2*	130.75	Caramel, musty, fungal
	99	95	Carrot	*PC2*	624.14	
	99	95	BSG	*PC1*	11.04	
	99	95	BSG	*PC2*	687.33	
	99	95	KFS	*PC2*	588.31	
	99	95	KFS	*PC1*	7.72	
	97	95	Olive	*PC2*	138.46	
	100	95	Onion	*PC1*	17.89	
	100	95	Onion	*PC2*	816.65	

	98	95	Orange	*PC2*	58.68	
Methyl isovalerate	95	74	KFS	*PC2*	1294.67	Fruity
	94	74	Onion	*PC2*	139.85	
	94	74	RG	*PC2*	395.00	
Methyl salicylate	97	120	Orange	*AN*	15.49	Wintergreen mint,
	96	120	Orange	*PC2*	16.38	root beer
Methyl valerate	96	74	Carrot	*PC2*	44.37	Sweet, fruity
Methyleugenol	97	178	Apple	*PC1*	845.19	Spicy, clove
	97	178	Apple	*PC2*	41.88	
Methyleugenol	97	178	Orange	*PC1*	48.28	Clove
Nonanoic acid, methyl ester	95	74	Carrot	*PC2*	90.73	Pear, tropical, waxy
Phenyl acetaldehyde	100	91	Carrot	*AN*	3.15	Honey, floral
	100	91	Carrot	*AO*	1.52	
	96	91	BSG	*AO*	3.47	
	100	91	BSG	*PC2*	77.04	

	96	91	Olive	*PC2*	5.73	
Phenylethyl alcohol	100	91	BSG	*PC2*	77.04	Rose
Propanoic acid, 2-methyl-	98 98	43 43	Carrot Carrot	*AO* *AN*	187.92 62.03	Rancid butter
Pyrazine, 2,5-dimethyl-	94 94	42 42	Carrot Carrot	*AN* *AO*	3.68 2.95	Nutty, musty
Pyrazine, 2,5-dimethyl-	97	42	Onion	*AN*	2.83	Roasted, chocolate
Vanillin	100	151	Olive	*PC1*	1.14	Vanilla

Legend: Four filamentous fungi: **AN=*Aspergillus niger*, AO= *Aspergillus oryzae*, PC1= *Penicillium camemberti*, and *PC2=Pycnoporus cinnabarinus and nine substrates:* Apple=apple pomace, Carrot=carrot pomace, Grain=spent brewer's grain, KFS=kiwifruit skins, Olive=olive cake, Onion=onion pulp, Orange=orange pomace, Red grape=red grape marc and SB grape=Sauvignon blanc grape marc. Fold-change is compared to the unfermented negative control of the respective substrate.**

Many other compounds were also tentatively identified but had lower match percentages or were not flavour or fragrance compounds. Several volatile compounds were produced by specific fungi irrespective of the substrate they were cultivated on. For example, methyl-2-furoate was produced by *P. camenbertii* and *P. cinnabarinus* on apple, carrot, onion, olive, grain, kiwifruit and orange-based substrates. Others were produced by all four fungi on a range of different substrates e.g. 3-octanol and 3-octanone.

Four compounds of interest were quantified from the fermentations: benzoic acid, methyl ester; phenylacetaldehyde; 1-octen-3-ol; and phenylethyl alcohol. These were extracted from the fermented substrate and are presented in **Table 6.**

Table 6. Yield of commercially relevant flavour and aroma chemicals produced from fermented substrates

Compound	Value ($/kg)*	Annual consumption (kg)**	Substrate	Microorganism	Yield (mg)***
Benzoic acid, methyl ester	$335*	590	Orange	*Aspergillus niger*	0.173±0.0003
Phenylacetaldehyde	$450*	106	SBG	*Pycnorporus cinnabarinus*	1.493±0.384
1-Octen-3-ol	$4800*	250	Onion	*Aspergillus oryzae*	1.297±0.107
Phenylethyl alcohol	$500*	1240	SBG	*Pycnorporus cinnabarinus*	0.970±0.242

*Value per kilogram obtained through personal communication with Jeffrey Buco at Excellentia International. Prices quoted in US dollars correct as of September 2017.

**Annual consumption of compound as a flavour additive only *(George, 2005)*. Excludes other uses e.g. fragrance and cosmetics industry

***Yield represents average dry weight of compound produced per kilogram of fermented wet weight substrate ± 2 standard deviations and n=9. Substrates: Orange=orange pomace, SBG=spent brewer's grain, Onion=onion pulp.

The production of all four compounds was confirmed by comparing their mass spectra to pure standards. Yield was determined from the wet weight fermented substrate and was scaled up to reflect how much compound would theoretically be produced and extracted from one kilogram of fermented substrate. While yield was very low for benzoic acid-methyl ester (0.173 mg/kg), yields of 1-octen-3-ol (1.493 mg/ kg), phenylacetaldehyde (1.297 mg/kg) and phenylethyl alcohol (0.970 mg/kg) were considerably higher.

2.4 Discussion

Several hundred volatile compounds were produced by the fermentation of agro-industrial by-products with four different filamentous fungi. During screening of the 36 fermentations many compounds were identified − several with potential value in the flavour and fragrance industry (**Table 5**). There are many factors that require careful consideration. While none of the flavour and fragrance compounds quantified were produced in high quantities; yields of benzoic acid methyl ester was extremely low, whereas phenylethyl alcohol, 1-octen-3-ol and phenylacetaldehyde had higher yields. While yield is an important factor from a commercial perspective, the premium prices of natural compounds often far surpasses that of their synthetic alternatives. In that light, identifying potential valuable products in our fermentations was an important first step of investigating their potential production from fruit and vegetable by-products.

Thousands of tonnes of phenylethyl alcohol is consumed across the food, beverage, cosmetics and fragrance industries every year. Most of this is synthetically produced from benzene, styrene or toluene due to the extremely low yield of natural product from its traditional source − rose petals (Etschmann et al., 2002; George, 2005). For example, the yield of rose essential oil from rose petals is 0.03-0.04% and comprises up to 60% phenylethyl alcohol (Baydar, 2006; Etschmann et al., 2002). Our yield was approximately five times less than this.

However, spent brewer's grain is a very cheap substrate ($300/tonne) while rose petals cost upwards of $3000/tonne. With further optimisation, fermentative production of phenylethyl alcohol from grain could be a competitive, natural source of this alcohol.

1-Octen-3-ol was originally discovered in a Japanese mushroom, *Armillaria matsutake* (George, 2005; Maga, 1981; Murahashi, 1938). Currently, it is synthesised through a reaction between magnesium amyl bromide and acrolein (George, 2005). However, there is demand for natural 1-octen-3-ol as a mushroom flavouring in food products. As a high-value chemical ($4800/kg) with over 250 kilograms used annually in the food and beverage sector alone, our fermentation process could be a good option for the natural production of this compound (George, 2005). As all four fungi produced 1-octen-3-ol on many different substrates (**Table 5**), there is opportunity to further identify fungi that produce a higher yield. It is well known that many fungal species produce 1-octen-3-ol as part of their volatilome including *Penicillium*, *Aspergillus*, and many edible mushroom species (Borjesson et al., 1992; De Carvalho et al., 2011; Husson et al., 2005; Kaminski et al., 1974; Zawirska-Wojtasiak, 2004). Zawirska-Wojtasiak (2004) investigated natural 1-octen-3-ol extracted from several edible mushroom varieties finding high optical purity and yields of over 6 mg/100g in various types of edible mushrooms. These could be good candidates to include in further trials using fruit and vegetable by-products to target and optimise fungal production of this compound.

Phenylacetaldehyde is a third volatile compound identified and quantified in fermented spent brewer's grain that has potential as a natural flavour and fragrance ingredient. It is currently produced chemically from benzaldehyde through a Darzen glycidic ester synthesis (George, 2005). However, it can also be produced through an expensive biotechnological route from phenylalanine and therefore carry a natural label. Phenylacetaldehyde is found as a component of many essential oils and contributes to the flavour of foods such as chocolate,

cheeses, coffee, fruits, wine and breads (Crafack et al., 2014; San Juan et al., 2013; Sánchez-Rodríguez et al., 2018; Sunarharum et al., 2014; Van Leuven et al., 2008).

The volatilomes of some fermented substrates e.g. red grape marc, Sauvignon blanc grape marc and kiwifruit skins had far fewer compounds compared to substrates such as carrot, apple and orange pomaces. As all substrates were used in an unsupplemented state (the only treatment was autoclaving to sterilise the substrates), this leaves the potential to investigate supplementation or fortification of the substrates with nutrients i.e. nitrogen/phosphorus/potassium and/or sugars. For example, as red grape marc is pre-fermented by yeast and kiwifruit skins are naturally very low in sugar, both substrates may perform better if supplemented with additional sugar. On the other hand, Sauvignon blanc grape marc is high in fermentable sugars however, it also has a high lignin content which can be slow to break down. A two-step fermentation using a lignin-degrading fungus like *Pycnoporus cinnabarinus* followed by a second fungus could be an option to help increase the available nutrients and produce more of a desired compound (Agosin and Odier, 1985).

As nine different substrates were investigated and of those, onion pulp, apple-, carrot-, and orange pomaces appeared to perform the best and produce the most volatiles, we recommend any further screening experiments should focus on these substrates where available. Further experiments could also include investigating different fungal strains for their potential to produce high-value 1-octen-3-ol. Many edible mushrooms − especially *Agaricus bisporus* produce high quantities of 1-octen-3-ol and should be investigated for their potential to ferment agro-industrial by-products like onion pulp. As onions are very high in linoleic acid which is a precursor in the pathway to producing 1-octen-3-ol, this could be a very promising combination of substrate and fermenting fungi (Bello et al., 2013).

Solid-state fermentation is still in its infancy compared with industrial liquid-state fermentations. While there is potential to produce more complex secondary metabolites by using SSF, there are still problems with the large surface area required, air circulation and making homogenous and consistent fermentations as well as a heightened risk of contamination (Soccol et al., 2017). There are also several downstream challenges associated with extracting volatile compounds from the fermented substrate. While a distillate or extraction into ethanol could be feasible, distillation and/or purification can be expensive and challenging. Many essential oils and extracts contain mixtures of hundreds of compounds that convey a desired flavour or fragrance which is readily accepted by many consumers. In this study, all the fungi are GRAS organisms and the fruit and vegetable by-products are food grade. Provided our compound of interest is not masked or otherwise negatively affected by the presence of co-produced compounds, there is a good case to bring our compound to market as an essence, extract or essential oil derived from the fermented substrate. This is the case for many natural fragrance and flavour additives, for example rose oil, which contains up to 60% phenylethyl alcohol and vanilla extract, which contains only 0.1-0.25% vanillin (Baydar, 2006; Lawall and Forman, 1914; Walton et al., 2003).

2.5 Conclusions

We have screened 36 different fermentations in an exploratory study investigating four filamentous fungi for their potential to produce valuable flavour and aroma compounds from nine unsupplemented agro-industrial by-products. Hundreds of these volatiles were identified and four compounds of interest were quantified. While preliminary yields are low, the low-cost substrates and high-value products derived from the fermentation process present a compelling case to proceed with optimisation of the fermentation parameters and fungal strains to determine if yield and productivity can be improved. There are also many more candidate compounds that could be further investigated (**Table 5**). Further screening experiments should

focus on substrates that produced the most volatile compounds overall: apple pomace, carrot pomace, orange pomace and onion pulp.

CHAPTER III

3 SUBMERGED-STATE FERMENTATION OF FRUIT AND VEGETABLE BY-PRODUCTS TO SCREEN FOR PRODUCTION OF VALUABLE VOLATILE CHEMICALS USING A NON-SACCHAROMYCES YEAST

Melodie A. Lindsay [a], Ninna Granucci [b], David R. Greenwood [a] and Silas G. Villas-Bôas [a,b]

[a] School of Biological Sciences, University of Auckland, 3A Symonds Street, Auckland 1142, New Zealand.

[b] Greenspot Technologies, Toulouse White Biotechnology, 3 rue Ariane, Ramonville-Saint Agne 31520, France.

3.1 Introduction

Strong consumer pressure on traditional supply chains of natural fragrances and flavours has created a demand for alternative, sustainable sources of natural additives. While many natural fragrance and flavours are historically sourced from herbs, spices, flowers, plants and animals, global demand often outstrips supply. A combination of adverse weather events, political events and unpredictable harvests, makes prices of these chemicals unstable and volatile. As synthetic alternatives have been rejected by the consumer base, there is an opportunity to fill demand with alternative natural sources of these compounds.

For centuries, microorganisms have been used to produce and enhance flavours in foods and beverages such as wine, cheese, chocolate, beer and many others (Button and Dutton, 2012; Goddard, 2016). There is a recent resurgence in the use of microorganisms to produce consumer goods, including flavour and fragrance compounds (Hugenholtz, 2013). However, rather than using traditional sugar feedstocks or fermented food products to add flavour as in wine fermentation or cheese making, there is an increasing awareness of underutilised agro-industrial by-products. While some is diverted to animal feed, most by-products are still directed to landfill, often at a cost to the producer. This makes these fruit and vegetable pomaces a promising candidate for a cheap, sustainable alternative feedstock. Currently, these by-products have been investigated to produce or extract high-value compounds including: dietary supplements, polyphenols, pectins and enzymes, antioxidants, and antimicrobials (Kowalska et al., 2017; Schieber et al., 2001; Silva et al., 2018). As many by-products are chemically complex and often have a high content of fermentable sugars, fibres, proteins, vitamins and minerals, this makes them an ideal candidate as a feedstock for the production of natural flavour and aroma compounds (Brar et al., 2013). We previously found that the by-products apple-, carrot-, and orange pomace were among the best out of nine substrates screened for production of volatile compounds through fermentation by filamentous fungi

(**Chapter 2**.). In addition, previous work observed a pleasant, floral aroma produced during solid-state fermentation of carrot pomace with *Brettanomyces bruxellensis* (Granucci, 2018). Following the fungal fermentations in **Chapter 2**, a shortlist of valuable volatile chemicals was compiled including several with floral notes e.g. phenylacetaldehyde and phenylethyl alcohol. These were identified at very low concentrations in solid-state fermentations and based on their global use and value, were identified as potential compounds of interest for a commercial market. Due to the favourable fermentation outcomes in the previous chapter, and anecdotal evidence of floral fragrance production in previous works, we have chosen apple, carrot and orange by-products for further screening for production of valuable volatile chemicals using the non-*Saccharomyces* yeast *Brettanomyces bruxellensis*.

B. bruxellensis (aka. *Dekkera bruxellensis*) was the first patented microorganism for its role in beer fermentations in the early 1920s (Wedral et al., 2010). Much later it was identified as a spoilage yeast in wine and is associated with the specific aroma compounds in spoiled wine: 2- ethyltetrahydropyridine and 2-acetyltetrahydropyridine (Snowdon et al., 2006; Wedral et al., 2010). These compounds give off a characteristic medicinal, metallic, spiced and/or "mousy" off-characteristics in wine (Renouf et al., 2007; Snowdon et al., 2006). On the other hand, *B. bruxellensis* is the main fermenting organism in Lambic style, Belgian beers, and a key microorganism in sourdough, olives and the fermented beverage kombucha (Steensels et al., 2015b). Accordingly, not only is *B. bruxellensis* considered a GRAS microorganism and safe for human consumption, it is also capable of producing flavour and aroma compounds−both desirable and undesirable depending on the fermentation substrate. *B. bruxellensis* is also well adapted to surviving in the harsh, environments of wine-making which include: low pH; high osmotic stress; high acidity; nitrogen limitation; and high ethanol content. Several of these are often encountered in both industrial-scale fermentations and are

characteristic of the agro-industrial by-products in this screening experiment (De Barros Pita et al., 2013; Schifferdecker et al., 2014).

There are three main fermentation strategies: solid-, liquid-, and submerged-state fermentation. Solid-state fermentation is carried out on solid particles in the absence of free water while liquid state fermentation is defined as a liquid media with no solid particles. Submerged-state fermentation is a variation of liquid-state fermentation where solid particles are suspended in a liquid solution. This strategy is ideal for screening the fruit and vegetable pomaces as *B. bruxellensis* is known to form biofilms and having solid particles allows for the cells to adhere

3.2 Materials and methods:

By-products from New Zealand industries

Three by-products from New Zealand food industries: Frucor Beverages Limited, Simply Squeezed Limited and R2D International Limited were sourced for use as substrates for submerged state fermentations. Apple pomace, orange pomace and carrot pomace were stored at -20 °C and protected from light and air in food grade, resealable freezer bags. In preparation for fermentation, the substrates were thawed at 4 °C overnight.

3.2.1 Submerged-state fermentations

The non-saccharomyces yeast *Brettanomyces bruxellensis* CCT 3467 was previously found to produce a pleasant aroma during fermentation (Granucci, 2018). Three submerged-state fermentations were carried out using apple, orange and carrot pomaces based on previously established methods (Granucci, 2018). Briefly, *Brettanomyces bruxellensis* was maintained on plates containing malt extract–yeast extract–glucose–peptone (MYGP) (3 g/L yeast extract, 3 g/L malt extract, 6 g/L peptone and 10 g/L glucose) agar at 30 °C. Preinoculum was prepared by inoculating 200 mL of MYGP broth with a single colony of *B.bruxellensis* in

1 L Erlenmeyer flasks fitted with cotton stoppers. Flasks were left at 30 °C with agitation at 200 rpm in darkness until the end of the exponential phase was reached (20-fold diluted sample reached an optical density of approximately 0.500 at 600nm). Aliquots (50 mL) of preinoculum was measured into 50 mL falcon tubes and centrifuged (3000 x g, 5 min) to form a yeast cell pellet. Residual media was poured off and the cell pellet washed with sterile saline (0.9 % NaCl) twice.

For the fermentations, sterile substrate (150 g. 85.5% humidity) was weighed into 1 L Erlenmeyer flasks along with sterile, distilled water (350 mL), sterilised by autoclaving (121 °C, 20 min) and inoculated with the preinoculum as described above. These were agitated (200 rpm) in the dark at 30°C. Five replicates for each substrate and corresponding sterile, negative controls were analysed in parallel.

Samples (10 mL) were taken at the beginning of the fermentation (Time=0 hours) and every 24 hours for 72 hours. Cell numbers were counted using a Neubauer chamber at each time point, contamination checked and reducing sugars quantified using the DNS method as outlined by Miller (1959).

After 72 hours, samples (20 mL) were aseptically transferred into glass vials and flushed with nitrogen to prevent oxidation of volatile compounds. The vials were sealed and rapidly frozen at -20 °C in darkness until GC-MS analysis.

3.2.2 Volatilome analysis

3.2.2.1 Conditions for headspace solid-phase microextraction, sample preparation and extraction

Samples were prepared in daily batches. For each run, stored fermentations were thawed at 4°C. Samples (2 mL) were transferred into amber SPME headspace vials (20 mL) and immediately fitted with a silicone/PTFE septum cap. All analyses were carried out using a

1 cm 50/30 um DVB/CAR/PDMS (Supelco) fibre. Preincubation was carried out for 10 min at 60°C and was immediately followed by a 10 min extraction. The temperature selected for the incubation was 60°C as this yielded more volatiles than a 30°C incubation. Desorption was performed for 1 min at 250°C, operating in splitless mode. Following desorption, the fibre was held for a further 5 min at 250°C to clean the fibre in preparation for the next sample.

3.2.2.2 Conditions for gas chromatography-mass spectrometry

Volatile compounds entrapped by the fibre were analysed by GC-MS by desorbing into a Shimadzu QP2010 Plus GC-MS system via a CTC analytics Combi PAL autosampler. GC-MS parameters are shown in **Table 7**.

Table 7. GC-MS parameters for head-space volatiles analysis

General settings		**GC-capillary column**		**Inlet**	
Ionization voltage:	70 eV	**Film thickness:**	0.25 µm	**Sampling time:**	1 min
Ion source temperature:	200 ˚C	**Diameter:**	0.25 mm	**Injection mode:**	Splitless
Detector voltage:	0.70-0.15 kV	**Length:**	30.0 m	**Injection temperature:**	250 ˚C
Interface temperature:	250 ˚C	**Flow:**	1.10 mL/min	**Flow:**	1 mL/min
Quadrupole temperature:	200 ˚C	**Carrier gas:**	Helium		
Ionization mode:	EI	**Type:**	Rtx®- 5Sil MS (Restek)		
Acquisition mode:	Scan				
***m/z* range:**	33-400				

Oven temperature conditions are listed in **Table 8** below.

Table 8. Oven temperature conditions for head-space volatiles analysis

Rate °C/min	Temperature °C	Hold Time (min)
-	35.0	0.00
10.00	80.0	0.00
2.00	160.0	0.00
10.00	260.0	0.00

3.2.2.3 GC-MS identification of volatile compounds and data analysis

Volatile compounds detected by GC-MS were identified from their retention times and mass spectra using a suite of libraries, software and inhouse R packages. First, a subset of the NIST2014 library was created using Enhanced Chemstation (Agilent). Then, AMDIS (Automated Mass Spectral Deconvolution and Identification system) was used to deconvolute and assign identifications to each peak. Automated peak integration was performed using an in-house R package 'MassOmics' and manual corrections were made to remove false identifications and retention time corrections. Compounds that increased in abundance or were produced *de novo* and had a match factor of over 90% to the NIST2014 library spectra were considered for further characterisation experiments.

3.2.2.4 GC-MS quantification of extracted volatile compounds

A compound of interest that had a rose-scented aroma was identified from the carrot pomace fermentation. From the initial screening, this compound was tentatively identified as phenylethyl alcohol. To confirm its identity, homogenised sample (2 mL) was aliquoted along with the internal standard 12-bromo-dodecanol (5µl, 10 mM solution) and sodium chloride (~100 mg) added. Tetrahydrofuran (1 mL) was then added and vigorously mixed for 2 min using a vortex mixer. Samples were sonicated for 30 min before centrifuging (3000g, 10 min). The organic phase was aspirated into GC-MS vials and kept at 4 °C in a cooling tray pending GC-MS analysis.

Gas chromatography-mass spectrometry conditions

Extracted volatile compounds were quantified using a GC-7890 gas chromatograph (Agilent Technologies, Santa Clara, CA) coupled to a MSD-5975 mass spectrometer (Agilent Technologies, Santa Clara, CA). The GC-MS parameters are listed in **Table 9**.

Table 9. Oven temperature programmes for quantification of extracted volatile compounds

General settings		**GC-capillary column**		**Inlet**	
Ionization voltage:	70 eV	**Film thickness:**	0.25 µm	**Injection volume:**	1µL
Ion source temperature:	230 ˚C	**Diameter:**	0.25 mm	**Injection mode:**	Splitless
Detector voltage:	0.70-0.15 kV	**Length:**	30.0 m	**Injection temperature:**	200 ˚C
Interface temperature:	250 ˚C	**Flow:**	1.10 mL/min	**Flow:**	1 mL/min
Quadrupole temperature:	200 ˚C	**Carrier gas:**	Helium		
Ionization mode:	EI	**Type:**	Rtx®- 5Sil MS (Restek)		
Acquisition mode:	Scan				
***m/z* range:**	38-550				

3.2.2.5 GC-MS confirmation and quantification of phenylethyl alcohol

The rose-scented compound was confirmed as being phenylethyl alcohol by comparing both retention time and ion fragmentation patterns of its mass spectrum with the pure chemical standard. A calibration curve was constructed for each compound of interest using pure chemical standards obtained from Sigma Aldrich and analysed in the same run as the extracted samples. Phenylethyl alcohol was quantified using normalised peak heights that were compared with the calibration curve.

3.3 Results

3.3.1 Fermentation performance

Cell numbers associated with each substrate was used as a qualitative measure of fermentation performance. However, examination by bright-field light microscopy showed that *B. bruxellensis* tightly associated with the pomace particles during fermentation, and absolute

cell numbers were obviously skewed by this. As cells could not be separated from substrate particles to estimate biomass, cell counting of the entire sample was performed. After 72 hours all three fermentations appeared to reach completion as indicated by cell numbers (**Figure 2.**).

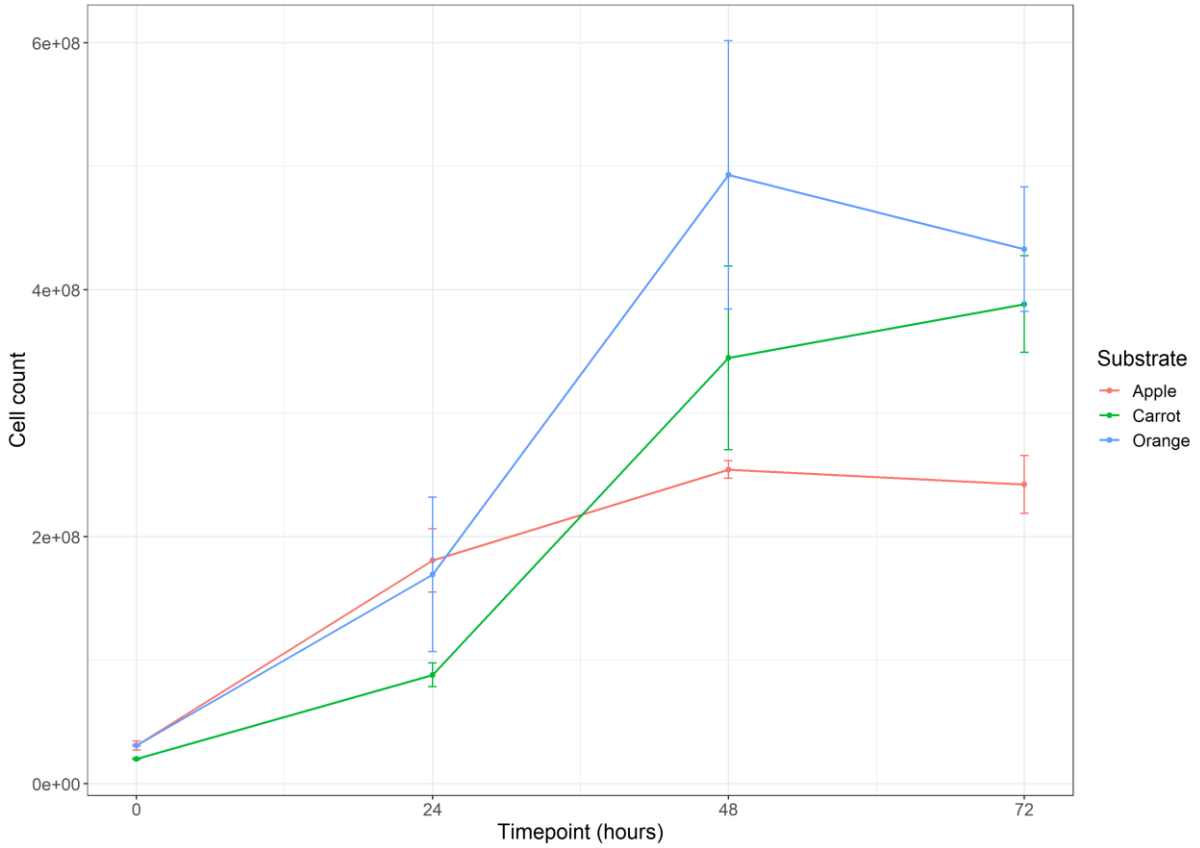


Figure 2. Average numbers of *Brettanomyces bruxellensis* cells counted during submerged-state fermentation of apple-, carrot-, and orange pomaces. Error bars show the standard deviation between replicates (n=5).

Reducing sugar concentrations were used as a second measure of fermentation performance (**Figure 3.**)

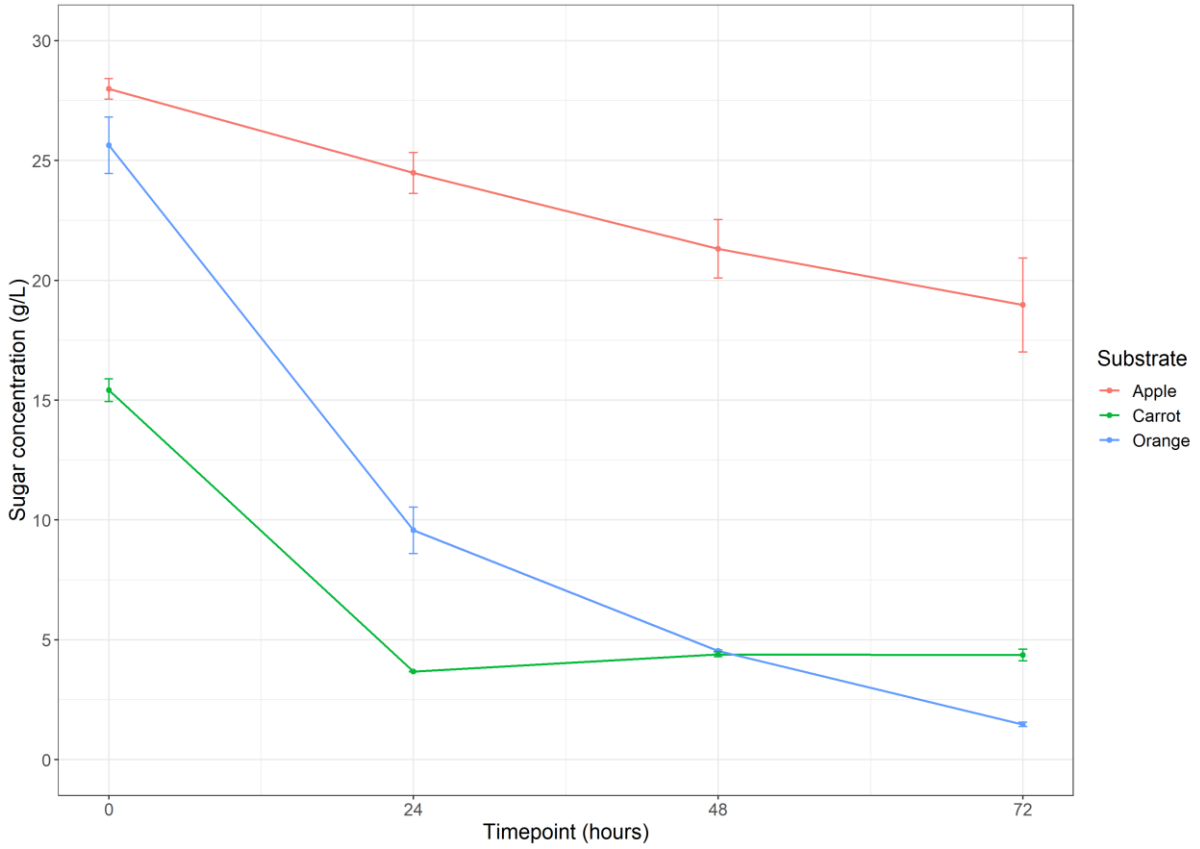


Figure 3. Average soluble reducing sugar concentrations in apple, carrot-, and orange pomace during fermentation with *Brettanomyces bruxellensis*. Error bars show standard deviation between replicates (n=5).

Profiling of volatile compounds

Over 800 volatile compounds were identified following fermentation of apple pomace (189 compounds), carrot pomace (327 compounds), and orange pomace (332 compounds). However, only about half of the compounds increased in abundance or were produced *de novo* after fermentation: apple pomace (106 compounds), carrot pomace (160 compounds) and orange pomace (142 compounds). Carrot pomace produced the most compounds through fermentation. Compounds of interest produced in the three fermentations that had match factors of over 90% to the NIST2014 spectral library were further investigated for their organoleptic

properties. Compounds that increased in abundance compared with an unfermented control are shown in **Table 10**.

Table 10. Organoleptic qualities of potentially industrially-relevant volatile compounds produced during submerged-state fermentations of three fruit and vegetable by-products using *Brettanomyces bruxellensis*.

Compound	Match %	Reference ion	Substrate	Fold change	Descriptor
1-Butanol, 2-methyl-	97	57	Carrot	887.82	Black truffle
1-Butanol, 3-methyl-	100	55	Carrot	797.93	Fusel, banana
	94	55	Orange	230.85	
1-Butanol, 3-methyl-, acetate	97	43	Apple	5.17	Banana, fruity
Acetic acid	99	43	Apple	6.12	Vinegar
Acetic acid, 2-phenylethyl ester	97	104	Apple	22.44	Floral, honey
	95	104	Orange	313.01	
Benzyl alcohol	98	79	Apple	4.96	Sweet, floral, balsamic
Butanoic acid, 2-methyl-	98	74	Carrot	392.19	Acidic, cheesy
	95	74	Orange	543.54	
Butanoic acid, 2-methyl-, ethyl ester	98	57	Orange	256.57	Fruity, green apple
Decanoic acid, ethyl ester	97	88	Apple	6.35	Waxy, sweet, fruity
Decanoic acid, methyl ester	100	74	Carrot	2623.43	Oily, wine-like

Dodecanoic acid, ethyl ester	97	88	Orange	68.75	Sweet, waxy
Ethyl Acetate	99	43	Apple	5.90	Ethereal, fruity
Heptanoic acid, ethyl ester	95	88	Orange	103.77	Pineapple
Heptanoic acid, methyl ester	100	74	Carrot	370.38	Orris, currant,
Hexanoic acid, ethyl ester	93	88	Orange	87.26	Sweet, fruity, pineapple
Hexanoic acid, methyl ester	100	74	Carrot	206.73	Pineapple
Isopentyl hexanoate	99	70	Carrot	107.59	Apple, pineapple
Methyl valerate	99	74	Carrot	899.52	Fruity
Nonanoic acid, ethyl ester	95	88	Apple	55.69	Waxy, fruity
Nonanoic acid, methyl ester	94	74	Carrot	351.76	Wine, coconut
Octanal, 7-hydroxy-3,7-dimethyl-	96	59	Apple	15.43	Floral, lily, green
Pentadecanoic acid, 3-methylbutyl ester	92	70	Orange	57.45	Waxy, banana
Phenol, 4-ethyl-2-methoxy-	90 97	137 137	Apple Orange	91.11 8358.92	Smoky, spicy, clove, wine taint

Phenylethyl Alcohol	93	91	Carrot	325.70	Floral, rose
Propanoic acid, 2-methyl-	95	43	Orange	295.86	Acidic, sour, cheesy

A compound tentatively identified as phenylethyl alcohol in the initial screening (see Chapter 1) was categorised as a compound of interest. The identity of the compound was confirmed as phenylethyl alcohol by GC-MS on the basis of its retention time and mass spectrum compared with a pure chemical standard.

Table 11 Phenylethyl alcohol value, consumption and yield on carrot pomace fermented with *Brettanomyces bruxellensis*

Compound	**Value ($/kg)***	**Annual consumption (tonnes)****	**Substrate**	**Yield (mg)*****
Phenylethyl alcohol	$500*	14 000	Carrot pomace	50.96 ± 0.718

*Value per kilogram of pure, natural fragrance chemical (personal communication Jeffrey Buco of Excellentia International). Prices quoted in US dollars and correct as of September 2017.

**Estimate for fragrance and flavours industries (Schwab et al., 2008)

***Yield as average dry weight of compound produced per kilogram of dry weight carrot pomace ± 2 standard deviations and n=9.

Yield was determined from the wet weight of carrot pomace used for the fermentation and was scaled up to reflect how much compound would theoretically be produced and extracted from one kilogram of fermented substrate.

3.4 Discussion

Over 400 volatile compounds were produced by submerged-state fermentation of three different fruit and vegetable pomaces using *B. bruxellensis*. Many of the most interesting compounds from an organoleptic point of view compounds were esters and had fruity, floral or sweet notes. All three fermentations utilised the free reducing sugars available in the pomaces. (**Figure 3.**) However, none were completely exhausted. While the cell counting data appeared to support the fermentation reaching stationary phase, it is possible that the

fermentation became “stuck” due to limitation of some other nutrients. In winemaking, this is a common phenomenon when there is inadequate yeast available nitrogen (Beltran et al., 2005; Deed et al., 2011). This leads to the cessation of cell division due to insufficient nitrogen, even in the presence of high titres of fermentable sugars. This may have occurred in the case of carrot pomace and apple pomace fermentations. Alternatively, there may be a mixture of sugars present in the pomaces where one sugar is preferentially utilised over another. In this case, the stationary phase that appears to be present may instead be a second lag phase. Accordingly, the fermentation duration could potentially be extended.

The most significant compound of interest was phenylethyl alcohol. Compared with the solid-state fermentation using spent brewer’s grain and *Pycnoporus cinnabarinus* (**Chapter 2, Table 6**), the submerged-state fermentation using carrot pomace and *B. bruxellensis* produced over three times more phenylethyl alcohol from the same wet weight of substrate (**Table 11**).

Phenylethyl alcohol is commonly found in household cleaning products, deodorizers, soaps, cosmetics and perfumes as a fragrance ingredient and preservative. Additionally, over one tonne is consumed in food and beverage products annually. Most is cheaply produced through chemical synthesis using benzene, styrene or toluene. However, phenylethyl alcohol produced by this method cannot be labelled as “natural”. Natural phenylethyl alcohol is traditionally extracted from rose petals in the form of rose essential oil (~60% phenylethyl alcohol) with an extremely low yield (0.03-0.04%) (Baydar, 2006; Etschmann et al., 2002). Several biotechnological attempts have also been made to produce phenylethyl alcohol from the amino acid L-phenylalanine, with titres of over 2 g/L achieved in media (Etschmann et al., 2002; Mei et al., 2009). Although titres achieved through other biotechnological methods are higher than are described here, carrot pomace may still be a competitive option given the high price of L-phenylalanine and other sugar feedstocks. Rose petals, the current natural source of

phenylethyl alcohol, cost thousands of dollars per tonne and the extraction process is both energy and labour intensive (Baydar, 2006). On the other hand, carrot pomace is an extremely cheap and very abundant substrate with over 20 thousand tonnes produced annually by the New Zealand juicing industry alone (Farmex New Zealand, personal communication). Accordingly, further optimisation of the carrot pomace fermentation could identify a promising alternative source of natural phenylethyl alcohol.

The solid-state fermentation described in **Chapter 2** using brewer's spent grain showed an initial promising yield of phenylethyl alcohol (0.970 mg/kg); however, the yield from the submerged-state fermentation using carrot pomace far exceeded this (3.693 mg/kg). Compared with solid-state fermentation, submerged-state fermentation is already much more industrially advanced and there is existing scale-up technology, e.g. bioreactors, large tank fermenters including continuous, batch-, and fed-batch systems (Yusef, 1999). An optimised, submerged-state fermentation using carrot pomace could be both industrially feasible and financially competitive if yield can be improved.

There are several other yeasts already known to produce phenylethyl alcohol, but this is the first report of *B. bruxellensis* being used to produce phenylethyl alcohol for use as a fragrance and flavour additive from the agro-industrial waste product, carrot pomace. With this in mind, further optimisation experiments should investigate the potential of other yeasts to produce phenylethyl alcohol from carrot pomace to determine if yields can be improved by changing only the fermenting organism. Several other yeast have been investigated for their ability to produce phenylethyl alcohol: *Kluyveromyves marxianus, Saccharomyces cerevisiae* and the opportunistic pathogen *Candida albicans* (Etschmann et al., 2002; Ghosh et al., 2008). These yeasts can produce phenylethyl alcohol by two different methods. First, by bioconversion of phenylalanine into phenylethyl alcohol via the Ehrlich pathway and second, *de novo* through glycolysis and the Shikimate pathway (**Figures 4 and 5**) (Akita et al., 1990;

Ayrapaa, 1965; B. Kim et al., 2014; Liu, 2015). These three yeasts would be an interesting starting point to investigate if yield can be improved by selecting a different fermenting organism already known to produce phenylethyl alcohol and determining their ability to use carrot pomace as a growth medium. While most yeasts have been investigated for their potential to convert phenylalanine into phenylethyl alcohol, it would be more attractive to investigate whether they can produce phenylethyl alcohol either from phenylalanine present in the carrot pomace, or *de novo* using sugars available in carrot pomace as the substrate.

3.5 Conclusion

Three different agro-industrial by-products were screened; apple-, orange-, and carrot pomace, fermented by *B. bruxellensis,* a non-*Saccharomyces* yeast, in a submerged-state fermentation. Over 800 volatile compounds were identified in the fermented and unfermented pomaces, and over 400 volatile compounds that were produced *de novo* or increased in abundance as a result of the fermentation. One compound of interest, phenylethyl alcohol, was produced in a yield of over 3 mg/ kg of unsupplemented carrot pomace as the only substrate in a submerged-state fermentation. This had already been identified as a compound of interest (**Chapter 2**) and the yield in the present experiment was over three times higher. As a result, phenylethyl alcohol was selected as the compound of interest to optimise throughout the remainder of this thesis.

CHAPTER IV

4 SUBMERGED-STATE FERMENTATION OF CARROT POMACE USING SIX YEAST STRAINS FOR THE PRODUCTION OF PHENYLETHYL ALCOHOL

Melodie A. Lindsay [a], Ninna Granucci [b], David R. Greenwood [a] and Silas G. Villas-Bôas [a,b]

[a] School of Biological Sciences, University of Auckland, 3A Symonds Street, Auckland 1142, New Zealand.

[b] Greenspot Technologies, Toulouse White Biotechnology, 3 rue Ariane, Ramonville-Saint Agne 31520, France.

4.1 Introduction

Phenylethyl alcohol is a rose-scented fragrance additive commonly used in perfumes, household cleaning products, cosmetics, soaps, deodorizers, food items and beverages (Chreptowicz et al., 2016; Etschmann et al., 2002; Martínez-Avila et al., 2018). Over 14 000 tonnes is consumed annually making it one of the most popular fragrance additives (Schwab et al., 2008). Most phenylethyl alcohol is produced synthetically however, there is a consumer movement to change this in favour of natural alternatives (Carlquist et al., 2015). Currently, most natural phenylethyl alcohol is produced through the distillation of rose petals to obtain an essential oil. While the essential oil contains approximately 60% phenylethyl alcohol, it takes several tonnes of rose petals to produce one kilogram of essential oil (Baydar, 2006). This makes the end-product very expensive (>$1000 US/kg) and in short supply. As a result, there is a demand for alternative natural sources of phenylethyl alcohol.

While a number microorganisms can produce phenylethyl alcohol including *Aspergillus niger, Aspergillus oryzae* and *Enterobacter* spp., yeasts are the most well-known and studied (Lomascolo et al., 2001; Masuo et al., 2015; Zhang et al., 2014). A range of yeast including: *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Yarrowia lipolytica, Candida* spp., *Kluyveromyces* spp., *Pichia* spp., and *Metschnikowia* spp. produce phenylethyl alcohol (Chreptowicz et al., 2018; Fabre et al., 1998; Martínez-Avila et al., 2018).

Phenylethyl alcohol is produced by yeasts either *de novo* from sugar through the shikimate pathway (**Figure 4.**), or from the amino acid precursor – phenylalanine through the Ehrlich pathway (**Figure 5.**) (Akita et al., 1990; Ayrapaa, 1965; B. Kim et al., 2014; Liu, 2015). To produce phenylethyl alcohol via the Ehrlich pathway, there are three main steps: 1) transamination of L-phenylalanine to phenylpyruvate, 2) decarboxylation of phenylpyruvate to phenylacetaldehyde and 3) reduction of phenylacetaldehyde to phenylethyl alcohol (Etschmann et al., 2003; T. Y. Kim et al., 2014).

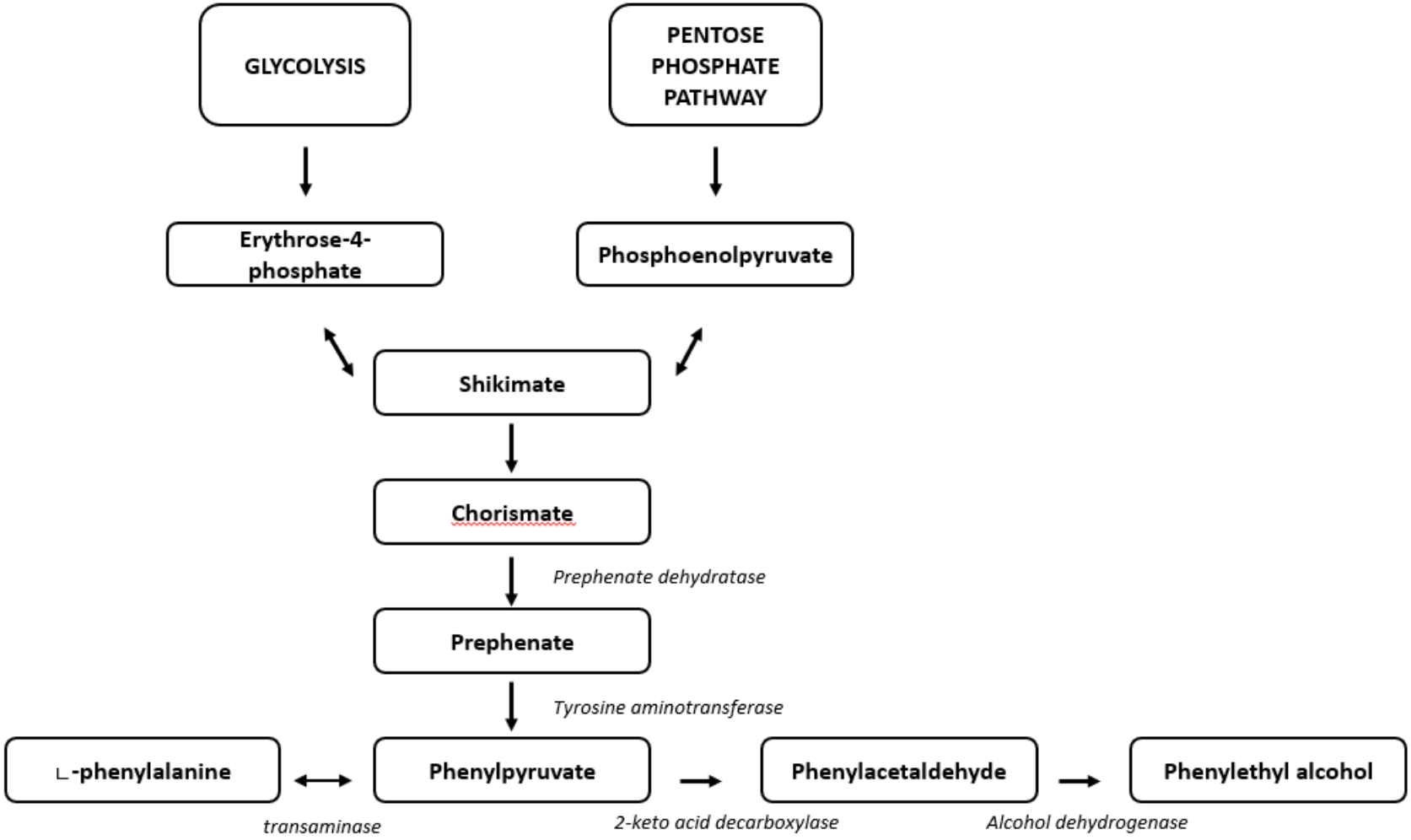


Figure 4. *De novo* production of phenylethyl alcohol via the Shikimate pathway.

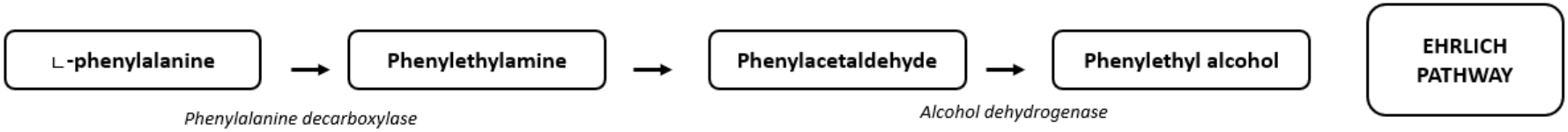


Figure 5. Production of phenylethyl alcohol from the precursor L-phenylalanine via the Ehrlich pathway.

Several biotechnological routes to produce natural phenylethyl alcohol already exploit this pathway to convert phenylalanine into phenylethyl alcohol (Celinska et al., 2013; Chantasuban et al., 2018; Chreptowicz and Mierzejewska, 2018; Fabre et al., 1998; Siddiqui et al., 2012). The most well studied yeasts for this are *S. cerevisiae* and *Kluyveromyces marxianus*. *S. cerevisiae* is known to produce up to 4.8 g/L of phenylethyl alcohol in liquid-state fermentation while *K. marxianus* produces only half as much (Adler et al., 2010; Fabre et al., 1998; B. Kim et al., 2014). Most media are glucose-based, and supplemented with high levels of phenylalanine − a precursor of phenylethyl alcohol. This is currently the most established and efficient route for production of phenylethyl alcohol. However, L-phenylalanine is an expensive precursor and there are still limitations due to toxicity of phenylethyl alcohol to the yeast cell (Chreptowicz et al., 2018; Etschmann et al., 2003; Fabre et al., 1998).

A second route to producing phenylethyl alcohol is through the Shikimate pathway (**Figure 4.**) whereby yeast can produce phenylethyl alcohol *de novo* from carbohydrates. This pathway uses phosphoenolpyruvate from glycolysis and erythrose-4-phosphate from the pentose-phosphate pathway which are converted to phenylpyruvate. Phenylpyruvate is then decarboxylated to phenylacetaldehyde and then reduced to phenylethyl alcohol as with the Ehrlich pathway (Carlquist et al., 2015). As carrot pomace contains both free phenylalanine and sugars, there are two potential pathways by which yeasts could produce phenylethyl alcohol (Granucci, 2018).

In Chapter 2, *Brettanomyces bruxellensis* was identified as a good producer of phenylethyl alcohol when fermenting the agro-industrial by-product − carrot pomace. As many yeasts produce phenylethyl alcohol, and *B. bruxellensis* is not well renown for this property,

five other yeast species already known to produce phenylethyl alcohol were selected, to compare their ability to produce phenylethyl alcohol using carrot pomace. These include three *Candida* spp., along with *Kluyveromyces marxianus* and *Saccharomyces cerevisiae.S*

This chapter compares the production of phenylethyl alcohol across five yeast strains: *S. cerevisiae*, *K. marxianus*, *C. albicans*, *C. lipolytica*, *C. utilis*, and *B. bruxellensis* to determine an optimal strain of yeast that can produce phenylethyl alcohol when growing on carrot pomace.

4.2 Materials and Methods

4.2.1 Yeast strains

Six yeast strains were selected to ferment carrot pomace to determine an optimal strain for phenylethyl alcohol production: *Brettanomyces bruxellensis* CCT3467, *Saccharomyces cerevisiae* EC1118, *Kluyveromyces marxianus* P1/SVB-Y103, *Candida albicans* MEN SVB-Y25, *Candida utilis* DSM70167, and *Candida lipolytica* ICMP 14995. Submerged-state fermentations were carried out as in **Chapter 2** and summarised below.

4.2.2 Submerged-state fermentations

Carrot pomace sourced from RD2 International was stored at -20 °C and protected from light and air in food grade, resealable freezer bags. In preparation for fermentation, the pomace was thawed at 4 °C overnight.

Six yeast strains were maintained on agar plates containing malt extract–yeast extract–glucose–peptone (MYGP) (3 g/L yeast extract, 3 g/L malt extract, 6 g/L peptone, 10 g/L glucose and 15 g/L agar) at 30 °C. Preinoculum was prepared by inoculating 200 mL of MYGP broth with a single yeast colony in Erlenmeyer flasks (1 L) fitted with cotton stoppers. Flasks were left at 30 °C with agitation at 200 rpm in darkness. Preinoculum was grown for 2-days and corrected to a starting optical density of 0.300 (20-fold diluted sample at 600nm). Aliquots

of 20 mL preinoculum were measured into 50 mL falcon tubes and pelleted by centrifugation (3000 g, 10 min). Residual media was poured off and the resulting cell pellet washed twice with sterile saline (0.9 % w/v NaCl).

For the fermentations, carrot pomace (60 g, 85.5% moisture) was weighed into 500 mL silanised round-bottom flasks along with distilled water (120 mL). Flasks were fitted with cotton stoppers and sterilised by autoclaving (121 °C, 20 min). These were inoculated with the preinoculum resuspended in sterile saline (0.9 % w/v NaCl) for a total fermentation volume of 200 mL. These were agitated (200 rpm) in the dark at 30°C. Three replicates for each yeast strain and corresponding sterile, negative controls were analysed in parallel.

Samples (10 mL) were taken at the beginning of the fermentation (Time = 0 hours) and every 24 hours for six days. At each time point, samples were checked for contamination and an attempt was made to count cell numbers per millilitre of fermented carrot pomace. However, the yeast cells associated very tightly with particles of carrot and results were inconsistent. Due to this, samples were stored at -20 °C for analysis of total sugars and protein content to monitor the fermentation progress as well as to determine phenylethyl alcohol concentration at each time point.

4.2.3 Determination of sugars composition and concentration

Trimethyl silyl (TMS) derivatization was used to characterise sugars and determine their concentration at each time point based on the method of Villas-Bôas et al. (2006). Samples were prepared in daily batches. Briefly, filtered samples (20 µL) were mixed with the internal standard D-ribitol (20 µL, 10 mM), dried using a rotary vacuum dryer and kept in a desiccator under vacuum over P_5O_5 overnight. Dried samples were resuspended in a methoxyamine hydrochloride solution in pyridine (80 µL, 2 g/100 mL), and incubated at 30°C for 90 min. N-Methyl-N- (trimethylsilyl)-trifluoroacetamide (MSTFA) (80 µL) was added, incubated at 37°C

for 30 min, and analysed by GC-MS as described by Villas-Bôas *et al.* (2006). A calibration curve of sugar standards (concentrations ranging from 0.0625 mM to 30 mM) was prepared using the same method and run with each batch of samples.

4.2.4 GC-MS sugars identification and quantification

The TMS derivatized samples were analysed using a GC-7890 gas chromatograph (Agilent Technologies, Santa Clara, CA) coupled to a MSD-5975 mass spectrometer (Agilent Technologies, Santa Clara, CA) with a quadrupole mass selective detector. Samples were introduced via direct injection followed by electron impact (EI) ionization operated at 70 eV. A Zebron ZB-1701 column (30 m) (Phenomenex) with internal diameter 250 µm and film thickness 0.15 µm was used with a 5m guard column. The MS was operated in scan mode with a solvent delay of 5.15 min and mass range 38-659 a.m.u. at 1.47 scans/s.

Sugars were identified by comparing the retention time and mass spectra of sugar standards run in each batch of samples. AMDIS (Automated Mass Spectral Deconvolution and Identification system) was used to deconvolute and assign identifications based on an in-house TMS mass spectral library. Automated integration of peak height was performed using an in-house R package 'MassOmics' and manual corrections were made to remove false identifications and correct for retention time shifts. Peak heights were normalised by D-ribitol and sugars quantified using the sugar calibration curves.

4.2.5 Protein quantification for estimation of biomass

Protein content was estimated at each time point using a method adapted from the Bradford method (1976). Briefly, each sample (500 µL) was mixed with 0.7 M NaOH (200 µL) and heated at 100 °C for 20 min to disrupt yeast cell membranes and release protein. Samples were cooled to room temperature and then centrifuged (12 000 rpm, 5 min) to separate carrot particles from the supernatant. The supernatant was diluted 10-fold with NaOH (0.2 M) and

loaded into microtiter plate wells (20 µL). Coomassie (Bradford) protein assay reagent (200 µL) was added and mixed well. A calibration curve of bovine serum albumin (BSA) was prepared in the same way as the samples (concentration range from 10 µg/mL to 1000 µg/mL in 0.2 M NaOH). Plates were read using a Spectramax iD3 plate reader (595 nm) within 45 min. Protein content of the fermented carrot pomace was quantified by using the calibration curve.

4.2.6 Rapid GC-MS quantification of phenylethyl alcohol

Phenylethyl alcohol was extracted from fermented carrot pomace using a two-step extraction process with ethyl acetate. Briefly, each sample (1000 µL) was mixed with internal standard d4-methanol (10 µL). Approximately 300 mg anhydrous NaCl was added until saturation. Ethyl acetate (350 µL) was added, vortexed and sonicated for 3 min. Samples were then centrifuged at 2000 *g* and the non-aqueous phase transferred to a separate vial. A second extraction was performed by adding ethyl acetate (150 µL) to the same sample. This was vortexed and sonicated (3 min). The upper non-aqueous phase was pooled with the first and any excess water removed with sodium sulfate (~50 mg). The dry sample was then transferred to an amber GC-MS vial for analysis. For quantification, a calibration curve of phenylethyl alcohol standards was prepared in ethyl acetate and run with each batch of samples.

A rapid GC-MS method was adapted from the method by Pinu and Villas- Bôas (2017) and targeted to phenylethyl alcohol. The GC-MS parameters are described below (**Table 12**).

Table 12. GC-MS parameters for rapid quantification of phenylethyl alcohol

General settings		GC-capillary column		Inlet	
Ionization voltage:	70 eV	**Film thickness:**	0.15 µm	**Injection mode:**	Split
Ion source temperature:	230 ˚C	**Diameter:**	250 µm	**Injection temperature:**	180 ˚C
Detector voltage:	0.70-0.15 kV	**Length:**	30.0 m	**Split Flow:**	97.701 mL/min
Interface temperature:	250 ˚C	**Flow:**	0.97701 mL/min	**Split ratio:**	100:1
Quadrupole temperature:	150 ˚C	**Carrier gas:**	Helium		
Ionization mode:	Electron Impact	**Type:**	Phenomenex Zebron ZB-1701		
Acquisition mode:	SIM				
m/z:	33.1, 35.1, 91.0, 122.0				

The GC oven temperature was initially held at 50 °C for 1 min then raised to 200 °C at a rate of 40 °C/min. Total run time of the method was 4.75 min. Phenylethyl alcohol was quantified using normalised peak heights and compared with the calibration curve.

4.3 Results

4.3.1 Fermentation performance

Cell numbers in the substrate were initially used as a qualitative measure of fermentation performance. However, this was found to be prohibitively time-consuming and unreliable (**Supplementary figures 1-4**). Accordingly, total sugars and protein content was used to monitor cell growth and estimate fermentation end points.

4.3.2 Sugar composition and quantification

Four different sugars or sugar derivatives were identified in carrot pomace: mannitol, fructose, ribose and arabinose. There was an unexpected absence of glucose and sucrose. In all

six fermentations, ribose and arabinose were detected at a concentration below 0.75 mM but were not quantifiable beyond that. Based on normalised peak heights, neither sugar changed in concentration during fermentation. However, the sugar alcohol mannitol, and fructose were used to different extents depending on the yeast strain. Fructose and mannitol concentrations are shown for each organism at three time points (**Figures 6** and **7.**).

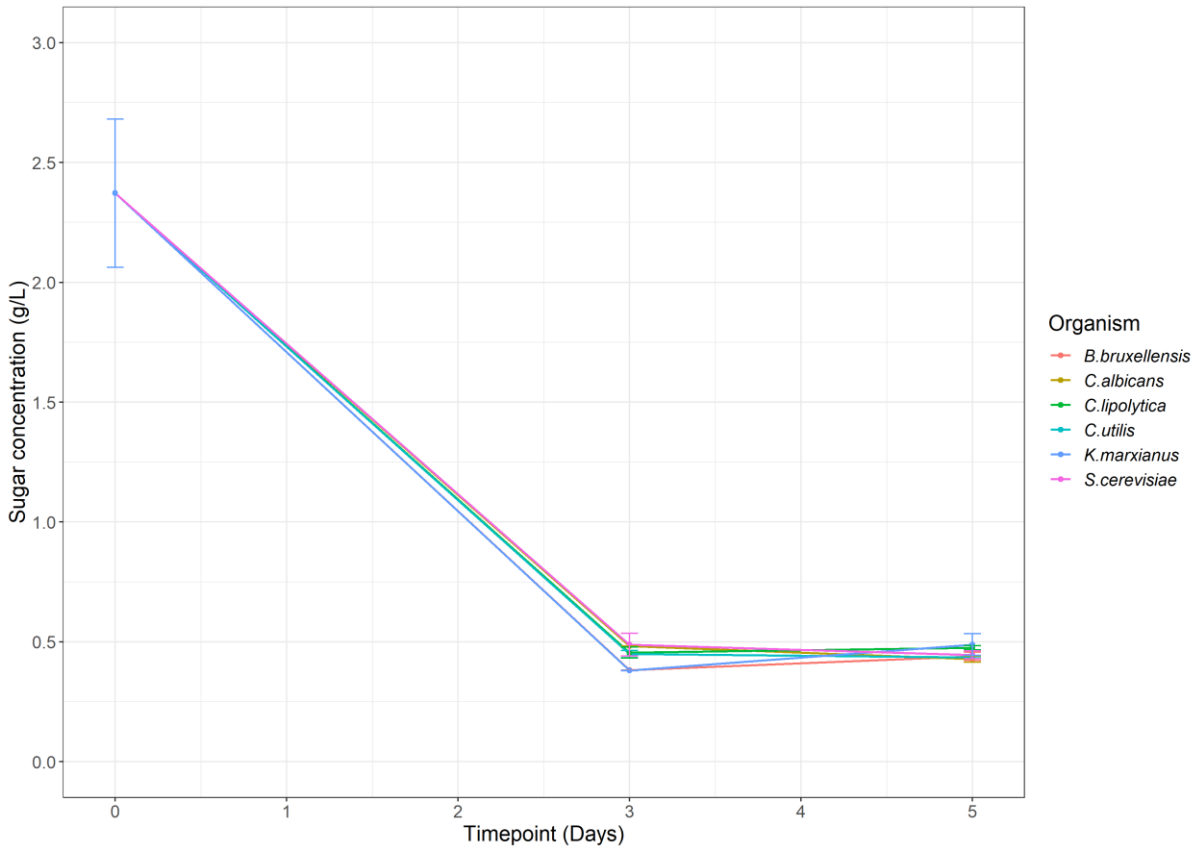


Figure 6. Average fructose concentration of *Brettanomyces bruxellensis, Candida albicans, Candida lipolytica, Candida utilis, Kluyveromyces marxianus* and *Saccharomyces cerevisiae* when grown in a submerged-state fermentation of carrot pomace over 5-days. N = 5.

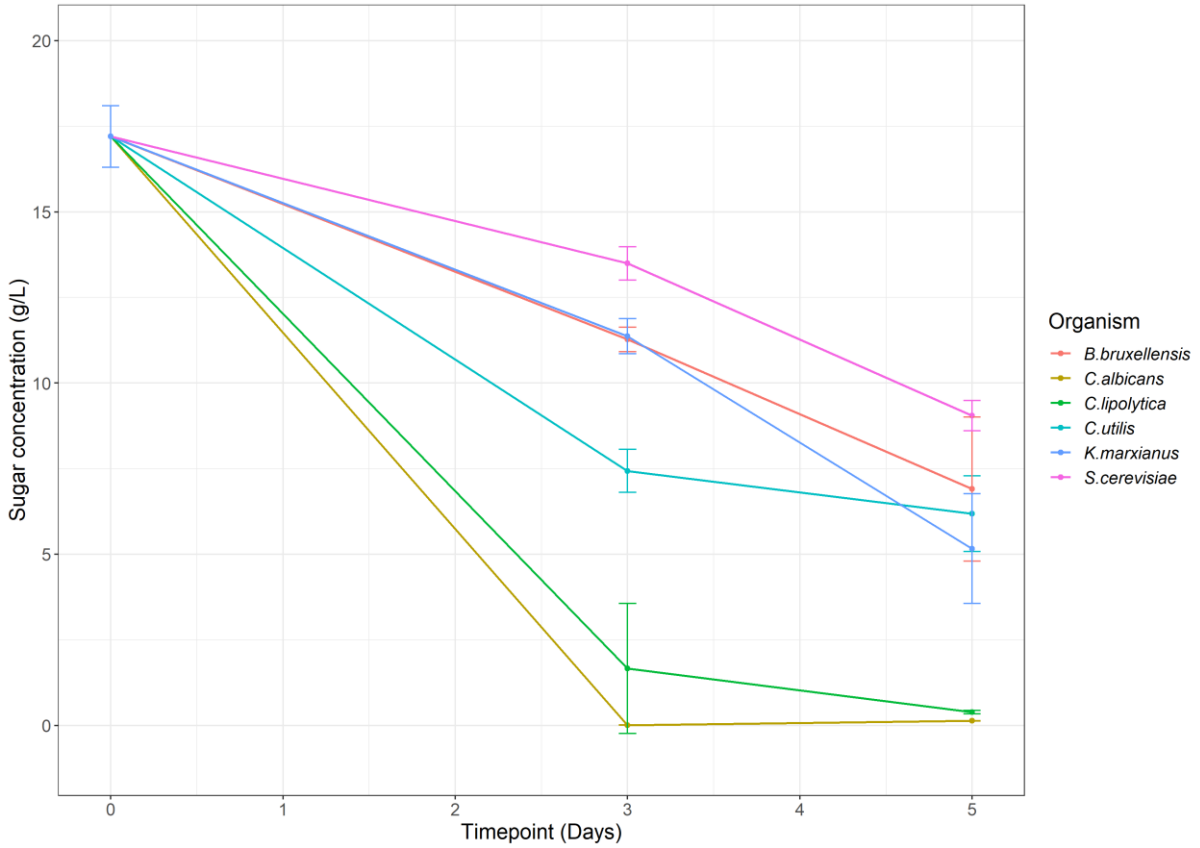


Figure 7. Average mannitol concentration of *Brettanomyces bruxellensis, Candida albicans, Candida lipolytica, Candida utilis, Kluyveromyces marxianus* and *Saccharomyces cerevisiae* when grown in a submerged fermentation of carrot pomace over 5-days. N = 5.

Before fermentation, fructose was detected at a concentration of 2.4 mM. This was completely exhausted by all six yeasts within three days. Mannitol (17.2 mM), on the other hand was used to different extents by the yeasts. *C. albicans* and *C. lipolytica* were the only yeasts that completely exhausted mannitol in the substrate while *C. utilis* used just over half (6.2 mM remaining after 5 days fermentation). The other three yeast strains: *B. bruxellensis; K. marxianus;* and *S. cerevisiae*, also used approximately half of the available mannitol.

4.3.3 Protein concentration

Protein concentration was measured as an indirect estimate of yeast biomass to monitor the fermentation (**Figure 8**).

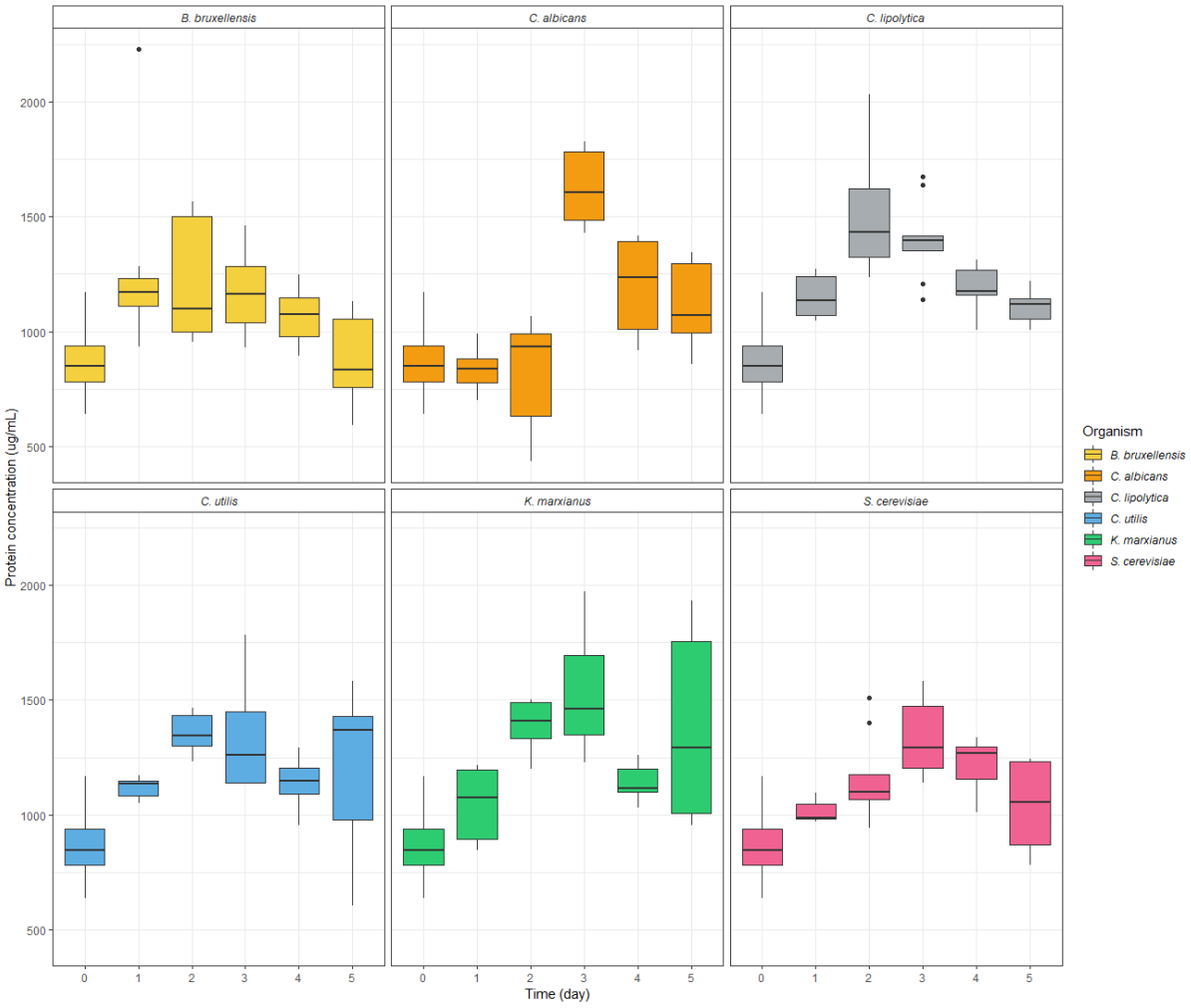


Figure 8. Box plots showing total protein concentration (µg/mL) of carrot pomace at 24-hourly time points for five days of fermentation using six yeast strains: *Brettanomyces bruxellensis*, *Candida albicans*, *Candida lipolytica*, *Candida utilis*, *Kluyveromyces marxianus*, and *Saccharomyces cerevisiae*. (n=9-45).

All six yeasts showed a similar trend in protein concentration where protein concentration increased in the first 3 days. However, protein concentration decreased in the last days of the fermentation. Samples were highly variable and tended to become more variable as the fermentation progressed. For all fermentations, protein concentration stopped increasing from about 3 days into the fermentation.

4.3.4 Phenylethyl alcohol production and yield

Phenylethyl alcohol was produced to different extents by the different yeast strains (**Figure 9**). *C. albicans* produced the most phenylethyl alcohol of all six yeast strains after 5-

6 days (1937 mg/kg) while *K. marxianus* (256 mg/kg) and *C. lipolytica* (172 mg/kg) produced the least. After five days, *B. bruxellensis* (469 mg/kg) and *S. cerevisiae* (564 mg/kg) produced approximately the same amount of phenylethyl alcohol. *C. utilis* was the highest producer of phenylethyl alcohol for the first three days but at the fermentation end point, was the second highest producer (1197 mg/kg) behind *C. albicans*. Phenylethyl alcohol yield on carrot pomace are displayed in **Table 13.** as both a dry weight of phenylethyl alcohol in mg/kg dry pomace substrate and as a percentage on dry carrot pomace.

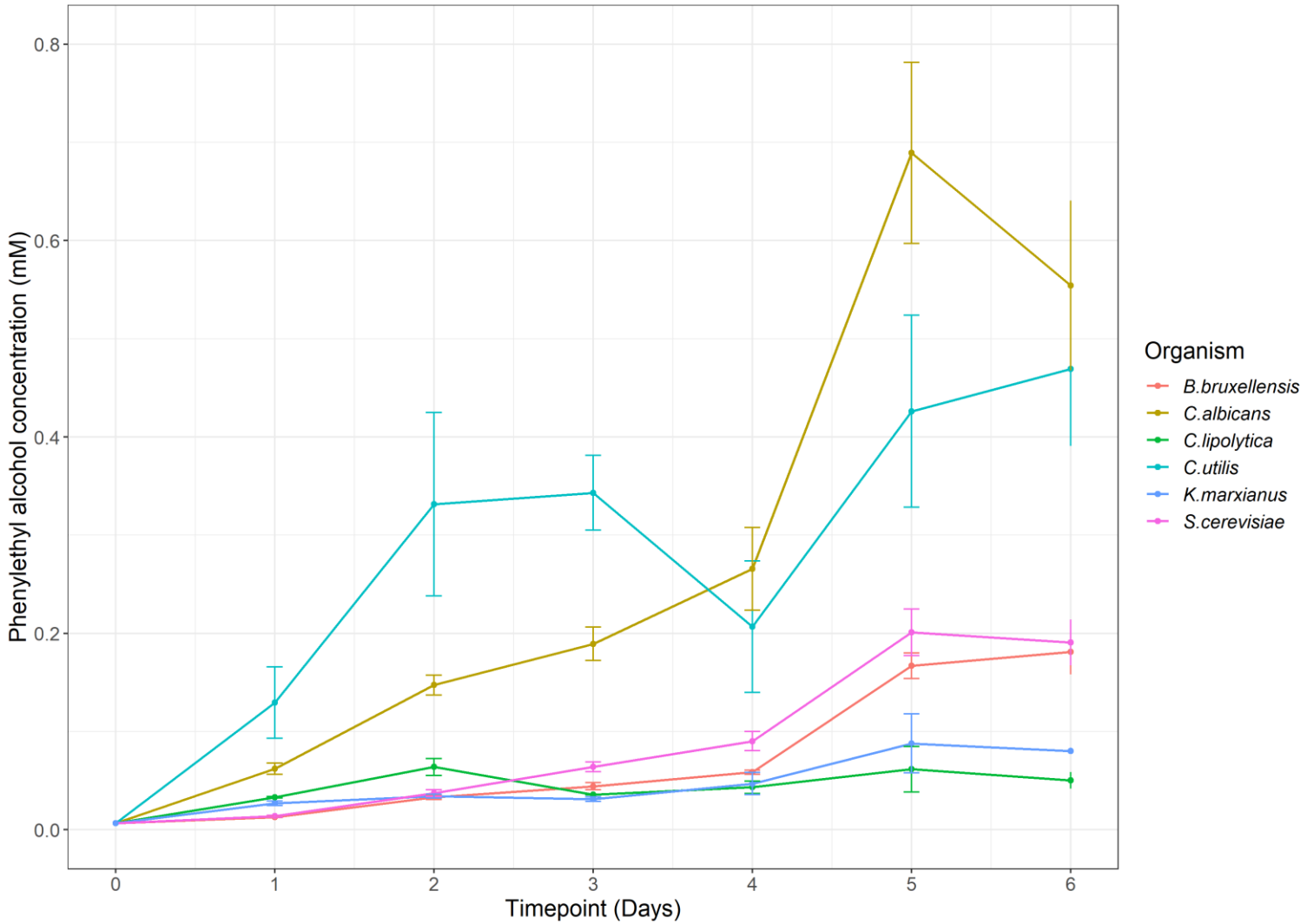


Figure 9. Average phenylethyl alcohol concentration during fermentation of carrot pomace by six yeast strains: *Candida utilis, Candida albicans, Candida lipolyica, Brettanomyces bruxellensis, Saccharomyces cerevisiae* and *Kluyveromyces marxianus* for six days: Error bars show ± 1 standard deviation between replicates (n=3-5).

Table 13. Phenylethyl alcohol yield on carrot pomace for six yeast strains

	Candida utilis		*Candida albicans*		*Candida lipolytica*		*Bretannomyces bruxellensis*		*Saccharomyces cerevisiae*		*Kluyveromyces marxianus*	
	Yield (mg)	Yield % w/w	Yield (mg/kg)	Yield % w/w	Yield (mg/kg)	Yield % w/w	Yield (mg/kg)	Yield % w/w	Yield (mg/kg)	Yield % w/w	Yield (mg/kg)	Yield % w/w
0	18.46	0.00	18.46	0.00	18.46	0.00	18.46	0.00	18.46	0.00	18.46	0.00
1	363	0.04	174	0.02	92	0.01	35	0.00	39	0.00	75	0.01
2	931	0.09	414	0.04	180	0.02	92	0.01	104	0.01	96	0.01
3	964	0.10	532	0.05	100	0.01	124	0.01	180	0.02	87	0.01
4	581	0.06	746	0.07	121	0.01	164	0.02	253	0.03	132	0.01
5	1198	0.12	1937	0.19	173	0.02	469	0.05	565	0.06	247	0.02
6	1318	0.13	1557	0.16	141	0.01	509	0.05	535	0.05	225	0.02

Legend: Yield mg = yield of phenylethyl alcohol per kilogram of dry carrot pomace, Yield % = phenylethyl alcohol yield on dry carrot pomace.

4.4 Discussion

Cell-counting was initially attempted to assess fermentation end point (**Supplementary Figures 1-4.**). However, cell numbers per millilitre of substrate were variable and examination under bright-field light microscopy showed that yeasts tightly associate with the carrot pomace particles during fermentation. Therefore, cell numbers counted were skewed by this phenomenon. Consequently, sugar concentration and protein concentration were measured to monitor the fermentation progression.

Based on previous work, sucrose, fructose and glucose was expected to be the main sugars present in carrot pomace (Granucci, 2018). However, of these three sugars, only fructose (2.4 mM) was detected. Three other sugars: mannitol (17.2 mM), arabinose (<0.75 mM) and galactose (<0.75 mM), were unexpectedly detected. It was suspected that the carrot pomace had begun fermenting prior to receiving it in the laboratory. The pomace had a distinct "sour" smell upon receipt, and it had been stored at 4 °C for a short period of time before transport. These observations combined with the sugar data make it highly likely that the pomace was fermented before receipt, probably by psychrotrophic bacteria. Mannitol is known to be produced by lactic acid bacteria and *Leuconostoc* spp. from the fermentation of glucose, fructose and sucrose (Giglio and Mccleskey, 1953; Saha and Racine, 2011). Based on previous work by Granucci (2018), all three sugars – glucose, fructose and sucrose were present in fresh carrot pomace on previous occasions. This is an important factor to consider for any downstream commercial applications of using carrot pomace as there must be adequate quality control and testing to ensure the pomace is kept in appropriate conditions, so it is not spoiled, toxic or otherwise it will negatively affect the fermentation process.

As the pomace was severely limited in sugars and only fructose and the sugar alcohol – mannitol were available for growth and fermentation, it was unsurprising that all yeasts

quickly exhausted fructose. Mannitol was used to varying degrees depending on the yeast strain used. *C. albicans* and *C. lipolytica* were able to completely exhaust mannitol within 5 days of fermentation (**Figure 7.**). Interestingly, *Candida* spp. are able to utilise glucose, sucrose, maltose, fructose, mannose and xylose in a mixture of sugars without any additional lag phases. However, this was not the case for mannitol. In a mixture of glucose and mannitol, glucose was completely exhausted first followed by a clear diauxic shift to using mannitol. This lag phase was likely due to the presence of dedicated mannitol transporters in *Candida* spp. which are repressed by the presence of glucose and possibly the other preferred sugars above, and induced by mannitol (Niimi et al., n.d.). Interestingly, *C. utilis* did not deplete mannitol which began to plateau. It may have required another day to completely use up mannitol or, it may be due to the limitation of other nutrients e.g. nitrogen which could have stalled the fermentation progression. One other factor to consider in the utilisation of mannitol by *Candida* spp. is that some species can produce enzymes that catalyse the oxidation of mannitol (Chakravorty et al., 1962). It has been suggested that this allows conversion of mannitol back into fructose (Chakravorty et al., 1962). It could be valuable to investigate concentrations of both mannitol and fructose at more frequent time intervals to determine if any of the *Candida* spp. are indeed converting mannitol back into fructose to complete the fermentation.

K. marxianus, *S. cerevisiae* and *B. bruxellensis* all depleted mannitol but to a lesser extent than the *Candida* spp. (**Figure 7.**). This was an expected observation as none of these three strains are well known for their ability to utilise mannitol as a carbon source. Different strains of these yeast are found to utilise mannitol to different extents in literature for example, some yeast cannot uptake mannitol at all (Boulton, 2019). As mannitol concentration decreased as the fermentation progressed for all yeasts, this is clearly not the case for this fermentation. *S. cerevisiae* was shown to have a 14-21 day lag period in order to transition to utilising mannitol (Boulton, 2019). This suggests that mannitol is not a favourable carbon source for

this yeast. On the other hand, many yeast strains can uptake mannitol but either cannot metabolise it, or can only use mannitol for growth but not for fermentation e.g. to produce ethanol (Boulton, 2019; Lee and Lee, 2011; Maxwell and Spoerl, 1971). It is difficult to determine which of the latter is the case for *S. cerevisiae, K. marxianus* and *B. bruxellensis* but as mannitol is decreasing constantly for all three strains throughout the fermentation, it is arguable that they are able to use mannitol for growth and just need a longer fermentation time. As *S. cerevisiae, K. marxianus* and *B. bruxellensis* all produced significantly lower yields of phenylethyl alcohol, it is possible that they were only able to use mannitol for growth and not to produce secondary metabolites. Alternatively, they may be nutrient-limited as inferred by the decreasing protein levels/low biomass estimates from day three of the fermentation.

The low sugar content of the carrot pomace – especially as glucose and sucrose are absent entirely – suggests that an addition of a more favourable carbon source could favour biomass production and/or increase yields of phenylethyl alcohol. However, the carrot pomace may also be deficient in other vital nutrients (e.g. nitrogen). As an additional means of monitoring the fermentation, total soluble protein was measured every 24 hours. Protein increased for the first few days of fermentation. However, total protein began to decrease from around day three for all fermentations. There are likely several compounding factors at play here. The first factor could be nutrient limitations in carrot pomace. As the carrot pomace arrived in a pre-fermented condition and was already very low in sugars, it is likely that other nutrients were also depleted. From the protein levels, it appears that while yeast growth was sustained on fructose and possibly mannitol for the first few days of fermentation, yeast cells began to die off in the later stages of the fermentation. Viable yeast cells likely began degrading protein derived from dead cells once other nutrients ran out. This would account for the apparent decreases in protein content towards the end of fermentation. A second factor could be that the composition of protein in the sample can be variable. As the Bradford reagent

associates with specific amino acid groups, changes in composition of the proteins in the samples could contribute to the additional variation in protein content towards the end of fermentation. On this note, phenylethyl alcohol can be produced by yeast from the amino acid phenylalanine. This this is one of the main amino acids that the Bradford reagent interacts with, so it is possible that as phenylalanine was converted into phenylethyl alcohol, it further compounded the decreasing protein levels observed. Nevertheless, it appears that nutrient limitation in the carrot pomace has restricted biomass production for all yeast strains. As phenylethyl alcohol is reportedly associated with biomass production, it is possible that improving the nutrient content in the carrot pomace could improve phenylethyl alcohol production. Interestingly, from the phenylethyl alcohol production curves (**Figure 9**), it is evident that the maximum phenylethyl alcohol level has been produced by day 4-5 of the fermentation. Considering this, biomass may not be the only factor in phenylethyl alcohol production.

In this work, the fermentations were highly variable, especially towards the end of the fermentation. The small fermentation size for each time point and using samples of just 1 mL of carrot pomace from the fermentation may have misrepresented the total protein content of the entire sample. In addition, towards the end of the fermentation, the pomace becomes much less viscous. This may have skewed protein concentrations in the sample as the amount of carrot particles can vary significantly. To account for this, and to assess variation, up to 45 replicates for some fermentations were analysed. As variation across samples – both technical and biological was high, a larger fermentation volume with a larger sample size may allow use of the dry matter for alternative protein measurements (e.g. Kjeldahl method).

Phenylethyl alcohol was produced by all six yeast strains. *B. bruxellensis* was initially identified as a good producer of phenylethyl alcohol with a yield of 50.96 mg/kg dry carrot pomace after three days of fermentation (**Chapter 3**). After optimising the extraction method,

the same three-day fermentation produced 125 mg/kg phenylethyl alcohol. However, a five-day fermentation period yielded the highest levels of phenylethyl alcohol for most yeast strains in the present work. While *B. bruxellensis* produced 123.96 mg/kg phenylethyl alcohol, *C. albicans* (1937 mg/ kg) and *C. utilis* (1197 mg/kg) produced almost 20- and 10-fold more, respectively. Interestingly, they are both *Candida* spp. which are known to produce phenylethyl alcohol as a quorum-sensing molecule (Han et al., 2013). While many yeasts are known to produce phenylethyl alcohol as a quorum-sensing molecule, *C. albicans* is one of the most studied (Biswas and Morschhäuser, 2005; Chen and Fink, 2006; Csank and Haynes, 2000; Ghosh et al., 2008; Han et al., 2013). These yeasts produce phenylethyl alcohol in response to nitrogen starvation – a factor that may be limited in carrot pomace, especially towards the end of fermentation. Interestingly, *C. lipolytica* produced negligible amounts of phenylethyl alcohol which was unexpected. *C. lipolytica* is reported in literature to produce high titres of phenylethyl alcohol in synthetic medium containing phenylalanine as a precursor (Celinska et al., 2013). However, it is possible that carrot pomace was comparatively low in phenylalanine and *C. lipolytica* facilitates the conversion of this phenylethyl alcohol precursor rather than producing it *de novo* as may be the case for both *C, albicans* and *C. lipolytica*. In addition, *C. lipolytica* was able to completely exhaust both fructose and mannitol in which case, it potentially had less nutrient stress compared to the other yeasts.

Most biotechnological efforts to produce phenylethyl alcohol has focused on the bioconversion of phenylalanine to phenylethyl alcohol. While *S. cerevisiae, K. marxianua* and *C. lipolytica* may be well studied in regard to this, it is much more challenging to approach *de novo* production of phenylethyl alcohol. In the literature, these yeasts have produced high titres of phenylethyl alcohol using media rich in both glucose and phenylalanine. However, phenylalanine is an expensive precursor whilst agro-industrial by-products such as carrot pomace are extremely cheap and versatile. We have shown that phenylethyl alcohol can be

produced on unsupplemented carrot pomace. However, it will be important to establish if yields can be increased through supplementation of the substrate – without phenylalanine additions or, if phenylethyl alcohol is simply being produced through bioconversion of phenylalanine available to yeasts in the substrate. Given the distinct lack of phenylethyl alcohol production by all three well-known yeasts that facilitate phenylalanine bioconversion, *C. albicans* and *C. utilis* may be producing phenylethyl alcohol *de novo* through glycolysis and the Shikimate pathway (Chreptowicz et al., 2018; Fabre et al., 1998). This could be an essential factor in the biotechnological production of phenylethyl alcohol as without expensive precursor additions, it may keep production costs low enough to compete with other sources of natural phenylethyl alcohol.

While *C. albicans* produced the highest yield of phenylethyl alcohol on carrot pomace (0.19%), there are other factors to consider from an industrial point of view. One of the most important is assessing the GRAS status of a microorganism – especially as production is of a food additive. *C. albicans* is considered an opportunistic human pathogen and therefore does not have GRAS status (Personal communication, The Food and Drug Administration's (FDA) Food and Cosmetic Information Center, 2018). There are a few examples of other similarly classified-microorganisms e.g. *Escherichia coli* strains that have been granted GRAS status for specific applications under strict conditions of use which are available on the market e.g. rennet production for cheese making and coagulation of milk-products. In such cases, the microorganism may not be present in any form in the end product. However, there can also be health and safety risks associated with mass cultivation of an opportunistic pathogen which may be problematic to gain approval (Personal communication, FDA Food and Cosmetics Information Centre, 2018). This in itself can make the use of such microorganisms industrially unfavourable – despite high yields of a given product. Finally, while GRAS exemptions are also possible, this can be expensive and time-consuming as one must prove beyond all

reasonable doubt that the microorganism presents no threat to human (and animal) health. In this instance, considering a second candidate may be the more economically feasible choice of yeast e.g. *C. utilis* - capable of producing a high yield of phenylethyl alcohol (0.12%).

4.5 Conclusions

The carrot pomace used in this experiment was very low in sugars overall, and only low amounts of fructose and mannitol were available for production of biomass and secondary metabolites. All six yeast strains were capable of using fructose for both growth and fermentation. However, mannitol utilisation was more variable amongst the six yeast strains. Protein levels used to estimate biomass indicated that carrot pomace may be deficient in vital nutrients such as yeast-available nitrogen. Overall, *C. albicans* produced the highest yield of phenylethyl alcohol (0.19%) while *C. utilis* (0.12%) was a close second. The availability of GRAS status *C. utilis* combined with its relatively high production of phenylethyl alcohol on unsupplemented carrot pomace made it the best choice of yeast to continue with further optimisation trials.

Chapter V

5 FACTORIAL DESIGN OPTIMISATION OF CARROT POMACE FERMENTATION BY *CANDIDA UTILIS* FOR THE PRODUCTION OF PHENYLETHYL ALCOHOL.

Melodie A. Lindsay [a], Ninna Granucci [b], David R. Greenwood [a], Phillip J. Harris [a],

Gillian Lewis[a], and Silas G. Villas- Bôas [a,b]

[a] School of Biological Sciences, University of Auckland, 3A Symonds Street, Auckland 1142, New Zealand.

[b] Greenspot Technologies, Toulouse White Biotechnology, 3 rue Ariane, Ramonville-Saint Agne 31520, France.

5.1 Introduction

The rose-scented compound, phenylethyl alcohol is one of the most popular fragrance additives and is widely used in cosmetics, fragrances, deodorizers, soaps, cleaning products, beverages and other foodstuffs. Globally, over 14 000 tonnes is consumed globally per annum although most of this is synthetic. There is an increasing consumer demand for natural additives and as such, new methods for the production of natural phenylethyl alcohol.

Previous work (**Chapter 4**) identified *Candida utilis* as a good producer of phenylethyl alcohol when using carrot pomace as the sole substrate in a submerged fermentation. *C. utilis*, telemorph *Pichia jadinii* is a Crabtree negative, industrially important yeast – especially in the food industry (Henneberg 1921-cited in Buerth et al., 2016). It has the ability to grow on a variety of inexpensive and nutritionally limited substrates including pulp-waste liquor, pineapple waste, molasses, cassava peel, sugarcane bagasse and apple pomace (Christen et al., 1993; Ezekiel and Aworh, 2018; Granato Villas-Boâs et al., 2003; Rosma and Cheong, 2017; Tomita et al., 2012). Its lignocellulosic abilities play a key role in allowing it to use these kinds of agro-industrial wastes. *C. utilis* cells were historically used as a protein supplement and food additive while currently they are more popular for producing glutathione (Boze et al., 1992; Li et al., 2004; Tomita et al., 2012). That said, there are established industrial-scale fermentations for the cultivation and production of food additives using *C. utilis.*

In **Chapter 4**, we identified *C. utilis* as a good producer of phenylethyl alcohol using carrot pomace as the sole substrate in a submerged fermentation. The carrot pomace was found to be previously fermented, likely by psychotropic bacteria which had depleted the sugars and potentially other nutrients. As both sugar and protein levels from fermented the carrot pomace in **Chapter 4** were relatively low, the carrot pomace may be low in available nitrogen, sugars and other nutrients that could be limiting the growth and metabolism of *C. utilis*. This chapter addresses a factorial design process to identify supplementations to carrot pomace that could

optimise or improve the fermentative production of phenylethyl alcohol. Phenylethyl alcohol can be produced in two ways by yeasts 1) through bioconversion of phenylalanine to phenylethyl alcohol through the Ehrlich pathway or 2) *de novo* using phosphoenolpyruvate from glycolysis and erythrose-4-phosphate from the pentose phosphate pathway (Akita et al., 1990; Ayrapaa, 1965; B. Kim et al., 2014; Liu, 2015). It is well documented that additions of the precursor – phenylalanine improves phenylethyl alcohol production in yeasts (Celinska et al., 2013; Chantasuban et al., 2018; Chreptowicz and Mierzejewska, 2018; Fabre et al., 1998; Siddiqui et al., 2012). However, as phenylalanine is a relatively expensive precursor, there may be potential opportunities to increase its production and that of phenylethyl alcohol *de novo.*

Supplementations of ammonium, phosphate and sucrose were selected based on their potential to significantly affect cell growth and metabolism. All three of these components are essential for cell growth and deficiencies in each, and all three can stress or significantly alter the growth rate and metabolism of the yeast. Under stressing conditions, especially nitrogen starvation, aromatic alcohols are known to be released as quorum sensing molecules in many species of yeast including *Candida* spp. (Biswas and Morschhäuser, 2005; Chen and Fink, 2006; Csank and Haynes, 2000; Gimeno et al., 1992; Wickes et al., 1996). Phenylethyl alcohol is one of these aromatic alcohol quorum sensing molecules which triggers yeast to transition from spherical yeast cells to filamentous forms (Han et al., 2013). Some studies have linked phenylethyl alcohol production to biomass generation which is highly affected by both nitrate and sugar deficiencies; others suggest there are optimal amounts of sugar and phenylalanine precursors and that productivity is inhibited by toxicity of phenylethyl alcohol to the cells (Fabre et al., 1998; Rosma and Cheong, 2017). This chapter focuses on three factors: ammonium, phosphate and sucrose levels that will likely have an effect on the *de novo* production of phenylethyl alcohol.

5.2 Materials and methods

5.2.1 Yeast strain and substrate

Candida utilis DSM70167 was maintained on plates containing malt extract–yeast extract–glucose–peptone (MYGP) (3 g/L yeast extract, 3 g/L malt extract, 6 g/L peptone and 10 g/L glucose) agar at 30 °C. Carrot pomace sourced from RD2 International was stored at -20 °C and protected from light and air in food grade, resealable freezer bags. In preparation for fermentation, the pomace was thawed at 4 °C overnight.

5.2.2 Factorial design additions

Three supplements: Ammonium ($(NH_4)_2SO_4$), phosphate (KH_2PO_4) and sucrose were prepared in concentrated solution to add into carrot pomace fermentations in a factorial manner i.e. each supplement was added to carrot pomace both alone and in all possible combinations with the other two supplements (three supplements at two possible concentrations giving 2^3 possible combinations). A total of eight conditions were prepared as in **Table 14** below and an unfermented control. Stock solutions were filter-sterilised and final concentrations of ammonium, phosphate and sucrose additions in the resulting substrate are listed below.

Table 14. Factorial design of three supplements to carrot pomace: Nitrate, phosphate and sucrose

Condition:	N/A	S	SN	SP	NP	P	N	SNP
Sucrose	-	+	+	+	-	-	-	+
Nitrate	-	-	+	-	+	-	+	+
Phosphate	-	-	-	+	+	+	-	+

Legend: N/A=no addition, N=nitrogen 5 g/L, S=sucrose 10 g/L, P=phosphate 3 g/L according to physiological minimal media conditions Villas-Boas et al (2003). Where + is addition of N/P/S (“high-“condition) and – is no addition (“low-“ condition).

5.2.3 Submerged-state fermentations

Small-scale, submerged-state fermentations were performed based on the methods from Chapter 4. Briefly, preinoculum was prepared by inoculating 200 mL of MYGP broth

with a single colony of *C.utilis* in Erlenmeyer flasks (1 L) fitted with cotton stoppers. Flasks were left at 30 °C with agitation at 200 rpm in darkness. Preinoculum was prepared by inoculating 200 mL of MYGP broth with a single colony of *C. utilis* in Erlenmeyer flasks (1 L) fitted with cotton stoppers. Flasks were left at 30 °C with agitation at 200 rpm in darkness. Preinoculum was corrected to a starting optical density of 0.300 (20-fold diluted sample at 600nm). Aliquots of preinoculum (20 mL) were measured into 15 mL falcon tubes and pelleted by centrifugation (3000 g, 10 min). Residual media was poured off and the resulting cell pellet washed twice with sterile saline (0.9 % NaCl).

For the fermentations, carrot pomace (60 g, 85.5% moisture) was weighed into 500 mL silanised round-bottom flasks along with 120 mL distilled water. Flasks were fitted with cotton stoppers and sterilised by autoclaving (121 °C, 20 min). These were inoculated with the preinoculum resuspended in sterile saline (0.9 % NaCl) to a total fermentation volume of 200 mL. These were agitated (200 rpm) in the dark at 30°C.

Nitrate, phosphate and sucrose supplementations were made up in concentrated solution and filter-sterilised (0.22 µm). Additions were made to the sterilised carrot substrate to a final concentration in the substrate as listed in **Table 14**. Fermentations were agitated (200 rpm) in the dark at 30°C for 5 days as determined from **Chapter 4** phenylethyl alcohol production curves. At the end of the fermentation, the entire flask was harvested and rapidly cooled to -20 °C to stop the fermentation. Samples were stored protected from both air and light until chemical analysis.

5.2.4 Initial nitrogen and phosphate quantification of carrot pomace

Initial nitrogen and total phosphorus levels in the unfermented carrot pomace slurry were quantified at R J Hill Laboratories Limited, Hamilton, New Zealand. Samples were air dried at 35 °C and sieved through a screen (<2mm). Basic methods were provided as below.

5.2.4.1 Total recoverable phosphorus

Dried and sieved sample was acid digested (nitric/hydrochloric acid). ICP-MS was then used to determine total elemental phosphorus

5.2.4.2 Total nitrogen

5.2.4.3 Ammonium-nitrate

Dried and sieved sample was digested (KCl, 2M) and phenol/hypochlorite colorimetry used to determine total recoverable phosphorus using discrete analyser, standard method: APHA 4500-NH_3 F (modified) 23rd edition, 2017.

5.2.4.4 Nitrite

Flow injection analysis (FIA) determined using substrate "as received" following extraction with KCl (2M) using standard method: APHA 4500-NO_3^- I 23rd edition, 2017.

5.2.4.5 Nitrate

Calculation performed: (Nitrate-N + Nitrite-N) – (Nitrite-N)

5.2.4.6 Nitrate-N and Nitrite-N measurement

Automated cadmium reduction using substrate "as received". FIA determination of 2M KCl extracted sample. Standard method APHA 4500-NO_3^-I (modified) 23rd edition, 2017.

5.2.5 Derivatization by silylation for determination of sugars composition and concentration

Trimethyl silyl (TMS) derivatization was used to characterise sugars and determine their concentration at each time point based on the method of Villas-Bôas et al. (2006). Samples were prepared in daily batches. Briefly, filtered samples (20 μL) were mixed with the internal standard D-ribitol (20 μL, 10 mM), dried using a rotary vacuum dryer and kept in a desiccator under vacuum over P_5O_5 overnight. Dried samples were resuspended in a methoxyamine hydrochloride solution in pyridine (80 μL, 2 g/100 mL), and incubated at 30°C for 90 min. N-

Methyl-N- (trimethylsilyl)-trifluoroacetamide (MSTFA) (80 µL) was added, incubated at 37°C for 30 min, and analysed by GC-MS as described by Villas-Bôas *et al.* (2006). A calibration curve of sugar standards (concentrations ranging from 0.0625 mM to 30 mM) was prepared using the same method and run with each batch of samples.

5.2.6 GC-MS sugars identification and quantification

The TMS derivatized samples were analysed using a GC-7890 gas chromatograph (Agilent Technologies, Santa Clara, CA) coupled to a MSD-5975 mass spectrometer (Agilent Technologies, Santa Clara, CA) with a quadrupole mass selective detector (EI) operated at 70 eV. A Zebron ZB-1701 column (30 m) (Phenomenex) with internal diameter 250 µm and film thickness 0.15 µm was used with a 5m guard column. The MS was operated in scan mode with a solvent delay of 5.15 min and mass range 38-659 a.m.u. at 1.47 scans/s.

Sugars were identified by comparing to retention time and mass spectra of sugar standards run in each batch of samples. AMDIS (Automated Mass Spectral Deconvolution and Identification system) was used to deconvolute and assign identifications of each peak. Automated peak integration was performed using an in-house R package 'MassOmics' and manual amendments were made to remove false identifications and apply retention time corrections. Peak heights were normalised by D-ribitol and sugars quantified by comparing to the sugar calibration curves.

5.2.7 Rapid GC-MS quantification of phenylethyl alcohol

Phenylethyl alcohol was extracted from fermented carrot pomace using a two-step extraction process with ethyl acetate. Briefly, each sample (1000 µL) was mixed with internal standard D4-methanol (10 µL). Approximately 300 mg anhydrous NaCl was added until saturation. Ethyl acetate (350 µL) was added, vortexed and sonicated for 3 min. Samples were then centrifuged at 2000 x *g* and the non-aqueous phase transferred to a separate vial. A second

extraction was performed by adding ethyl acetate (150 µL) to the same sample. This was vortexed and sonicated (3 min). The upper non-aqueous phase was pooled with the first and any excess water removed with sodium sulfate (~50 mg). The dry sample was then transferred to an amber GC-MS vial for analysis. For quantification, a calibration curve of phenylethyl alcohol standards was prepared in ethyl acetate and run with each batch of samples.

A rapid GC-MS method was adapted from the method by Pinu and Villas-Boas and targeted to phenylethyl alcohol. The GC-MS parameters are described below (**Table 15**).

Table 15. GC-MS parameters for rapid quantification of phenylethyl alcohol

General settings		**GC-capillary column**		**Inlet**	
Ionization voltage:	70 eV	**Film thickness:**	0.15 µm	**Injection mode:**	Split
Ion source temperature:	230 ˚C	**Diameter:**	250 µm	**Injection temperature:**	180 ˚C
Detector voltage:	0.70-0.15 kV	**Length:**	30.0 m	**Split Flow:**	97.701 mL/min
Interface temperature:	250 ˚C	**Flow:**	0.97701 mL/min	**Split ratio:**	100:1
Quadrupole temperature:	150 ˚C	**Carrier gas:**	Helium		
Ionization mode:	EI	**Type:**	Phenomenex Zebron		
Acquisition mode:	SIM		ZB-1701		
***m/z*:**	33.1, 35.1, 91.0, 122.0				

The GC oven temperature was initially held at 50 °C for 1 min then raised to 200 °C at a rate of 40 °C/min. Total run time of the method was 4.75 min. Phenylethyl alcohol was quantified using normalised peak heights that were compared with the calibration curve.

5.2.8 Protein quantification for estimation of biomass

Protein content was estimated at each time point using a method adapted from the Bradford method (1976). Briefly, each sample (500 µL) was mixed with 0.7M NaOH (200µL) and heated at 100 °C for 20 min to disrupt yeast cell membranes and release protein. Samples

were cooled to room temperature and then centrifuged (12 000 rpm, 5 min) to separate carrot particles from the supernatant. The supernatant was diluted 10-fold with NaOH (0.2 M) and loaded into microtiter plate wells (20 µL). Coomassie (Bradford) protein assay reagent (Thermo Scientific^{TM}) (200 µL) was added and mixed well. A calibration curve of bovine serum albumin (BSA) was prepared in the same way as the samples (concentration range from 10 µg/mL to 1000 µg/mL in 0.2 M NaOH). Plates were read using a Spectramax iD3 plate reader (595 nm) within 45 min. Protein content of the fermented carrot pomace was quantified by comparing to the calibration curve.

5.3 Results

5.3.1 Total nitrogen and phosphorus of carrot pomace

Total nitrogen and phosphorus in the original carrot pomace was almost negligible (**Table 16**). Considering minimal media for yeast growth contains 5 g/L ammonium nitrate and 3 g/L phosphate, the unsupplemented carrot pomace was extremely deficient in both nitrogen and phosphorus sources for yeast growth.

Table 16. Nitrogen and phosphorus composition of dry weight carrot pomace, "no addition" carrot pomace substrate and minimal medium.

	Average mg/kg dry weight carrot pomace	**Average mg/ L in N/A* condition**	**Average mg/L in minimal medium**
Ammonium-N	323 mg/kg	2.9 mg/L	5000 mg/L
Nitrite-N	<13 mg/kg	<0.12 mg/L	-
Nitrate-N	<18 mg/kg	<0.16 mg/L	-
Nitrate-N + Nitrite-N	<13 mg/kg	<0.12 mg/L	-
Total phosphorus	1536 mg/kg	13.8 mg/L	3000 mg/L
Total protein	-	1210 mg/L	-

* N/A = no addition

5.3.2 Fermentation performance

All samples were checked for contamination using bright field microscopy after five days of fermentation. All samples showed yeast growth. However, as cells associated tightly with carrot pomace particles, cell-counting could not be used to determine fermentation performance.

5.3.3 Sugar composition and quantification of fermented carrot pomace

The sugar composition of carrot pomace before fermentation is detailed in **Chapter 4** (**Section 4.3.2**). Briefly, sucrose was not detected in unfermented samples while fructose and mannitol were present in very low amounts. Arabinose and ribose were present in trace amounts, were not quantifiable and any change in their concentration was not detected during fermentation. In the present study, for samples with sucrose additions, the initial carrot substrate was prepared for submerged fermentation with 10 g/L sucrose. Sugar concentrations after fermentation are detailed in **Table 17**. Sucrose was completely exhausted in all fermented samples while there were trace amounts of residual fructose. Mannitol was not completely exhausted in any of the fermentations. Levels of both arabinose and ribose were not quantifiable (<0.75 mM) and any changes in concentration were not detected. These are excluded from the table.

Table 17 Sugar concentration in carrot pomace after fermentation by *Candida utilis* under different conditions

	N/A	S	SN	SP	NP	P	N	SNP
Fructose (mM)	0.527	0.512	0.483	0.482	0.485	0.513	0.498	0.480
%RSD	4.60	3.23	0.11	1.10	0.70	1.16	3.39	1.98
Mannitol (mM)	6.35	5.03	5.73	4.44	6.53	6.01	8.14	4.25
%RSD	8.18	8.89	25.37	13.39	21.69	3.53	9.32	8.20
Sucrose (mM)	ND	ND	ND	ND	ND	ND	ND	ND

Legend: N/A=no addition, N=nitrogen 5 g/L, S=sucrose 10 g/L, P=phosphate 3 g/L, ND = not detected. RSD=relative standard deviation, n=3

5.3.4 Phenylethyl alcohol and protein concentration.

Protein and phenylethyl alcohol concentrations are shown for each of the eight fermentation conditions in **Figure 10**. Protein concentration was not significantly different in unfermented compared to fermented carrot pomace with no additions. However, when comparing fermented carrot pomaces, there were three statistically significant differences in protein concentration (p-value ≤ 0.05) between conditions, all involving nitrogen additions (**Table 18.**). Nitrogen addition alone was not enough to significantly increase protein concentration while all other nitrogen additions (NP, SN, SNP) resulted in increased protein concentration (**Table 18.** and **Figure 10.**). Sucrose, nitrogen, and phosphate supplementation (SNP) increased protein concentration (2086 µg/mL) the most – almost twice the amount of protein in the unsupplemented condition (1093 µg/mL). This suggests a higher biomass in the final fermented carrot pomace where nitrogen is added. High protein/biomass was not associated with high phenylethyl alcohol concentration (**Figure 10.**). Nitrogen supplementation was associated with low phenylethyl alcohol concentrations for all conditions with nitrogen additions (SNP, SN, NP, N). For all of these conditions, phenylethyl alcohol concentration was significantly lower than the unsupplemented condition, and also lower than all other addition conditions (**Table 19.**). Sucrose supplementation (S) condition showed the highest phenylethyl alcohol yield of all conditions (11.14 g/kg). Phosphate supplementation (SP and P conditions) appears to slightly decrease the yield of phenylethyl alcohol compared to similar conditions lacking phosphate (S and SP conditions) however these results are not statistically significant. Overall, the highest yield of phenylethyl alcohol was associated with high sugar and low nitrogen conditions and high protein does not correlate with high phenylethyl alcohol yield.

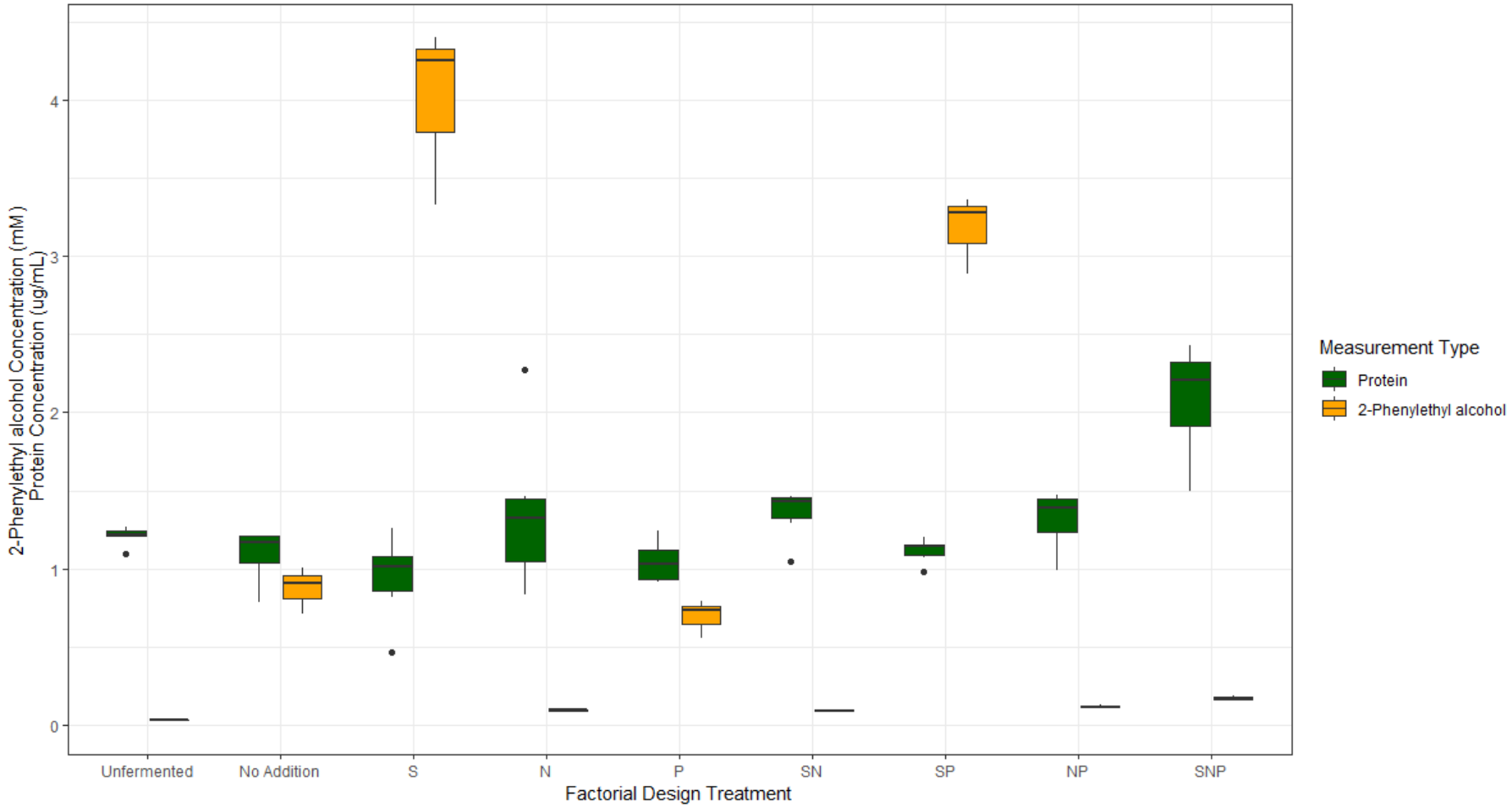


Figure 10. Box plot comparing total protein concentration and total phenylethyl alcohol concentration of carrot pomace fermentations after 5-days of fermentation with *Candida utilis* across nine fermentation conditions: N/A=no addition, N=ammonium 5 g/L, S=sucrose 10 g/L, P=phosphate 3 g/L and unfermented = unfermented carrot pomace. Number of replicates: N=3-6

Table 18. Protein concentration of fermented and unfermented carrot pomace

	N/A	S	SN	SP	NP	P	N	SNP	Control
Average protein (ug/mL)	1093	1367	1316	1353	1116	1044	943	2086	1210
%rsd	15.68	37.05	14.32	12.14	6.98	12.66	29.11	16.93	4.92
***p*-value**	-	0.233	0.0288	0.0115	0.385	0.297	0.141	5.09E-05	0.0732

Legend: N/A=no addition, N=ammonium 5 g/L, S=sucrose 10 g/L, P=phosphate 3 g/L, Control= unfermented carrot pomace, %rsd = relative standard deviation

Table 19. Phenylethyl alcohol yield on carrot pomace

	N/A	S	SN	SP	NP	P	N	SNP
Average 2-PE yield (g/kg)	2.376	11.136	0.174	8.837	0.242	1.865	0.175	0.393
%rsd	16.90	14.55	7.16	7.85	9.54	17.63	7.70	8.91
***p*-value**	-	4.22E-04	3.94E-04	8.15E-05	4.48E-04	0.0882	3.95E-04	5.98E-04

Legend: N/A=no addition, N=ammonium 5 g/L, S=sucrose 10 g/L, P=phosphate 3 g/L. Yield is based on g phenylethyl alcohol produced from one kilogram of dry weight carrot pomace. %rsd = relative standard deviation

5.4 Discussion

Phenylethyl alcohol production was associated with nitrogen starvation in the fermentation conditions trialled. The initial carrot pomace was low in yeast-available nitrogen and provided the perfect substrate to test the effects of nitrogen supplementation on the fermentation performance and production of phenylethyl alcohol. In many yeasts, low nitrogen is a stress-factor that can result in a switch from a spherical yeast cell form to a hyphal, or filamentous form (Biswas and Morschhäuser, 2005; Chen and Fink, 2006; Csank and Haynes, 2000; Gimeno et al., 1992; Wickes et al., 1996). This can be triggered by the release of quorum sensing molecules – often aromatic alcohols. In *Candida* spp. one of these aromatic alcohols is phenylethyl alcohol (Chen and Fink, 2006; Csank and Haynes, 2000; Han et al., 2013). In the present study, nitrogen limitation played the largest role in affecting phenylethyl alcohol production. This was interesting as other studies have associated biomass production with phenylethyl alcohol production and nitrogen supplementation is a popular choice for increasing biomass (Carrau et al., 2008; Fabre et al., 1998; Rosma and Cheong, 2017). This is regularly done in the wine industry to solve "stuck" fermentations while agro-industrial by-products also often lack appropriate nitrogen titres and require supplementation (Carrau et al., 2008; Martínez-Moreno, Quirós, Morales, & Gonzalez, 2014; Rosma et al., 2007; Varela, Pizarro, & Agosin, 2004; Vilanova, Siebert, & Henschke, 2012). While nitrogen addition significantly increased protein concentration and, as an indirect estimate – biomass, phenylethyl alcohol production was adversely affected in all cases. While nitrogen addition clearly favoured biomass production, it was unexpected that nitrogen additions would result in a decrease of phenylethyl alcohol compared to the "no addition" condition because several studies have linked increased biomass and optimal nitrogen concentration with an increase in phenylethyl alcohol (Carrau et al., 2008; Martínez-Moreno et al., 2014; Vilanova et al., 2012). That said, the present study only investigated nitrogen starvation versus an adequate/high nitrogen

condition. Further optimisation of nitrogen supplementation conditions may uncover a smaller nitrogen addition to be beneficial to phenylethyl alcohol concentration as the biomass could be increased before nitrogen starvation conditions trigger the production of quorum sensing molecules − including phenylethyl alcohol.

Carrot pomace was very low in total phosphorus (**Table 16.**) Hess *et al* (2006) found that steady-state yeast cell numbers dropped off from 40 million cells/mL to less than 2 million cells/mL when a limiting concentration of 3 mM phosphate (285 g/L) was supplied in the culture medium. By contrast, above 4 mM phosphate (380 mg/L) supplied in the medium maintained steady-state cell numbers of 40 million cells/mL. A second group, Petti *et* al (2011) defined phosphate-limited medium containing just 13.3 mg/L phosphate which is similar to the levels found in carrot pomace (<13 mg/L total phosphorus). Phosphate supplementation conditions appeared to have a negative effect on phenylethyl alcohol production but to a much lesser extent when compared to nitrogen supplementation. In general, conditions with low phosphate were associated with higher levels of phenylethyl alcohol compared to phosphate addition conditions. Low phosphate levels have been associated with a low growth rate in yeast cells which can favour lipid production and accumulation (Schulze, 1956). Schulze also found that low nitrogen in addition to low phosphate exacerbates lipid accumulation. Low phosphate levels has also been associated with decreased sugar consumption by yeast and an increased survival rate/ low cell death rate (Petti et al., 2011). Saldanha, Brauer, & Botstein (2004) similarly found that low phosphate leads to a rapid cessation of cell division and cell budding in over 95% of yeast cells in stationary phase. In this case, low phosphate potentially affects the survival of *C, utilis* in progressively harsh conditions – especially as the fermentation progresses and phenylethyl alcohol accumulates. Saldanha *et al* (2004) also found that genes for phosphate metabolism were increased in expression during phosphate starvation while Stincone *et al* (2015)similarly found Pentose Phosphate (PP) pathway activity is generally

increased under stressing conditions. Another group found that low phosphate conditions triggered a higher accumulation of glycolysis intermediates and PP pathway intermediates in yeast (Franco et al., 1984). As phosphoenolpyruvate from glycolysis and erythrose-4-phosphate from the PP pathway are both required for *de novo* production of phenylethyl alcohol (**Figure 4.**), the low phosphorus condition potentially enables higher production of phenylethyl alcohol due to the increased flux through the PP pathway (Cadière et al., 2011).

Sucrose supplementation played a major role in phenylethyl alcohol production but only in nitrogen-limited conditions. The effect of sucrose addition was highest during both phosphate and nitrogen-starvation. As cell division is prevented by limiting these factors, the sucrose present can be channelled to the production of secondary metabolites and quorum sensing molecules that signal nitrogen-starvation. This includes phenylethyl alcohol which was produced at a high yield (i.e. 1.1%) on supplemented carrot pomace when sucrose was high and both nitrogen and phosphate limited. This is a much higher yield than the traditional source – Damascus rose petals which have a yield of just 0.03-0.04%. The cost of carrot pomace is also much less than that of rose petals at ~$300/ tonne compared to $3000-$4000 per tonne of rose petals. Carrot pomace usually contains glucose, fructose, xylose and sucrose (Sharma et al., 2012). The non-reducing sugars component (estimated sucrose concentration) is 1.02-1.18% w/w in a commercial set-up (Sharma et al., 2012). Consequently, any sucrose supplementations may be minimal – or could be added after an initial biomass is established and nitrogen/phosphate levels depleted. Interestingly, as biomass production was not associated with phenylethyl alcohol production in this study, but is implicated in its production in others, there may be an optimal concentration for nitrogen supplementation not covered by the present study. Lower supplementation of nitrogen e.g. around 500 mg/L may be beneficial to increasing initial biomass while later additions of sucrose should be investigated to identify if even higher yields can be achieved, or productivity boosted.

5.5 Conclusion

Phenylethyl alcohol was produced at a high yield when carrot pomace was supplemented with sucrose. Nitrogen limitation and phosphate limitation were both beneficial to the production of phenylethyl alcohol while biomass production did not favour production. This was likely due to the impact of starvation conditions favouring the production of quorum sensing molecules in the yeast as well as low cell division rates allowing sucrose to be channelled towards the production of these secondary metabolites. Compared to rose petal yields for the production of rose oil and phenylethyl alcohol of just 0.03-0.04%, *C. utilis* fermentation of carrot pomace produced a 1.11% yield. Further optimisation of nitrogen supplementation and sucrose additions at different points of the fermentation could allow for an optimal method for the production of natural phenylethyl alcohol on a commercial scale.

Chapter VI

6 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

6.1 General discussion

The current consumer market is increasingly demanding sustainable, ethical and natural ingredients. Flavour and fragrance ingredients are not immune to these changes and as a result, consumption often outstrips global supply. Since synthetic alternatives are poorly received by the consumer population, novel sources of natural ingredients are highly sought after. Biotechnological approaches to harness the fermentative capacity of microorganisms is a developing field – especially with regards to using alternative substrates like agro-industrial by-products as a starting point.

While there have been several attempts to produce specific flavour and fragrance ingredients through fermentation such as vanillin and diacetyl, there are considerably fewer studies that have followed a hypothesis-generating approach to identify valuable compounds of interest in a fermentation and to optimise the fermentation parameters to suit industrial production. The first two chapters of this work screened a combined 39 fermentations using five microorganisms and nine agro-industrial by-products. Hundreds of volatile compounds were produced during fermentation. However, quantification of large numbers of compounds such as those produced across a diverse range of substrates is challenging. As HS-SPME is not quantitative, only a handful of industrially relevant compounds were selected for further analysis. Fold-change increase was the only basis on which any inference on the quantities of volatiles produced so, there is merit in reviewing other compounds produced in these first screening experiments in order to establish other high-value compounds that may have been outcompeted for adsorption onto the SPME fibre. The solid-state fermentations using filamentous fungi had potential with a high production of volatile flavour and aroma compounds. Despite the technology for solid-state fermentation being underdeveloped when compared to liquid- and submerged-state fermentations, there could be uncaptured value in other volatile chemicals produced by those fermentations. The one compound selected –

phenylethyl alcohol had a high commercial value, and high yield on carrot pomace. In addition, the submerged-state fermentation process is already established on an industrial scale. As a result, efforts could be focussed on optimisation of the fermentation parameters to increase yield of phenylethyl alcohol, rather than assessing criteria and equipment required to scale up a solid-state fermentation. This pre-existing technology means the upstream costs for producing phenylethyl alcohol from carrot pomace ($300/tonne) could be significantly lower than extraction from rose petals ($3000/tonne). As industrial-scale infrastructure is already established for similar fermentations and optimised fermentation processes can be highly cost-effective, most of the ensuing costs would be realised during downstream processing and product recovery.

Yield of phenylethyl alcohol on carrot pomace started out at just 0.005% of dry carrot pomace using *B. bruxellensis* to ferment a slurry of carrot pomace and water over the course of a three-day fermentation. This paled in comparison to the current distillation process to extract phenylethyl alcohol from rose petals which yields 0.03-0.04%. However, the cost of rose petals is over $3000 per tonne, while carrot pomace is at least ten times cheaper. An optimised extraction process using ethyl acetate instead of tetrahydrofuran yielded 0.012% on the same fermentation while extending the fermentation time to six days further increased yield to 0.05% (**Chapter 4, Section 4.3.4**). In **Chapter 4**, by changing yeast strain, yield was boosted to 0.12% using a five-day fermentation with *C. utilis* and to 0.19% using *C. albicans*.

GRAS status of microorganisms is a complex regulation to navigate. While many microorganisms enjoy GRAS status based on a history of safe use and consumption other less ubiquitous microorganisms must be proven safe. Some potential human pathogens such as *E. coli* are used to produce food additives, but the process must be proven beyond reasonable doubt to be incapable of causing human harm. There are provisions in place to gain an exemption for specific strains that outline strict fermentation parameters and conditions as well

as ensuring the microorganism is not present in the final product but doing so can be prohibitively costly and time-consuming. In addition, there can be problems with health and safety regulations not only with the end product, but with mass cultivation of a potential human pathogen. As a result, while GRAS status can be applied for, and the fermentation processes potentially modified to fit the stringent regulations, it is often much more attractive and cost-effective to select an existing GRAS microorganism. In this case, *C. utilis* was selected instead of the higher producer *C. albicans* as it is already used as a cell-based protein additive as well as to produce glutamine for the food industry.

Once the yeast strain was selected, there were still a number of parameters to modify in the fermentation – especially in the carrot pomace substrate. The carrot pomace used was very low in sucrose, yeast available nitrogen and total phosphorus (**Chapter 4, section 4.3.2, Chapter 5 section 5.3.1**). Therefore, supplementations of each of these in a factorial design experiment were made (**Chapter 5**). In this way, each supplementation was tested alone and in combination with all other factors to determine which had the most effect on phenylethyl alcohol production. Sucrose addition produced the highest amount of phenylethyl alcohol but only when nitrogen and phosphate were limited.

A supplementation that was specifically excluded was the addition of the phenylethyl alcohol precursor phenylalanine. This is an expensive precursor – especially when compared to sucrose of other agro-industrial by-products and exploits the Ehrlich pathway to facilitate a simple conversion from phenylalanine to phenylethyl alcohol. By contrast, this research has targeted *de novo* production of phenylethyl alcohol from glycolysis and the Shikimate pathway and has produced high yields.

Nitrogen and phosphate starvation appeared to limit cell division as protein measurements remained extremely low in these fermentations. With abundant sucrose, while

biomass production was extremely low, phenylethyl alcohol production was much higher reaching a yield of 1.1%. Compared to rose petal yields, this is around 30 times higher and compared to the initial three-day fermentation using *B. bruxellensis*, almost 220 times higher. As sucrose was unexpectedly absent from the carrot pomace due to a suspected pre-fermentation by psychrotrophic bacteria, other carrot pomace may still be an ideal candidate for production of phenylethyl alcohol without sucrose supplementation. Alternatively, as sucrose is a generally cheap feedstock and abundant in other agro-industrial by-products, mixing other substrates with carrot pomace could be an attractive option. As nitrogen-limitation is extremely important to favour production of phenylethyl alcohol, other residues such as pineapple waste which is extremely low in nitrogen but high in sugars could be an option.

There a few drawbacks to using agro-industrial by-products as a substrate for fermentations that should be addressed. The first is seasonality of the by-product. Some waste products are highly abundant at certain times of the year, while almost non-existent at other times. In addition, they are often highly perishable as found with the carrot pomace which arrived pre-fermented by psychrotrophic bacteria. It is very expensive to store these agro-industrial by-products – especially if refrigeration or freezing temperatures are required. Seasonality would compound the expense as large quantities would need to be processed all at one time or, stored at cost for prolonged periods of time. This is an important factor to consider for scale-up purposes as extremely large amounts of substrate may need to be processed in a short amount of time, whilst fermentation vessels and equipment could remain unused for long periods of time. With that in mind, industrial-scale fermenters for the process should be generic, or versatile enough to produce other goods if production is only seasonal. Finally, when working with agro-industrial by-products, quality control will be paramount. Different cultivars or produce from different areas or even seasons can have different compositions

which could affect the fermentation. It will be important to establish if the fermentation is robust enough to resist variation introduced by substrate composition.

Finally, it is important to consider scale-up and especially downstream processing for the fermentative production of phenylethyl alcohol using carrot pomace. One important factor for both scale-up and downstream processing is to establish a better way of monitoring the fermentation progression. While cell-counting was not appropriate, protein measurements may have more success. Unfortunately, the sample sizes in the current research did not permit more than a few millilitres of sample for each assay. The draw for measuring protein using a colorimetric-based assay were the small sample size and fast and straight-forward sample preparation. Upwards of 500 samples were analysed in any given experiment so the method had to be relatively high throughput. Measuring ergosterol may be a more consistent method for estimating biomass. However, as carrot pomace is an extremely complex and variable substrate and the fermentation changes the viscosity of the pomace, it may not be much more successful. The Kjeldahl method may be a good way to measure total protein and infer biomass production. However, it requires a larger sample, and more preparation steps to extract and digest protein in the sample. To effectively monitor the fermentation, it would be most valuable to define the fermentation parameters using a larger fermentation size and then take larger samples to quantify protein using dried and milled samples. The Kjeldahl method would be a good candidate for this and the larger sample size should reduce variation significantly.

Downstream processing – specifically product recovery has not been addressed in this work and represents a larger portion of production costs compared to the upstream processing involved. While scale-up technology is well established for submerged-state fermentations of up to $100m^3$, product recovery could be challenging. The most efficient and cost-effective method to recover volatile aroma compounds like phenylethyl alcohol could be through a simple solvent extraction. There are several food grade solvents like ethanol or as established

in **Chapter 4**, ethyl acetate which could be an attractive option to make an extract containing phenylethyl alcohol. A second, more expensive option is through distillation – in the same way that rose petals are processed for the recovery of rose oil. Phenylethyl alcohol is not degraded in the steam distillation process and industrial-scale methods are already established. An important consideration for both of these methods is that other volatile aroma compounds will be collected along-side phenylethyl alcohol. The overall aroma profile of the fermented carrot pomace is floral and pleasant which suggests any co-products do not significantly, or adversely affect the overall aroma profile. However, it will be important to establish if any toxic or otherwise undesirable co- or degradation products are collected along with phenylethyl alcohol.

In both cases, purification may be required in order to remove undesirable co-products. This can be expensive and cannot likely be directly applied to the fermented carrot pomace, even with membrane assisted or *in-situ* product recovery techniques as the carrot particles could interfere with the purification. In any case, the most attractive product recovery process will likely be a distillation or solvent extraction based on price and product recovery yields as well as any purification costs associated. As rose-petals have both a high commodity price and high downstream processing costs associated with distillation, carrot pomace fermentation and subsequent extraction of phenylethyl alcohol could be a competitive, cheaper, natural alternative.

6.2 Future perspectives

One limitation of the current work is the concentration range of the supplementations – especially nitrogen. Only two concentrations “high” and “low” were assessed. The high concentration was ideal for promoting growth of yeast and producing biomass while the low concentration was severely restrictive. It is possible that additions of limited amounts of nitrogen e.g. ~500 mg/L could help to promote initial biomass production. In addition, a later

addition of sucrose could potentially then be utilised more efficiently to produce phenylethyl alcohol. Low phosphate on the other hand, seemed ubiquitously required to promote phenylethyl alcohol production and its addition did not significantly affect biomass production if sufficient nitrogen was present.

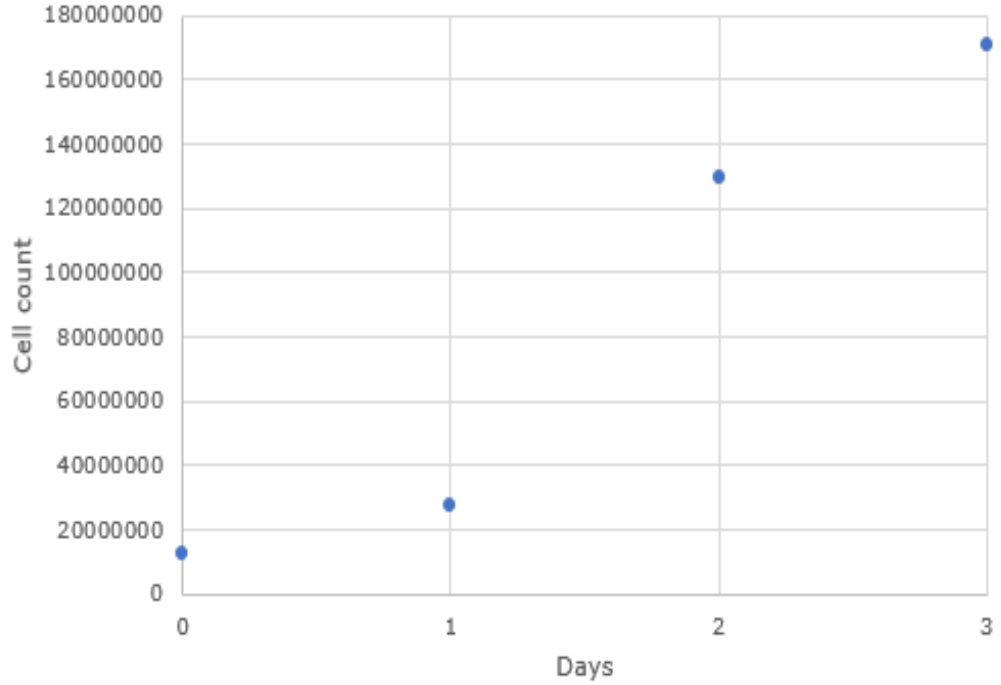
Secondly, this work exclusively worked with aerobic fermentation. It is important to investigate the production of phenylethyl alcohol under anaerobic conditions. Ghosh *et al* (2008) reported *C. albicans* produces double the amount of aromatic alcohols when under anaerobic conditions compared to aerobic conditions. However, it is unclear if the same if true for *C. utilis*. In any case, it could be a worthwhile and rewarding pursuit to investigate different nitrogen additions as well as anaerobic fermentation using *C. utilis*.

Phenylethyl alcohol toxicity to *C. utilis* was not established in this research. While many other studies have been limited by the accumulation of phenylethyl alcohol in the media, it has not been established for this fermentation. Phenylethyl alcohol accumulation could be self-inhibiting at concentrations of around 5 g/L in the growth medium according to other studies using *S. cerevisiae* and *K. marxianus*. While nitrogen and phosphate limitation also limit cell division, there is evidence that low phosphate conditions trigger cells to increase in cell size, accumulate lipids and cease cell division all the while increasing flux through the pentose phosphate pathway (Cadière et al., 2011; Saldanha et al., 2004; Schulze, 1956). With this in mind, it would be interesting and potentially valuable to establish if *C. utilis* is a more robust candidate under these conditions and can withstand a higher concentration of phenylethyl alcohol.

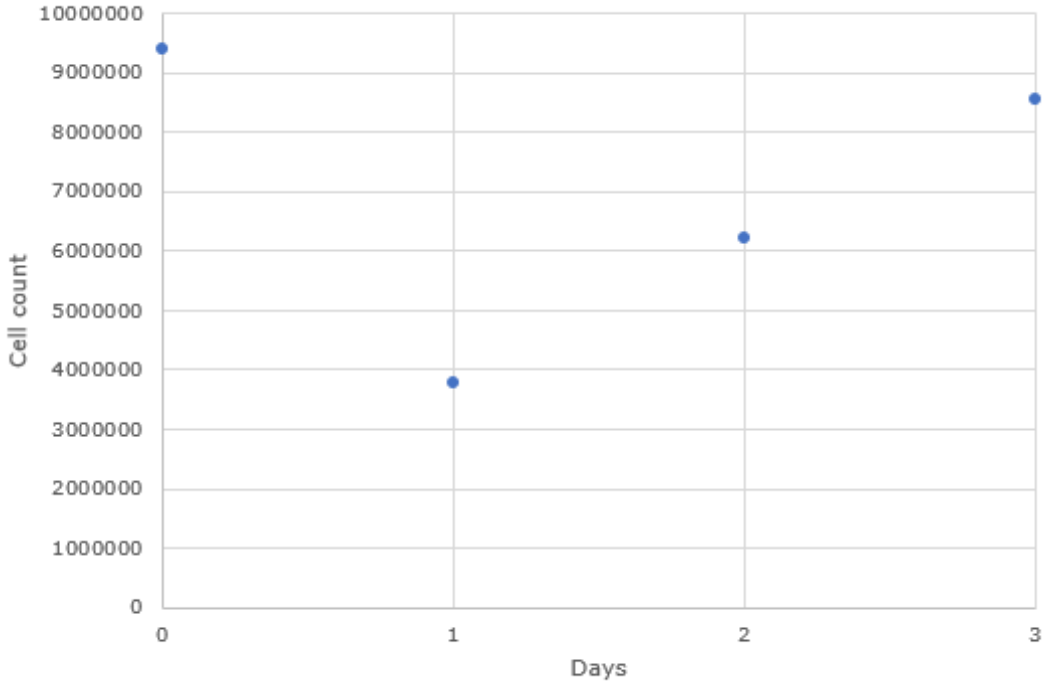
Finally, one of the most exciting prospects for the work in this thesis is to scale-up production to at least pilot plant scale. This would allow processing of large quantities of carrot pomace and investigation into some of the downstream processing methods that would yield a

viable commercial product. This is one of the key steps to establishing if the fermentation process described is appropriate for industrial scale production of phenylethyl alcohol and truly determining if it is commercially viable.

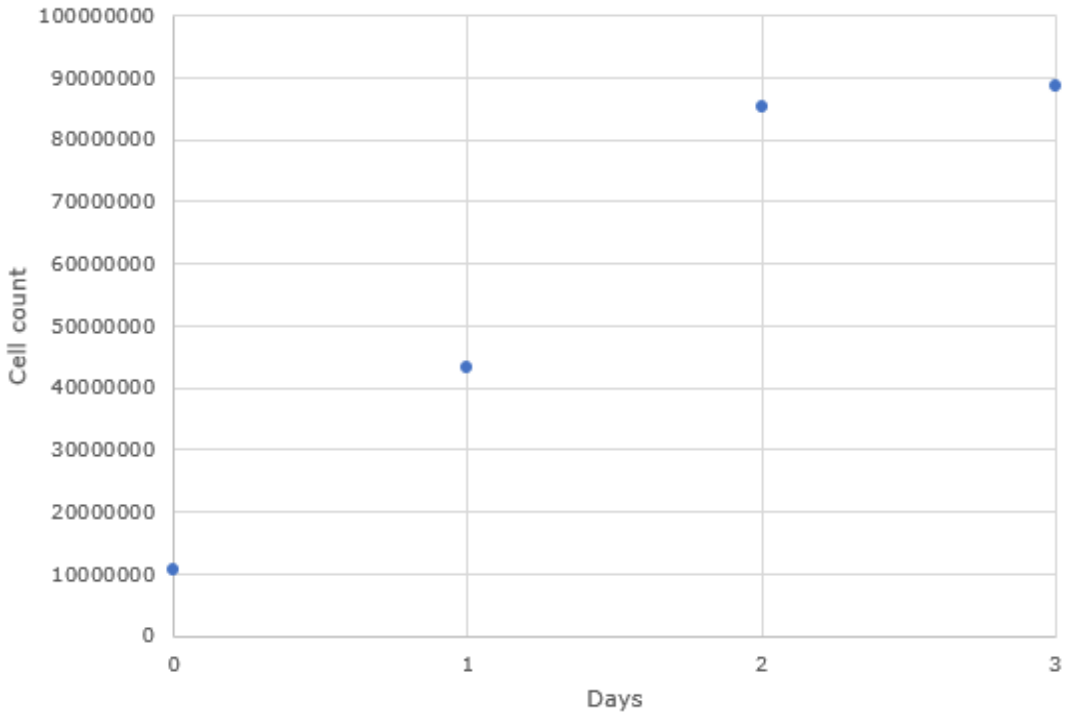
Supplementary figures



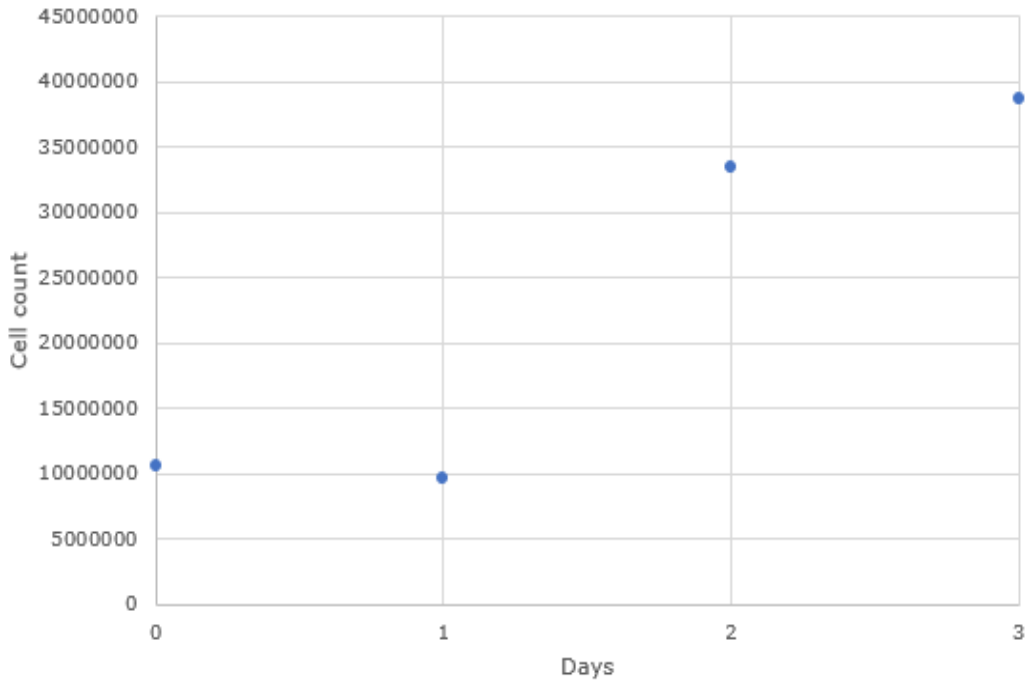
Supplemetary Figure 11. ***Brettanomyces bruxellensis*** **cell count per day when fermenting carrot pomace**



Supplementary Figure 12. ***Saccharomyces cerevisiae*** **cell count per day when fermenting carrot pomace**



Supplementary Figure 13. ***Kluyveromyces marxianus*** **cell count per day when fermenting carrot pomace**



Supplementary Figure 14. ***Candida albicans*** **cell count per day when fermenting carrot pomace**

References

Adler, P., Hugen, T., Wiewiora, M., Kunz, B., 2010. Modeling of an integrated fermentation/membrane extraction process for the production of 2-phenylethanol and 2-phenylethylacetate. Enzyme Microb. Technol. 48, 285–292.

Agosin, E., Odier, E., 1985. Solid-state fermentation, lignin degradation and resulting digestibility of wheat straw fermented by selected white-rot fungi. Appl Microbiol Biotechnol 21, 397–403.

Aishvarya, V., Jena, J., Pradhan, N., Panda, P.K., Sukla, L.B., 2015. Microbial processing for valorization of horticultural wastes., in: Environmental Microbial Biotechnology. Springer, Cham, pp. 203–221.

Akita, O., Ida, T., Obata, T., Hara, S., 1990. Mutants of *Saccharomyces cerevisiae* Producing a Large Quantity of B-Phenethyl Alcohol and -Phenethyl Acetate. J. Ferment. Bioeng. 69, 125–128.

Alissandrakis, E., Tarantilis, P.A., Harizanis, P.C., Polissiou, M., 2007. Aroma investigation of unifloral Greek citrus honey using solid-phase microextraction coupled to gas chromatographic-mass spectrometric analysis. Food Chem. 100, 396–404.

Ayrapaa, T., 1965. The formation of phenlethyl alcohol from 14C-labelled phenylalanine. J. Inst. Brew. 71, 341–347.

Barba, C., Thomas-Danguin, T., Guichard, E., 2017. Comparison of stir bar sorptive extraction in the liquid and vapour phases, solvent-assisted flavour evaporation and headspace solid-phase microextraction for the (non)-targeted analysis of volatiles in fruit juice. LWT - Food Sci. Technol. 85, 334–344.

Baydar, H., 2006. Oil-bearing rose (R*osa damascena* Mill .) Cultivation and rose oil industry

in Turkey. Ind. Crops Prod. 14, 13–17.

Beekwilder, J., Van der Meer, I.M., Sibbbesen, O., Broekgaarden, M., Qvist, I., Mikkelsen, J.D., Hall, R.D., 2007. Microbial production of natural raspberry ketone. Biotechnol. J. 2, 1270–1279.

Behera, S.S., Ray, R.C., 2016. Solid state fermentation for production of microbial cellulases: Recent advances and improvement strategies. Int. J. Biol. Macromol. 86, 656–669.

Bello, M.O., Olabanji, I.O., Abdul-Hammed, M., Okunade, T.D., 2013. Characterization of domestic onion wastes and bulb (*Allium cepa* L.): Fatty acids and metal contents. Int. Food Res. J. 20, 2153–2158.

Beltran, G., Esteve-Zarzoso, B., Rozes, N., Mas, A., Guillamon, J.M., 2005. Influence of the Timing of Nitrogen Additions during Synthetic Grape Must Fermentations on Fermentation Kinetics and Nitrogen Consumption. J. Agric. Food Chem. 53, 996–1002.

Benítez, V., Mollá, E., Martín-Cabrejas, M.A., Aguilera, Y., López-Andréu, F.J., Cools, K., Terry, L.A., Esteban, R.M., 2011. Characterization of Industrial Onion Wastes (Allium cepa L.): Dietary Fibre and Bioactive Compounds. Plant Foods Hum. Nutr. 66, 48–57.

Berger, R.G., 2015. Biotechnology as a source of natural volatile flavours. Curr. Opin. Food Sci. 1, 38–43.

Berger, R.G. (Ed.), 2007. Flavours and Fragrances: Chemistry, Bioprocessing and Sustainability, I. ed. Springer-Verlag Berlin Heidelberg, Heidelberg.

Berger, R.G., 1995. From fermentation to white biotechnology : how microbial catalysts generate flavours, in: Modifying Flavour in Food. Woodhead Publishing, pp. 65–95.

Berovic, M., Legisa, M., 2007. Citric acid production. Biotechnol. Annu. Rev. 13, 303–343.

Bicas, J.L., Pastore, G.M., 2007. Isolation and screening of d-limonene-resistant microorganisms. Brazilian J. Microbiol. 38, 563–567.

Bicchi, C., Iori, C., Rubiolo, P., Sandra, P., 2002. Headspace sorptive extraction (HSSE), stir bar sorptive extraction (SBSE), and solid phase microextraction (SPME) applied to the analysis of roasted Arabica coffee and coffee brew. J. Agric. Food Chem. 50, 449–459.

Biswas, K., Morschhäuser, J., 2005. The Mep2p ammonium permease controls nitrogen starvation-induced filamentous growth in *Candida albicans*. Mol. Microbiol. 56, 649–669.

Blomqvist, J., Eberhard, T., Schnürer, J., Passoth, V., 2010. Fermentation characteristics of *Dekkera bruxellensi*s strains. Appl. Microb. CELL Physiol. 87, 1487–1497.

Boker, A., Fischer, M., Berger, R.G., 2001. Raspberry Ketone from Submerged Cultured Cells of the *Basidiomycete Nidula niveo-tomentosa*. Biotechnol. Prog. 17, 568–572.

Boratyński, F., Szczepańska, E., Grudniewska, A., Gniłka, R., Olejniczak, T., 2018. Improving of hydrolases biosythesis by solid-state fermentation of *Penicillium camemberti* on rapeseed cake. Sci. Rep. 8, 10157.

Borjesson, T., Stollman, U., Schnurer2, J., 1992. Volatile Metabolites Produced by Six Fungal Species Compared with Other Indicators of Fungal Growth on Cereal Grains Downloaded from. Appl. Enviromental Microbiol. 58, 2599–2605.

Bornscheuer, U.T., Huisman, G.W., Kazlauskas, R.J., Lutz, S., Moore, J.C., Robins, & K., 2012. Engineering the third wave of biocatalysis. Nature 485, 185–194.

Bosse, A.K., Fraatz, M. a, Zorn, H., 2013. Formation of complex natural flavours by biotransformation of apple pomace with basidiomycetes. Food Chem. 141, 2952–9.

Boulton, R.A., 2019. Growth and Metabolism of Mannitol by Strains of *Saccharomyces*

cerevisiae, Journal of General Microbiology.

Boze, H., Moulin, G., Galzy, P., 1992. Production of Food and Fodder Yeasts. Crit. Rev. Biotechnol. 12, 65–86.

Brar, S.K., Dhillon, G.S., Soccol, C.R. (Eds.), 2013. Biotransformation of waste biomass into high value biochemicals. Springer Science & Business Media.

Bruhlmann, Fredi; Blomburg, R., 2016. Process for making flavour and fragrant compounds. WO 2016/126892 A1.

Brunati, M., Marinelli, F., Bertolini, C., Gandolfi, R., Daffonchio, D., Molinari, F., 2004. Biotransformations of cinnamic and ferulic acid with actinomycetes. Enzyme Microb. Technol. 34, 3–9.

Buerth, C., Tielker, D., Ernst, J.F., 2016. Candida utilis and Cyberlindnera (Pichia) jadinii: yeast relatives with expanding applications. Appl. Microbiol. Biotechnol.

Button, J.E., Dutton, R.J., 2012. Cheese microbes. Curr. Biol. 22, R587–R589.

Cacho, J.I., Campillo, N., Viñas, P., Hernández-Córdoba, M., 2015. Evaluation of three headspace sorptive extraction coatings for the determination of volatile terpenes in honey using gas chromatography-mass spectrometry. J. Chromatogr. A 1399, 18–24.

Cadière, A., Ortiz-Julien, A., Camarasa, C., Dequin, S., 2011. Evolutionary engineered *Saccharomyces cerevisiae* wine yeast strains with increased in vivo flux through the pentose phosphate pathway. Metab. Eng. 13, 263–271.

Caldeira, M., Rodrigues, F., Perestrelo, R., Marques, J.C., Câmara, J.S., 2007. Comparison of two extraction methods for evaluation of volatile constituents patterns in commercial whiskeys. Elucidation of the main odour-active compounds. Talanta 74, 78–90.

Camarasa, C., Chiron, H., Daboussi, F., Della Valle, G., Dumas, C., Farines, V., Floury, J., Gagnaire, V., Gorret, N., Leonil, J., Mouret, J.-R., O'donohue, M.J., Sablayrolles, J.-M., Salmon, J.-M., Saulnier, L., Truan, G., O'donohue, M.J., 2018. INRA's research in industrial biotechnology_ For food, chemicals, materials and fuels. Innov. Food Sci. Emerg. Technol. 46, 140–152.

Carlquist, M., Gibson, B., Yuceer, Y.K., Paraskevopoulou, A., Sandell, M., Angelov, A.I., Gotcheva, V., Angelov, A.D., Etschmann, M., Billerbeck, G.M. de, Lidén, G., 2015. Process engineering for bioflavour production with metabolically active yeasts – a mini-review. Yeast 32, 123–143.

Carlson, Ting Liu; Peters, E.M.J.., 1997. Low pH lactic acid fermentation. US 6,475,759 B1.

Carrau, F.M., Medina, K., Farina, L., Boido, E., Henschke, P.A., Dellacassa, E., 2008. Production of fermentation aroma compounds by *Saccharomyces cerevisiae* wine yeasts: e¡ects of yeast assimilable nitrogen on two model strains.

Castilho, L.R., Mitchell, D.A., Freire, D.M.G., 2009. Production of polyhydroxyalkanoates (PHAs) from waste materials and by-products by submerged and solid-state fermentation. Bioresour. Technol. 100, 5996–6009.

Caven-Quantrill, D.J., Buglass, A.J., 2006. Comparison of micro-scale simultaneous distillation-extraction and stir bar sorptive extraction for the determination of volatile organic constituents of grape juice. J. Chromatogr. A 1117, 121–131.

Celinska, E., Kubiak, P., Bialas, W., Dziadas, M., Grajek, W., 2013. *Yarrowia lipolytica*: the novel and promising 2-phenylethanol producer. J. Ind. Microbiol. Biotechnol. 40, 389–392.

Chakravorty, M., Veiga, L.A., Bacila, M., Horecker, B.L., 1962. Pentose Metabolism in

Candida: The diphosphopyridine nucleotide-specific polyol dehyrogenase of *Candida utilis*, The Journal of Biological Chemistry.

Chantasuban, T., Santomauro, F., Gore-Lloyd, D., Parsons, S., Henk, D., Scott, R.J., Chuck, C., 2018. Elevated production of the aromatic fragrance molecule, 2-phenylethanol, using *Metschnikowia pulcherrima* through both *de novo* and *ex novo* conversion in batch and continuous modes. J. Chem. Technol. Biotechnol. 93, 2118–2130.

Check Hayden, E., 2014. Synthetic-biology firms shift focus. Nature 505, 598–598.

Cheetham, P.S.J., 2002. Enzymes for Flavor Production, in: Flickinger, M.C. (Ed.), Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, and Bioseparation. pp. 1–27.

Chen, H., Fink, G.R., 2006. Feedback control of morphogenesis in fungi by aromatic alcohols. Genes Dev. 20, 1150–61.

Chen, J., 2017. Freeze−Thaw Sample Preparation Method Improves Detection of Volatile Compounds in Insects Using Headspace Solid-Phase Microextraction. Anal. Chem. 89, 8366–8371.

Choi, J.-M., Han, S.-S., Kim, H.-S., 2015. Industrial applications of enzyme biocatalysis: Current status and future aspects. Biotechnol. Adv. 33, 1443–1454.

Chreptowicz, K., Mierzejewska, J., 2018. Enhanced bioproduction of 2-phenylethanol in a biphasic system with rapeseed oil. N. Biotechnol. 42.

Chreptowicz, K., Sternicka, M.K., Kowalska, P.D., Mierzejewska, J., 2018. Screening of yeasts for the production of 2-phenylethanol (rose aroma) in organic waste-based media. Lett. Appl. Microbiol. 66, 153–160.

Chreptowicz, K., Wielechowska, M., Główczyk-Zubek, J., Rybak, E., Mierzejewska, J., 2016.

Production of natural 2-phenylethanol: From biotransformation to purified product. Food Bioprod. Process. 100, 275–281.

Christen, P., Aljria, R., Vega, C., Villegas, E., Revah, S., 1993. Growth of *Candida utilis* in solid state fermentation. Biotechnol. Adv. 11, 549–557.

Christen, P., Bramorski, A., Revah, S., Soccol, C.R., 2000. Characterization of volatile compounds produced by *Rhizopus* strains grown on agro-industrial solid wastes. Bioresour. Technol. 71, 211–215.

Christen, P., Meza, J.C., Revah, S., 1997. Fruity aroma production in solid state fermentation by *Ceratocystis fimbriata*: influence of the substrate type and the presence of precursors. Mycol. Res. 101, 911–919.

Contini, M., Esti, M., 2006. Effect of the matrix volatile composition in the headspace solid-phase microextraction analysis of extra virgin olive oil. Food Chem. 94, 143–150.

Couto, S.R., Sanromán, M.Á., 2006. Application of solid-state fermentation to food industry-A review. J. Food Eng. 76, 291–302.

Crafack, M., Keul, H., Eskildsen, C.E., Petersen, M.A., Saerens, S., Blennow, A., Skovmand-Larsen, M., Swiegers, J.H., Petersen, G.B., Heimdal, H., Nielsen, D.S., 2014. Impact of starter cultures and fermentation techniques on the volatile aroma and sensory profile of chocolate. Food Res. Int. 63, 306–316.

Csank, C., Haynes, K., 2000. *Candida glabrata* displays pseudohyphal growth. FEMS Microbiol. Lett. 189, 115–120.

De Barros Pita, W., Leite, F.C.B., De Souza Liberal, A.T., Simões, D.A., De Morais, M.A., 2011. The ability to use nitrate confers advantage to *Dekkera bruxellensis* over *S. cerevisiae* and can explain its adaptation to industrial fermentation processes. Antonie van

Leeuwenhoek, Int. J. Gen. Mol. Microbiol. 100, 99–107.

De Barros Pita, W., Tiukova, I., Leite, F.C.B., Passoth, V., Simões, D.A., De Morais, M.A., 2013. The influence of nitrate on the physiology of the yeast *Dekkera bruxellensis* grown under oxygen limitation. Yeast 30, 111–117.

De Carvalho, C.C.C.R., 2010. Enzymatic and whole cell catalysis: Finding new strategies for old processes. Biotechnol. Adv. 29, 75–83.

De Carvalho, D.S., Dionísio, A.P., Santos, R. Dos, Boguzs, S., Godoy, H.T., Pastore, G.M., 2011. Production of 1-octen-3-ol by Neurospora species isolated from beiju in different culture medium. Ital. Oral Surg. 1, 1694–1699.

de Lourdes Cardeal, Z., Guimarães, E.M., Parreira, F.V., 2005. Analysis of volatile compounds in some typical Brazilian fruits and juices by SPME-GC method. Food Addit. Contam. 22, 508–513.

Deed, N.K., van Vuuren, H.J.J., Gardner, R.C., 2011. Effects of nitrogen catabolite repression and di-ammonium phosphate addition during wine fermentation by a commercial strain of *S. cerevisiae.* Appl. Microbiol. Biotechnol. 89, 1537–49.

Demyttenaere, J.C.R., Sánchez Martínez, J.I., Verhé, R., Sandra, P., De Kimpe, N., 2003. Analysis of volatiles of malt whisky by solid-phase microextraction and stir bar sorptive extraction. J. Chromatogr. A 985, 221–232.

Dhillon, G.S., Brar, S.K., Verma, M., Tyagi, R.D., 2011. Utilization of different agro-industrial wastes for sustainable bioproduction of citric acid by *Aspergillus niger*. Biochem. Eng. J. 54, 83–92.

Dhillon, G.S., Kaur, S., Brar, S.K., 2013. Perspective of apple processing wastes as low-cost substrates for bioproduction of high value products: A review. Renew. Sustain. Energy

Rev. 27, 789–805.

Dong, L., Liu, R., Dong, H., Piao, Y., Hu, X., Li, C., Cong, L., Zhao, C., 2015. Volatile metabolite profiling of malt contaminated by *Fusarium poae* during malting. J. Cereal Sci. 66, 37–45.

Dörnenburg, H., Knorr, D., 1996. Production of phenolic flavor compounds with cultured cells and tissues of vanilla species. Food Biotechnol. 10, 75–92.

Duboff, Shirley A.; Kwon, Steven S.; Vadehra, D. V., 1992. Diacetyl production. W/O 5,236,833.

Ducki, S., Miralles-Garcia, J., Zumbé, A., Tornero, A., Storey, D.M., 2008. Evaluation of solid-phase micro-extraction coupled to gas chromatography-mass spectrometry for the headspace analysis of volatile compounds in cocoa products. Talanta 74, 1166–1174.

Erbaş, S., Baydar, H., 2016. Variation in scent compounds of oil-bearing rose (*Rosa damascena* Mill.) Produced by headspace solid phase microextraction, hydrodistillation and solvent extraction. Rec. Nat. Prod. 10, 555–565.

Etschmann, M., Bluemke, W., Sell, D., Schrader, J., 2002. Biotechnological production of 2-phenylethanol. Appl. Microbiol. Biotechnol. 59, 1–8.

Etschmann, M.M.W., Sell, D., Schrader, J., 2003. Screening of yeasts for the production of the aroma compound 2-phenylethanol in a molasses-based medium. Biotechnol. Lett. 25, 531–536.

Ezekiel, O., Aworh, O., 2018. Simultaneous saccharification and cultivation of *Candida utilis* on cassava peel. Innov. Food Sci. Emerg. Technol. 49, 184–191.

Fabre, C.E., Blanc, P.J., Goma, G., 1998. Production of 2-Phenylethyl Alcohol by *Kluyveromyces marxianus*. Biotechnol. Prog. 14, 270–274.

Fadel, H.H.M., Mahmoud, M.G., Asker, M.M.S., Lotfy, S.N., 2014. Characterization and evaluation of coconut aroma produced by *Trichoderma viride* EMCC-107 in solid state fermentation on sugarcane bagasse. Electron. J. Biotechnol. 18, 5–9.

Feron, G., Mauvais, G., Martin, F., Sé Mon, E., Blin-Perrin, C., 2007. Microbial production of 4-hydroxybenzylidene acetone, the direct precursor of raspberry ketone. Lett. Appl. Microbiol. 45, 29–35.

Fraatz, M.A., Berger, R.G., Zorn, H., 2009. Nootkatone—a biotechnological challenge. Appl. Microbiol. Biotechnol. 83, 35–41.

Franco, C.M.M., Smith, J.E., Berry, D.R., 1984. Effect of Nitrogen and Phosphate on the Levels of Intermediates in Bakers' Yeast Grown in Continuous Culture. J. Gen. Microbiol. 130, 246–2472.

Furusawa, M., Hashimoto, T., Noma, Y., Asakawa, Y., 2005. Highly Efficient Production of Nootkatone, the Grapefruit Aroma from Valencene, by Biotransformation. Chem. Pharm. Bull 53, 1513–1514.

Gamero, A., Wesselink, W., 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.

Gatfield, I.-L., Hilmer, J.-M., Bornscheuer, U., Schmidt, R., Vorlová, S., 2004. Process for the preparation of L-Menthol. US 6706500 B2.

George, B.A., 2005. Fernaroli's handbook of flavor ingredients, Fifth. ed. CRC Press, Boca Raton, Florida.

Gherghel, S., Morgan, R. M., Arrebola-Liébanas, J., Romero-González, R., Blackman, C. S., Garrido-Frenich, A., & Parkin, I.P., 2018. Development of a HS-SPME/GC–MS method

for the analysis of volatile organic compounds from fabrics for forensic reconstruction applications. Forensic Sci. Int. 290, 207–218.

Ghosh, S., Kebaara, B.W., Atkin, A.L., Nickerson, K.W., 2008. Regulation of aromatic alcohol production in *Candida albicans*. Appl. Environ. Microbiol. 74, 7211–7218.

Giglio, D.M., Mccleskey, C.S., 1953. The fermentation of sucrose by Leuconostoc mesenteroides. J. Bacteriol. 65, 75–78.

Gimeno, C.J., Ljungdahl, P.O., Styles, C.A., Fink, G.R., 1992. Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: Regulation by starvation and RAS. Cell 68, 1077–1090.

Givaudan, 2016. Investor presentation, in: Reinforcing a Leadership Position-Outperforming the Market.

Goddard, M.R., 2016. Microbiology: Mixing Wine, Chocolate, and Coffee. Curr. Biol. Dispatches 26, R275–R277.

Goldsmith, N., Hansen, E.H., Meyer, J.-P., Brianza, F., 2015. The process for producing vanillin. 2015/121379 A3.

González-Mas, M.C., Rambla, J.L., Alamar, M.C., Gutiérrez, A., Granell, A., 2011. Comparative analysis of the volatile fraction of fruit juice from different citrus species. PLoS One 6, 1–11.

Gounaris, Y., 2010. Biotechnology for the production of essential oils, flavours and volatile isolates. A review. Flavour Fragr. J. 25, 367–386.

Granato Villas-Boâs, S., Esposito, E., Matos de Mendonca, M., 2003. Bioconversion of apple pomace into a nutritionally enriched substrate by *Candida utilis* and *Pleurotus ostreatus.* World J. Microbiol. Biotechnol. 19, 461–467.

Granato Villas-Bôas, S., Esposito, E., Mitchell, D.A., 2002. Microbial conversion of lignocellulosic residues for production of animal feeds. Anim. Feed Sci. Technol. 98, 1–12.

Granucci, N., 2018. Fruit residues: Low cost substrates for development of new food products. University of Auckland.

Groguenin, A., Waché, Y., Garcia, E.E., Aguedo, M., Husson, F., LeDall, M.T., Belin, J.M., 2004. Genetic engineering of the β-oxidation pathway in the yeast *Yarrowia lipolytica* to increase the production of aroma compounds. J. Mol. Catal. B Enzym. 28, 75–79.

Gross, B., Yonnet, G., Picque, D., Brunerie, P., Corrieu, G., Asther, M., 1990. Applied Microbiology Biotechnology Production of methylanthranilate by the basidiomycete *Pycnoporus cinnabarinus* (Karst.). Appl Microbiol Biotechnol 34, 387–391.

Haisman, D.R., Knight, D.J., 1967. The Enzymic Hydrolysis of Amygdalin. Biochem. J 103, 528–534.

Han, T.L., Tumanov, S., Cannon, R.D., Villas-Boas, S.G., 2013. Metabolic Response of *Candida albicans* to Phenylethyl Alcohol under Hyphae-Inducing Conditions. PLoS One 8, e71364.

Hanem, H., Fadel, M., Gomaa Mahmoud, M., Mohamed, M., Asker, S., Lotfy, S.N., 2015. Characterization and evaluation of coconut aroma produced by *Trichoderma viride* EMCC-107 in solid state fermentation on sugarcane bagasse. EJBT 18, 5–9.

Hansen, E.H., Moller, B.L., Kock, G.R., Bunner, C.M., Kristensen, C., Jensen, O.R., Okkels, F.T., Olsen, C.E., Motawia, M.S., Hansen, J., 2009. *De Novo* Biosynthesis of Vanillin in Fission Yeast *(Schizosaccharomyces pombe)* and Baker's Yeast (*Saccharomyces cerevisiae*). Appl. Environ. Microbiol. 75, 2765–2774.

Hellborg, L., Piškur, J., 2009. Complex Nature of the Genome in a Wine Spoilage Yeast, *Dekkera bruxellensis*. Eukaryot. Cell 8, 1739–1749.

Hess, D.C., Lu, W., Rabinowitz, J.D., Botstein, D., 2006. Ammonium Toxicity and Potassium Limitation in Yeast. PLoS Biol. 4, e351.

Hölker, U., Lenz, J., 2005. Solid-state fermentation-are there any biotechnological advantages? Curr. Opin. Microbiol. 8, 301–306.

Hong, Y.-C., Huang, L.-C., Reineccius, G.A., Harlander, S.K., Labuza, T.P., 1990. Production of aroma compounds from strawberry cell suspension cultures by addition of precursors. Plant Cell. Tissue Organ Cult. 21, 245–251.

Howard, K.L., Mike, J.H., Riesen, R., 2005. Validation of a solid-phase microextraction method for headspace analysis of wine aroma components. Am. J. Enol. Vitic. 56, 37–45.

Hugenholtz, J., 2013. Traditional biotechnology for new foods and beverages. Curr. Opin. Biotechnol. 24, 155–159.

Husson, F., Krumov, K.N., Cases, E., Cayot, P., Bisakowski, B., Kermasha, S., Belin, J.M., 2005. Influence of medium composition and structure on the biosynthesis of the natural flavour 1-octen-3-ol by *Penicillium camemberti.* Process Biochem. 40, 1395–1400.

Husson, F., Thomas, M., Kermasha, S., Belin, J.M., 2002. Effect of linoleic acid induction on the production of 1-octen-3-ol by the lipoxygenase and hydroperoxide lyase activities of *Penicillium camemberti*. J. Mol. Catal. B Enzym. 19–20, 363–369.

Jani, J., 2017. Flavour of the season likely to smell success.

Joginder Singr, B., Dincro, D., 1994. Molds as Protective Cultures for Raw Dry Sausages. J. Food Prot. 57, 928–930.

Johnstone, R., Herbert, C., 2002. Mass Spectrometry Basics, 1st ed. CRC Press.

Kaminski, E., Stawicki, S., Wasowicz, E., 1974. Volatile Flavor Compounds Produced by Molds of *Aspergillus, Penicillium,* and Fungi *imperfecti*. Appl. Microbiol. 27, 1001–4.

Karahadian, C., Josephson, D.B., Lindsay, R.C., 1985. Volatile Compounds from *Penicillium* sp. Contributing Musty-Earth Notes to Brie and Camembert Cheese Flavors. J. Food Sci 33, 435.

Kaur, P., Ghoshal, G., Jain, A., 2019. Bio-utilization of fruits and vegetables waste to produce β-carotene in solid-state fermentation: Characterization and antioxidant activity. Process Biochem. 76, 155–164.

Kim, B., Cho, B.-R., Hahn, J.-S., 2014. Metabolic engineering of *Saccharomyces cerevisiae* for the production of 2-phenylethanol via Ehrlich pathway. Biotechnol. Bioeng. 111, 115–124.

Kim, T.Y., Lee, S.W., Oh, M.K., 2014. Biosynthesis of 2-phenylethanol from glucose with genetically engineered *Kluyveromyces marxianus*. Enzyme Microb. Technol. 61, 44–47.

Kowalska, H., Czajkowska, K., Cichowska, J., Lenart, A., 2017. What's new in biopotential of fruit and vegetable by-products applied in the food processing industry. Trends Food Sci. Technol. 67, 150–159.

Lawall, C.H., Forman, L., 1914. The analysis of vanilla extract. Am. Pharmacbutical Assoc. 3, 25–28.

Lee, D., Lloyd, N.D.R., Pretorius, I.S., Borneman, A.R., 2016. Heterologous production of raspberry ketone in the wine yeast *Saccharomyces cerevisiae* via pathway engineering and synthetic enzyme fusion. Microb. Cell Fact. 15, 49.

Lee, S.-M., Lee, J.-H., 2011. Ethanol fermentation for main sugar components of brown-algae

using various yeasts. J. Ind. Eng. Chem. 18, 16–18.

Lesage-Meessen, L., Delattre, M., Haon, M., Thibault, J.F., Ceccaldi, B.C., Brunerie, P., Asther, M., 1996. A two-step bioconversion process for vanillin production from ferulic acid combining *Aspergillus niger* and *Pycnoporus cinnabarinus*. J. Biotechnol. 50, 107–113.

Li, X. lei, Kang, L., Hu, J. jing, Li, X. fei, Shen, X., 2008. Aroma Volatile Compound Analysis of SPME Headspace and Extract Samples from Crabapple (*Malus* sp.) Fruit Using GC-MS. Agric. Sci. China 7, 1451–1457.

Li, Y., Wei, G., Chen, J., 2004. Glutathione: a review on biotechnological production. Appl. Microbiol. Biotechnol. 66, 233–242.

Liu, S.Q., 2015. Impact of yeast and bacteria on beer appearance and flavour, in: Brewing Microbiology. Woodhead Publishing, pp. 357–374.

Lomascolo, A., Lesage-Meessen, L., Haon, M., Navarro, D., Antona, C., Faulds, C., Marcel, A., 2001. Evaluation of the potential of *Aspergillus niger* species for the bioconversion of L-phenylalanine into 2-phenylethanol. World J. Microbiol. Biotechnol. 17, 99–102.

Lomascolo, A., Lesage-Meessen, L., Labat, M., Navarro, D., Delattre, M.., Asther, M., 1999. Enhanced benzaldehyde formation by a monokaryotic strain of *Pycnoporus cinnabarinus* using a selective solid adsorbent in the culture medium. Can. J. Microbiol. 45, 653–657.

Lomascolo, A., Stentelaire, C., Asther, M., Lesage-Meessen, L., 1999. Basidiomycetes as new biotechnological tools to generate natural aromatic flavours for the food industry. Trends Biotechnol. 17, 282–289.

Lomascolo, A., Uzan-Boukhris, E., Herpoël-Gimbert, I., Sigoillot, J.-C., Lesage-Meessen, L., 2011. Peculiarities of *Pycnoporus* species for applications in biotechnology. Appl.

Microbiol. Biotechnol. 92, 1129–1149.

López, P., Huerga, M.A., Batlle, R., Nerin, C., 2006. Use of solid phase microextraction in diffusive sampling of the atmosphere generated by different essential oils. Anal. Chim. Acta 559, 97–104.

M.Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Madrera, R.R., Pando, R., Nana, B., En Su Arez Valles, B., 2015. Production and characterization of aroma compounds from apple pomace by solid-state fermentation with selected yeasts. LWT - Food Sci. Technol. 64, 1342–1353.

Maga, J.A., 1981. Mushroom Flavor. Am. Chem. Soc. 29, 1–4.

Mantzouridou, F.T., Paraskevopoulou, A., Lalou, S., 2015. Yeast flavour production by solid state fermentation of orange peel waste. Biochem. Eng. J. 101, 1–8.

Market Research Consulting Pvt Ltd, 2018. Flavors and Fragrances - Global Market Outlook (2017-2026).

Market research provier, E.I., 2018. Ingredients: Euromonitor International from trade interviews and industry sources.

Martin, V.J., Pitera, D.J., Withers, S.T., Newman, J.D., Keasling, J.D., 2003. Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. Nat. Biotechnol. 21, 796–802.

Martínez-Avila, O., Sánchez, A., Font, X., Barrena, R., 2018. Bioprocesses for 2-phenylethanol and 2-phenylethyl acetate production: current state and perspectives. Appl. Microbiol. Biotechnol. 102, 9991–10004.

Martínez-Moreno, R., Quirós, M., Morales, P., Gonzalez, R., 2014. New insights into the advantages of ammonium as a winemaking nutrient. Int. J. Food Microbiol. 177, 128–35.

Martínez, O., Sánchez, A., Font, X., Barrena, R., 2017. Valorization of sugarcane bagasse and sugar beet molasses using *Kluyveromyces marxianus* for producing value-added aroma compounds via solid-state fermentation. J. Clean. Prod. 158, 8–17.

Masuo, S., Osada, L., Zhou, S., Fujita, T., Takaya, N., 2015. *Aspergillus oryzae* pathways that convert phenylalanine into the flavor volatile 2-phenylethanol. Fungal Genet. Biol. 77, 22–30.

Maxwell, W.A., Spoerl, E., 1971. Mannitol Uptake by *Saccharomyces cerevisiae*, J. Bacteriol. 105, 753-758.

Mayer, E., 1958. Process for making vinegar. 2,997,424.

Mei Feng, X., Ostenfeld Larsen, T., Schnürer, J., 2007. Production of volatile compounds by *Rhizopus oligosporus* during soybean and barley tempeh fermentation. Int. J. Food Microbiol. 113, 133–141.

Mei, J., Min, H., Lü, Z., 2009. Enhanced biotransformation of l-phenylalanine to 2-phenylethanol using an in situ product adsorption technique. Process Biochem. 44, 886–890.

Micallef, N., 2014. The Multi-Faceted Nature of Innovation in Fragrances.

Michlmayr, H., Nauer, S., Brandes, W., Schümann, C., Kulbe, K.D., del Hierro, A.M., Eder, R., 2012. Release of wine monoterpenes from natural precursors by glycosidases from *Oenococcus oeni.* Food Chem. 135, 80–87.

Motedayen, N., Ismail, M.B.T., Nazarpour, F., 2013. Bioconversion of ferulic acid to vanillin by combined action of Aspergillus niger K8 and Phanerochaete crysosporium ATCC

24725. African J. Biotechnol. 12, 6618–6624.

Muheim, Andreas; Müller, Bruno; Münch, Thomas; Wetli, M., 1998. Process for the production of vanillin. EP 0 885 968 B3.

Murahashi, S., 1938. The odor of matsutake (*Armillaria matsutake* Ito er Imai) II. Sci. Pap. Inst. Phys. Chem. Res. 34, 155–172.

Nestl, B.M., Nebel, B.A., Hauer, B., Flitsch, S., Gotor, V., 2010. Recent progress in industrial biocatalysis This review comes from a themed issue on Biocatalysis and Biotransformation Edited. Curr. Opin. Chem. Biol. 15, 187–193.

Niedz, R.P., Moshonas, M.G., Peterson, B., Shapiro, J.P., Shaw, P.E., 1997. Analysis of sweet orange (*Citrus sinensis* (L.) Osbeck) callus cultures for volatile compounds by gas chromatography with mass selective detector. Plant Cell. Tissue Organ Cult. 51, 181–185.

Niimi, M., Tokunaga, M., Nakayama, H., n.d. Journal of Medical and Veterinary Mycology Regulation of mannitol catabolism in *Candida albicans*: evidence for cyclic AMP-independent glucose effect. J. Med. Vet. Mycol. 24, 211–217.

Ohsumi, C., Hayashit, T., Sano, K., 1993. Formation of Alliin in the Culture Tissues of *Allium sativum*. Oxidation of S-Allyl-Cysteine 33, 107–111.

Palmerín-Carreño, D.M., Rutiaga-Quiñones, O.M., Verde Calvo, J.R., Prado-Barragán, A., Huerta-Ochoa, S., 2015. Screening of microorganisms for bioconversion of (+)-valencene to (+)-nootkatone. LWT - Food Sci. Technol. 64, 788–793.

Pandey, A., 2003. Solid-state fermentation. Biochem. Eng. J. 13, 81–84.

Pandey, A., Soccol, C.R., 2008. Current developments in solid-state fermentation. Springer Science & Business Media.

Pandey, A., Soccol, C.R., Nigam, P., Soccol, V.T., 2000. Biotechnological potential of agro-industrial residues. I: Sugarcane bagasse. Bioresour. Technol. 74, 69–80.

Pel, H.J., De Winde, J.H., Archer, D.B., Dyer, P.S., Hofmann, G., Schaap, P.J., Turner, G., De Vries, R.P., Albang, R., Albermann, K., Andersen, M.R., Bendtsen, J.D., Benen, J.A.E., Van Den Berg, M., Breestraat, S., Caddick, M.X., Contreras, R., Cornell, M., Coutinho, P.M., Danchin, E.G.J., Debets, A.J.M., Dekker, P., Van Dijck, P.W.M., Van Dijk, A., Dijkhuizen, L., Driessen, A.J.M., D'Enfert, C., Geysens, S., Goosen, C., Groot, G.S.P., De Groot, P.W.J., Guillemette, T., Henrissat, B., Herweijer, M., Van Den Hombergh, J.P.T.W., Van Den Hondel, C.A.M.J.J., Van Der Heijden, R.T.J.M., Van Der Kaaij, R.M., Klis, F.M., Kools, H.J., Kubicek, C.P., Van Kuyk, P.A., Lauber, J., Lu, X., Van Der Maarel, M.J.E.C., Meulenberg, R., Menke, H., Mortimer, M.A., Nielsen, J., Oliver, S.G., Olsthoorn, M., Pal, K., Van Peij, N.N.M.E., Ram, A.F.J., Rinas, U., Roubos, J.A., Sagt, C.M.J., Schmoll, M., Sun, J., Ussery, D., Varga, J., Vervecken, W., Van De Vondervoort, P.J.J., Wedler, H., Wösten, H.A.B., Zeng, A.P., Van Ooyen, A.J.J., Visser, J., Stam, H., 2007. Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. Nat. Biotechnol. 25, 221–231.

Pellati, F., Benvenuti, S., Yoshizaki, F., Bertelli, D., Rossi, M.C., 2005. Headspace solid-phase microextraction-gas chromatography-mass spectrometry analysis of the volatile compounds of *Evodia* species fruits. J. Chromatogr. A 1087, 265–273.

Peñas, E., Martinez-Villaluenga, C., Frias, J., 2017. Sauerkraut: Production, Composition, and Health Benefits, in: Fermented Foods in Health and Disease Prevention. pp. 557–576.

Petti, A.A., Crutchfield, C.A., Rabinowitz, J.D., Botstein, D., 2011. Survival of starving yeast is correlated with oxidative stress response and nonrespiratory mitochondrial function. Proc. Natl. Acad. Sci. 108, E1089–E1098.

Pinu, F., Villas-boas, S.G., Pinu, F.R., Villas-boas, S.G., 2017. Rapid Quantification of Major Volatile Metabolites in Fermented Food and Beverages Using Gas Chromatography-Mass Spectrometry. Metabolites 7, 37.

Poliak, M., Kochman, M., Gordin, A., Amirav, A., 2006. A Comparison of SnifProbe and SPME for Aroma Sampling. Chromatographia 64, 487–493.

Procházka, E., Poláková, S., Piškur, J., Sulo, P., 2010. Mitochondrial genome from the facultative anaerobe and petite-positive yeast *Dekkera bruxellensis* contains the NADH dehydrogenase subunit genes. FEMS Yeast Res. 10, 545–557.

REGULATION (EC) No 1334/2008 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC, 2008. . The European Parliament and the Council of the European Union.

Reil, G., Berger, R.G., 1996. Accumulation of chlorophyll and essential oils in photomixotrophic cell cultures of *Citrus* sp. Zeitschrift fur Naturforsch. - Sect. C J. Biosci. 51, 657–666.

Renouf, V., Lonvaud-Funel, A., Coulon, J., 2007. The origin of *Brettanomyces bruxellensis* in wines: A review. J. Int. des Sci. la Vigne du Vin 41, 161–173.

Robinson, T., Singh, D., Nigam, P., 2001. Solid-state fermentation: a promising microbial technology for secondary metabolite production. Appl. Microbiol. Biotechnol. 55, 284–289.

Rodrigues, F., Caldeira, M., Câmara, J.S., 2008. Development of a dynamic headspace solid-phase microextraction procedure coupled to GC-qMSD for evaluation the chemical

profile in alcoholic beverages. Anal. Chim. Acta 609, 82–104.

Rosma, A., Cheong, M.W., 2017. Effects of nitrogen supplementation on yeast (*Candida utilis*) biomass production by using pineapple (Ananas comosus) waste extracted medium. Malays. J. Microbiol. 3, 19–26.

Rossi, S.C., Vandenberghe, L.P.S., Pereira, B.M.P., Gago, F.D., Rizzolo, J. a., Pandey, a., Soccol, C.R., Medeiros, a. B.P., 2009. Improving fruity aroma production by fungi in SSF using citric pulp. Food Res. Int. 42, 484–486.

Ruan, E.D., Aalhus, J.L., Juárez, M., Sabik, H., 2015. Analysis of Volatile and Flavor Compounds in Grilled Lean Beef by Stir Bar Sorptive Extraction and Thermal Desorption—Gas Chromatography Mass Spectrometry. Food Anal. Methods 8, 363–370.

Ruiz-Terán, F., Perez-Amador, I., López-Munguia, A., 2001. Enzymatic extraction and transformation of glucovanillin to vanillin from vanilla green pods. J. Agric. Food Chem. 49, 5207–5209.

Saha, B.C., Racine, F.M., 2011. Biotechnological production of mannitol and its applications. Appl. Microbiol. Biotechnol. 89, 879–891.

Saldanha, A.J., Brauer, M.J., Botstein, D., 2004. Nutritional Homeostasis in Batch and Steady-State Culture of Yeast. Mol. Biol. Cell 15, 4089–4104.

Sales, A., Paulino, B.N., Pastore, G.M., Lemos Bicas, J., 2018. Biogeneration of aroma compounds. Curr. Opin. Food Sci. 19, 77–84.

San Juan, F., Cacho, J., Ferreira, V., Escudero, A., 2013. Chapter 21. Differences in Chemical Composition of Aroma among Red Wines of Different Price Category. Flavour Sci. 117–122.

San Román, I., Bartolomé, L., Gee, W.S., Alonso, R.M., Beck, J.J., 2015. Comparison of ex

situ volatile emissions from intact and mechanically damaged walnuts. Food Res. Int. 72, 198–207.

Sánchez-Rodríguez, L., Ali, N.S., Cano-Lamadrid, M., Noguera-Artiaga, L., Lipan, L., Carbonell-Barrachina, Á.A., Sendra, E., 2018. Chapter 19. Flavors and Aromas.

Schieber, A., Stintzing, F.C., Carle, R., 2001. By-products of plant food processing as a source of functional compounds - Recent developments. Trends Food Sci. Technol. 12, 401–413.

Schifferdecker, A.J., Dashko, S., Ishchuk, O.P., Piškur, J., 2014. The wine and beer yeast *Dekkera bruxellensis*. Yeast 31, 323–332.

Schrader, J., 2007. Microbial Flavour Production, in: Berger, R.G. (Ed.), Flavours and Fragrances: Chemistry, Bioprocessing and Sustainability. Springer, pp. 507–574.

Schulze, K.L., 1956. The Effect of Phosphate Supply on the Rate of Growth and Fat Formation in Yeasts. Appl. Microbiol. 4, 207–210.

Schwab, W., 2007. Genetic Engineering of Plants and Microbial Cells for Flavour Production, in: Berger, R.G. (Ed.), Flavours and Fragrances: Chemistry, Bioprocessing and Sustainability. pp. 615–649.

Schwab, W., Davidovich-Rikanati, R., Lewinsohn, E., 2008. Biosynthesis of plant-derived flavor compounds. Plant J. 54, 712–732.

Sharma, K.D., Karki, S., Thakur, N.S., Attri, S., 2012. Chemical composition, functional properties and processing of carrot—a review. J. Food Sci. Technol. 49, 22–32.

Shepard, M.W., 1960. Method of producing citric acid by fermentation. 3,083,144.

Siddiqui, M.S., Thodey, K., Trenchard, I., Smolke, C.D., 2012. Advancing secondary metabolite biosynthesis in yeast with synthetic biology tools. FEMS Yeast Res. 12, 144–

170.

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

Silva, V., Igrejas, G., Falco, V., Santos, T.P., Torres, C., Oliveira, A.M.P., Pereira, J.E., Amaral, J.S., Poeta, P., 2018. Chemical composition, antioxidant and antimicrobial activity of phenolic compounds extracted from wine industry by-products. Food Control 92, 516–522.

Sindhwani, G., Uk, I., Aeri, V., 2012. Microbial transformation of eugenol to vanillin. J. Microbiol. Biotechnol. Res. 2, 313–318.

Smith, B.D., Divol, B., 2016. *Brettanomyces bruxellensis*, a survivalist prepared for the wine apocalypse and other beverages. Food Microbiol. 59, 161–175.

Snowdon, E.M., Bowyer, M.C., Grbin, P.R., Bowyer, P.K., 2006. Mousy Off-Flavor: A Review. J. Agric. Food Chem. 54, 6465–6474.

Soares, M., Christen, P., Pandey, A., Soccol, C.R., 2000. Fruity flavour production by *Ceratocystis fimbriata* grown on coffee husk in solid-state fermentation. Process Biochem. 35, 857–861.

Soccol, C.R., Ferreira Da Costa, E.S., Letti, A.J., Karp, S.G., Woiciechowski, A.L., Porto De Souza Vandenberghe, L., 2017. Recent developments and innovations in solid state fermentation. Biotechnol. Res. Innov. 1, 52–71.

Sowden, R.J., Yasmin, S., Rees, N.H., Bell, S.G., Wong, L.-L., 2005. Biotransformation of the sesquiterpene (+)-valencene by cytochrome P450cam and P450BM-3. Org. Biomol. Chem. 3, 57.

Sporkert, F.; Pragst, F., 2000. Use of headspace solid-phase microextraction (HS-SPME) in

hair analysis for organic compounds. Forensic Sci. Int. 107, 129–148.

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

Steensels, J., Daenen, L., Malcorps, P., Derdelinckx, G., Verachtert, H., Verstrepen, K.J., 2015b. *Brettanomyces* yeasts — From spoilage organisms to valuable contributors to industrial fermentations.

Stincone, A., Prigione, A., Cramer, T., Wamelink, M.M.C., Campbell, K., Cheung, E., Olin-Sandoval, V., Grüning, N.-M., Krüger, A., Tauqeer Alam, M., Keller, M.A., Breitenbach, M., Brindle, K.M., Rabinowitz, J.D., Ralser, M., 2015. The return of metabolism: biochemistry and physiology of the pentose phosphate pathway. Biol. Rev. 90, 927–963.

Styger, G., Prior, B., Bauer, F.F., 2011. Wine flavor and aroma. J. Ind. Microbiol. Biotechnol. 38, 1145–59.

Sunarharum, W.B., Williams, D.J., Smyth, H.E., 2014. Complexity of coffee flavor: A compositional and sensory perspective. FRIN 62, 315–325.

Sunesen, L.O., Stahnke, L.H., 2003. Mould starter cultures for dry sausages-selection, application and effects. Meat Sci. 65, 935–948.

Thom, C., 1906. Fungi in Cheese Ripening: Camambert and Roquefort. Washington.

Tomita, Y., Ikeo, K., Tamakawa, H., Gojobori, T., Ikushima, S., 2012. Genome and Transcriptome Analysis of the Food-Yeast *Candida utilis.* PLoS One 7, 37226.

Topakas, E., Kalogeris, E., Kekos, D., Macris, B.J., Christakopoulos, P., 2003. Bioconversion of ferulic acid into vanillic acid by the thermophilic fungus *Sporotrichum thermophile*. LWT - Food Sci. Technol. 36, 561–565.

Torre, P., Faveri, D.D.E., Perego, P., Ruzzi, M., Barghini, P., 2004. Bioconversion of ferulate into vanillin by *Escherichia coli* strain JM109 / pBB1 in an immobilized-cell reactor. Ann. Microbiol. 54, 517–527.

Torres, S., Baigorí, M.D., Swathy, S.L., Pandey, A., Castro, G.R., 2009. Enzymatic synthesis of banana flavour (isoamyl acetate) by *Bacillus licheniformis* S-86 esterase. Food Res. Int. 42, 454–460.

Toshinori, Matsui; Yukio, Nishimura; Ichijiro, Ide; Hiroshi, Okada; Iwao, Kameyama; Toshinao, T., 1962. Preparation of l-glutamic acid by fermentation. US3174908A.

Tripathi, U., Ramachandra Rao, S., Ravishankar, G.A., 2002. Biotransformation of phenylpropanoid compounds to vanilla flavor metabolites in cultures of *Haematococcus pluvialis*. Process Biochem. 38, 419–426.

U.S. Food and Drug Administration, 2018. CFR - Code of Federal Regulations Title 21.

Uscanga, M.G.A., Delia, M.-L., Strehaiano, P., 2000. Nutritional requirements of *Brettanomyces bruxellensis*: Growth and physiology in batch and chemostat cultures. Can. J. Microbiol. 46, 1046–1050.

Van Leuven, I., Caelenberg, T. Van, Dirinck, P., 2008. Aroma characterisation of Gouda-type cheeses. Int. Dairy J. 18, 790–800.

Varela, C., Pizarro, F., Agosin, E., 2004. Biomass Content Governs Fermentation Rate in Nitrogen-Deficient Wine Musts Downloaded from. Appl. Environ. Microbiol. 70, 3392–3400.

Viander, B., Aki, M.M., Palva, A., 2003. Impact of low salt concentration, salt quality on natural large-scale sauerkraut fermentation. Food Microbiol. 20, 391–395.

Vilanova, M., Siebert, T.E., Varela, C., Pretorius, I.S., Henschke, P.A., 2012. Effect of

ammonium nitrogen supplementation of grape juice on wine volatiles and non-volatiles composition of the aromatic grape variety Albariño. Food Chem. 133, 124–131.

Villas-Bôas, S.G., Mas, S., Åkesson, M., Smedsgaard, J., Nielsen, J., 2005. Mass spectrometry in metabolome analysis. Mass Spectrom. Rev. 24, 613–646.

Vojvodić, A., Komes, D., Vovk, I., Belščak-Cvitanović, A., Bušić, A., 2016. Compositional evaluation of selected agro-industrial wastes as valuable sources for the recovery of complex carbohydrates. Food Res. Int.

Waché, Y., Laroche, C., Bergmark, K., Møller-Andersen, C., Aguedo, M., Le Dall, M.T., Wang, H., Nicaud, J.M., Belin, J.M., 2000. Involvement of acyl coenzyme A oxidase isozymes in biotransformation of methyl ricinoleate into gamma-decalactone by *Yarrowia lipolytica*. Appl. Environ. Microbiol. 66, 1233–6.

Walton, N.J., Mayer, M.J., Narbad, A., 2003. Molecules of Interest Vanillin. Phytochemistry 63, 505–515.

Watson, J.T., Sparkman, O.D., 2013. Introduction to mass spectrometry: instrumentation, applications, and strategies for data interpretation. John Wiley & Sons., 4th ed. John Wiley & Sons, Ltd.

Wedral, D., Shewfelt, R., Frank, J., 2010. The challenge of *Brettanomyces* in wine. LWT - Food Sci. Technol. 43, 1474–1479.

Wickes, B.L., Mayorga, M.E., Edman, U., Edman, J.C., 1996. Dimorphism and haploid fruiting in *Cryptococcus neoformans*: association with the alpha-mating type. Proc. Natl. Acad. Sci. U. S. A. 93, 7327–31.

Widder, S., Lüntzel, C.S., Dittner, T., Pickenhagen, W., 2000. 3-mercapto-2-methylpentan-1-ol, a new powerful aroma compound. J. Agric. Food Chem. 48, 418–423.

Wilson, A.E., Sparks, D.L., Knott, K.K., Willard, S., Brown, A., 2018. Implementing solid phase microextraction (SPME) as a tool to detect volatile compounds produced by giant pandas in the environment. PLoS One 13, 1–18.

Wood, W.F., Brandes, J.A., Foy, B.D., Morgan, C.G., Mann, T.D., DeShazer, D.A., 2012. The maple syrup odour of the " candy cap" mushroom, *Lactarius fragilis* var. rubidus. Biochem. Syst. Ecol. 43, 51–53.

Woolfit, M., ˙bieta Rozpe, E., Piškur, J., Wolfe, K.H., 2007. Genome Survey Sequencing of the Wine Spoilage Yeast *Dekkera* (*Brettanomyces*) *bruxellensis*. Eukaryot. Cell 6, 721–733.

Yong, F.M., Wood, B.J.B., 1977. Biochemical changes in experimental soy sauce Moromi. J. Food Technol. 12, 263–273.

Yoshizaki, Y., Yamato, H., Takamine, K., Tamaki, H., Ito, K., Sameshima, Y., 2012. Analysis of Volatile Compounds in Shochu Koji, Sake Koji, and Steamed Rice by Gas Chromatography-Mass Spectrometry. J. Inst. Brew. 116, 49–55.

Yusef, C., 1999. Fermentation (Industrial) Basic Considerations. pp. 663–673.

Zabetakis, I., Gramshaw, J.W., 1998. 1,2-Propanediol in strawberries and its role as a flavour precursor. Food Chem. 61, 351–354.

Zabetakis, I., Holden, M.A., 1997. Strawberry flavour: Analysis and biosynthesis. J. Sci. Food Agric.

Zawirska-Wojtasiak, R., 2004. Optical purity of (R)-(−)-1-octen-3-ol in the aroma of various species of edible mushrooms. Food Chem. 86, 113–118.

Zhang, H., Cao, M., Jiang, X., Zou, H., Wang, C., Xu, X., Xian, M., 2014. De-novo synthesis of 2-phenylethanol by *Enterobacter* sp. CGMCC 5087. BMC Biotechnol. 14, 30.

Zheng, L., Zheng, P., Sun, Z., Bai, Y., Wang, J., Guo, X., 2007. Production of vanillin from waste residue of rice bran oil by *Aspergillus niger* and *Pycnoporus cinnabarinus.* Bioresour. Technol. 98, 1115–1119.

Zhu, H., Lu, Z., Cai, J., Li, J., Gao, L., 2009. Development of a headspace-SPME-GC/MS method to determine volatile organic compounds released from textiles. Polym. Test. 28, 521–527.

Zhu, Y., Tramper, J., 2013. Koji – where East meets West in fermentation. Biotechnol. Adv. 31, 1448–1457.