The utilisation of nitrogenous compounds by commercial non-*Saccharomyces* yeasts associated with wine

by

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Declaration

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Summary

Nitrogen is an essential element for yeast growth and metabolism. During winemaking, the uptake of nitrogen sources ensures the completion of alcoholic fermentation while participating in the production of aroma compounds. Preference for certain nitrogen sources has been determined for *Saccharomyces cerevisiae*, the main wine yeast, but not in other yeast species, collectively referred to as non-*Saccharomyces* yeasts. Yet, the latter are becoming increasingly used in industry in combination with *S. cerevisiae* and the preferences of these yeasts should also be investigated in order to ensure optimal fermentation ability and aroma compound production. This could unravel possible competition between yeast species and consequently potential needs for supplementation to avoid stuck fermentations and production of off-aroma compounds.

This study investigated the nitrogen source preferences of a number of commercial non-*Saccharomyces* yeasts (*Pichia kluyveri* Viniflora® FootZen™, *Torulaspora delbrueckii* Biodiva™ TD291, *Metschnikowia pulcherrima* Flavia® MP346 and *Lachancea thermotolerans* Viniflora® Concerto™) by conducting pure culture fermentations in a synthetic grape must medium with nitrogen sources present at equal assimilable nitrogen concentrations. Uptake varied between yeast species, however *P. kluyveri* and *M. pulcherrima* showed similar uptake trends, as did *L. thermotolerans* and *T. delbrueckii*. Nitrogen uptake was also proportional to CO_2 release, sugar consumption and yeast growth. *L. thermotolerans* and *T. delbrueckii* took up a greater amount of the nitrogen sources than *P. kluyveri* and *M. pulcherrima*, as well as at a faster rate. Variations in amino acid preferences between yeast species were observed. The presence of ammonium was shown to affect the % uptake of certain amino acids for the different species as well as the onset of amino acid uptake for *T. delbrueckii*. Sequential fermentations with grape must-like nitrogen concentrations revealed that *L. thermotolerans* and *T. delbrueckii* pose a larger problem regarding competition with *S. cerevisiae* compared to *P. kluyveri* and *M. pulcherrima*. This was reflected in fermentation kinetics of sequential fermentations as well as the levels of aroma compound production. This suggests that sequential fermentations with *L. thermotolerans* and *T. delbrueckii* may require higher nitrogen source supplementation than *P. kluyveri* and *M. pulcherrima* for *S. cerevisiae* to complete fermentation as well as increase the concentration of desirable aroma compounds. Some correlations were observed regarding nitrogen source uptake and aroma compounds produced, for example the uptake of threonine and resulting propanol production. The data showed that, after inoculation with *S. cerevisiae* Lalvin® EC1118, the population of non-*Saccharomyces* yeasts declined and the cells probably autolyse, thereby releasing nitrogen sources that can be taken up by *S. cerevisiae*. This study improved our knowledge of non-*Saccharomyces* yeasts' nitrogen source preferences, and

establishes some links between nitrogen source uptake and aroma compound production. Competition between yeasts was also demonstrated and linked with nitrogen uptake and fermentation ability.

Opsomming

Stikstof is ‘n essensiële element vir gis groei en gis metabolisme. Gedurende die wynmaak proses, verseker die opname van stikstof bronne die voltooiing van alkoholiese fermentasie terwyl die aroma verbindings geproduseer word. Voorkeur vir sekere stikstof bronne is vasgestel vir *S. cerevisiae*, die hoof wyngis, maar nie vir ander gis spesies, gegroepeer en bekend as nie-*Saccharomyces* giste, nie. Alhoewel, die laasgenoemde word al hoe meer gebruik in die industrie saam met *S. cerevisiae* en die voorkeur van hierdie giste moet ondersoek word om optimale fermentasie en produksie van aroma verbindings te verseker. Dit kan die potensieële kompetisie tussen gis spesies ontrafel en daarmee saam ook potensieële byvoegings behoeftes om staak fermentasies en produksie van nie-geurige aroma verbindings te verhoed.

Hierdie studie ondersoek die stikstofbron voorkeur van ‘n aantal kommersiële nie-*Saccharomyces* giste (*Pichia kluyveri* Viniflora® FootZen™, *Torulaspora delbrueckii* Biodiva™ TD291, *Metschnikowia pulherrima* Flavia® MP346 en *Lanchancea thermotolerans* Viniflora® Concerto™) deur reinkultuur fermentasies in sintetiese wynmedium met stikstofbronne by gelyke bruikbare stikstof konsentrasies te doen. Opname verskil tussen die verskillende gisspesies, alhoewel *P.kluyveri* en *M.pulcherrima* soortgelyke opname getoon het so ook het *L. thermotolerans* en *T.delbrueckii*. Stikstof opname was ook proporsioneel aan CO_2 vrystelling, suiker verbruik en gisgroei. *L.thermotolerans* en *T.delbrueckii* het ‘n groter aantal stikstofbronne opgeneem as *P.kluyverii* en *M.pulcherrima* asook teen ‘n vinniger tempo. Verskille in die stikstofbron voorkeur is tussen die giste waargeneem. Die teenwoordigheid van stikstof het die % van aminosure wat opgeneem word tussen die verskillende giste beinvloed asook die begin van aminosuur opname deur *T.delbrueckii*. Opeenvolgende fermentasies van druiwesap met gelyke stikstof konsentrasies toon dat *L.thermotelarns* en *T.delbrueckii* in groter kompetisie is met *S.cerevisiae* as *P.kluyveri* en *M.pulcherrima*. Hierdie kompetisie word getoon in die fermentasie kinetika van opeenvolgende fermentasies asook die vlakke van aromaverbinding produksie. Dit wil voorstel dat opeenvolgende fermentasies met *L.thermotolerans* en *T.delbrueckii* hoër stikstofbron byvoegings mag benodig as fermentasies met *P.kluyveri* en *M.pulcherrima,* vir *S.cerevisiae* om die fermentasie te voltooi asook om die konsentrasie van voorkeur aroma verbindings te verhoog.

Sommige ooreenkomste word gesien ivm die opname van stikstofbronne en die aroma verbindings geproduseer, byvoorbeeld die opname van treonien en die ooreenstemmende popanol produksie. Die data wys dat, na inkulasie met *S. cerevisiae,* die populasie van nie-*Saccharomyces* giste afneem en die selle moontlik outoliseer, waardeur stikstof bronne vrygestel en wat deur *S.cerevisiae* opgeneem kan word. Hierdie studie verbeter ons kennis van nie-*Saccharomyces* giste se stikstof voorkeure en bepaal skakels tussen stikstof bron opname en aroma verbinding produksie. Kompetisie tussen giste is en skakels met stikstof opname en fermentasie vermoë is ook bewys.

This thesis is dedicated to

My Family

Biographical sketch

Kelly Prior was born in East London on 28 April 1993. She matriculated from Clarendon Girls' High School in 2011 where after she obtained her BSc degree (Human Life Sciences) from Stellenbosch University in 2014. In 2015, she completed a BSc (Hons) degree in Wine Biotechnology at Stellenbosch University. Kelly then commenced with an MSc in Wine Biotechnology in 2016 at Stellenbosch University.

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Preface

This thesis is presented as a compilation of 4 chapters.

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Chapter 1

Introduction and project aims

Chapter 1: General introduction and project aims

1.1 Introduction

Various sources of nitrogen can be used by yeasts to ensure growth, such as amino acids, ammonium salts, proteins and acid amides (Swoboda 1922). The majority of nitrogenous compounds used in wine-making are ammonium and amino acids and these nitrogen sources impact fermentations kinetics, aroma compound production and biomass formation by yeasts (Jara et al. 2014; Tesnière et al. 2015). Indeed, the availability and nature of nitrogen sources affect yeast biosynthetic and transcriptional abilities as well as yeast metabolism (Broach 2012; Tesnière et al. 2015). Nevertheless, yeasts assimilate and use nitrogen sources differently depending on species and strain (Jones and Pierce 1964; de Koker 2015; Kemsawasd et al. 2015). It is thus important for research regarding nitrogen usage of yeasts to be conducted on a large number of species which show promise for use in the wine industry, as well as for different strains within those species due to this species and strain variation.

Nitrogen use by S*accharomyces cerevisiae* has been widely researched and its preference for certain nitrogen sources has been elucidated. Jones and Pierce (1964) found that the preferred nitrogen sources for *S. cerevisiae* were glutamic acid, aspartic acid, asparagine, glutamine, serine, threonine, lysine and arginine. These amino acids are deemed preferred because they are taken up before other amino acids if they are available in the medium used, which was a wort medium used for beer making in the latter study. Crépin et al. (2012), studying wine strains, found similar preferences. Poor nitrogen sources for *S. cerevisiae* include proline, urea and allantoin as yeast growth on these as sole nitrogen sources is slow (Crépin et al. 2012). Moreover, proline cannot be degraded in the absence of oxygen (Ribereau-Gayon et al. 2006), and urea and allantoin are not relevant for winemaking.

Certain non-*Saccharomyces* yeast species have been recently shown to contribute to the organoleptic profile of wine (Ciani et al. 2010; Beckner Whitener et al. 2015; Liu et al. 2016; Padilla et al. 2016; Varela et al. 2017; Belda et al. 2017). However, if non-*Saccharomyces* yeasts are inoculated into the grape must before or simultaneously with *S. cerevisiae*, these yeasts are likely to compete for nitrogen which is in limited supply in grape must. Nitrogen is not only critical for yeast survival as mentioned above, but also affects fermentation performance and aroma compound production. It is therefore important to determine their preferences for nitrogen in

order to ensure their best performance for fermentation, determine which nitrogen sources promote the most positive aroma compound formation results, as well as which yeasts may pose a problem with regard to competition for nitrogen sources with other yeasts. For example, Medina et al. (2012) noted more sluggish fermentations when *S. cerevisiae* was inoculated sequentially after a non-*Saccharomyces* yeast compared to co-inoculation. Kemsawasd et al. (2015) tested the effect of 19 single amino acid sources on growth and fermentation kinetics on various non-*Saccharomyces* yeasts. Different amino acids were found to promote growth best for the different yeasts studied. *T. delbrueckii* preferred arginine, asparagine, glutamine and isoleucine; *M. pulcherrima* preferred alanine and asparagine; *H. uvarum* preferred alanine and *L. thermotolerans* preferred serine (Kemsawasd et al. 2015). Therefore, from these results, it could be hypothesised that *T. delbrueckii* would be most problematic regarding competition for nitrogen sources with *S. cerevisiae*. Nevertheless, the latter study was not conducted under winemaking conditions and further investigation must therefore be carried out to assess whether competition actually occurs. Amino acid preferences are important with regard to aroma compound production as their catabolism can lead to the production of various desirable compounds.

Not much research has been conducted regarding aroma compound production by non-*Saccharomyces* yeasts, but some studies have attempted to generate knowledge in this regard. *Hanseniaspora* has been shown to be a low acetic acid producer, while *M. pulcherrima*, *C. zemplinina* and *L. thermotolerans* have been shown to produce elevated levels of higher alcohols (de Koker 2015; Padilla et al. 2016; Belda et al. 2017). Finally, *Candida, Hansenula, Pichia, Hanseniaspora, Rhodotorula, T. delbrueckii* and *K. gamospora* are good producers of esters (Beckner Whitener et al. 2015; Padilla et al. 2016). These general behaviours are nevertheless impacted by the availability of nitrogenous compounds and the inoculation scenario. Indeed, De Koker (2015) found that low YAN levels lead to high ester production and low fatty acid and higher alcohol production while Benito et al. (2016) established that sequential inoculation of *S. cerevisiae* into non-*Saccharomyces* fermentations can result in improved aroma compound production compared to when only pure *S. cerevisiae* is used. A similar outcome was generated for sequential fermentations with *H. vineae* and *S. cerevisiae,* when compared to pure *S. cerevisiae* fermentations as well as spontaneous fermentations (Medina et al. 2013). Limited nutrients for *S. cerevisiae* during sequential and co-inoculations with *H. vineae* and *M. pulcherrima* resulted in a reduction in positive sensorial quality (Medina et al. 2012). These results show that more attention

needs to be paid to non-*Saccharomyces* yeasts as they have great potential for wine improvement which can be undermined by nutrients availability and competition with *S. cerevisiae*.

1.2 Project aims

This study aimed to elucidate nitrogen source preferences of various commercial non-*Saccharomyces* yeasts by determining time and percentage of uptake in a medium with all amino acids present at equal assimilable nitrogen levels, in pure culture fermentations. Equal assimilable nitrogen source levels were used in order to determine which amino acids would be preferentially taken up if they were not limited to their typical concentrations found in grape must. One variable tested was that of the presence or absence of ammonium. Fermentation and growth kinetics were also monitored for these pure culture fermentations to determine the effect of nitrogen source uptake on these parameters for the different yeast species. The correlation between amino acid uptake, aroma compounds produced and fermentations kinetics was also investigated in a medium containing grape must-like nitrogen source concentrations (in order to simulate a more realistic fermentation environment and determine whether competition between yeasts was present), for pure and sequential fermentations with *S. cerevisiae*. The effect of filtering out the non-*Saccharomyces* yeasts before *S. cerevisiae* inoculation was also investigated.

The objectives of this study were:

1. To determine the individual amino acid preferences of various commercial non-*Saccharomyces* yeasts.
2. To investigate the correlation between nitrogen sources, fermentation kinetics and aroma compounds produced for pure cultures of non-*Saccharomyces* yeasts and with *S. cerevisiae* inoculated sequentially.
3. To investigate potential competition between non-*Saccharomyces* and *S. cerevisiae* yeasts during sequential fermentation.

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Chapter 2

Literature review

Amino acid uptake and metabolism of wine yeasts

Chapter 2: Amino acid uptake and metabolism of wine yeasts

2.1 Introduction

Yeasts play a key role in the production of food and beverages. For yeast, raw food substrates constitute environments that provide nutrients and support growth and reproduction. In the process, yeast may also produce metabolites of specific interest. For instance, yeast-driven alcoholic fermentation is a key step of wine production. Many individual nutrients are essential to sustain yeasts' life in a medium such as grape juice. These include lipids, carbohydrates (e.g. glucose and fructose), vitamins and nitrogenous compounds (Tesnière et al. 2015). Variations in availability and nature of these nutrients force the yeast cells to adapt accordingly (Tesnière et al. 2015). Nitrogen is vital for life and is an element of particular interest because it enters into the composition of vital molecules such as proteins and nucleic acids and the nature and availability of nitrogen sources therefore plays a critical role in the yeast biology (Tesnière et al. 2013). Grape juice provides essential nitrogenous compounds for the yeast. In this medium, ammonium and amino acids have been identified as the dominant nitrogen sources and their depletion negatively affects yeast biomass formation, fermentation kinetics and aroma compound production (Tesnière et al. 2015, Jara et al. 2014). A shortage of these nitrogenous compounds may even lead to the production of off-aroma compounds (e.g. hydrogen sulfide). Conversely, excess nitrogen can have negative consequences with respect to microbial stability during ageing, storage or bottling (Beltran et al. 2004). In most instances, the largest part of the alcoholic fermentation of grape juice is carried out in nitrogen-depleted conditions as all assimilable nitrogen sources are taken up during exponential phase, which usually corresponds to the first 2 days of fermentation, where after the cells enter stationary phase which is induced by this depletion of assimilable nitrogen (Tesnière et al. 2015).

The concentration of the ammonium cation, NH_4^+, which is one of the preferred nitrogen sources of *S. cerevisiae*, was thought to affect how quickly fermentation begins, as well as the fermentability of the grape must (Ribéreau-Gayon et al. 2006). However, recent data suggest that this nitrogen source is taken up later than many amino acids (Crépin et al. 2012; Crépin et al. 2014). Nevertheless, this form of nitrogen is most often completely utilised during the first few days of the fermentation process. If ammonium concentrations are deemed too low, winemakers

are allowed to supplement the grape must with diammonium phosphate ($(NH_4)_2HPO_4$) or diammonium sulfate ($(NH_4)_2SO_4$) in order to promote yeast growth (Ribereau-Gayon et al. 2006; Crépin et al. 2014). Fermentation performance, however, is not necessarily linked to ammonium concentration. Indeed, Gutiérrez et al. (2012) investigated this effect on various strains and found that the *S. cerevisiae* strain TTA, while having the worst growth levels, showed the best fermentation abilities. More research, however, is being conducted regarding other nitrogen sources, more specifically amino acids and how these compounds affect growth, fermentation and wine quality.

Specific amino acid catabolism can also contribute to more desirable aroma compounds produced including volatile fatty acids, esters and higher alcohols. For this purpose, it has been suggested that it is important to optimise amino acid composition to ensure that the most desirable wines are produced (Smit 2013). However, the exact link between amino acid composition and aroma production remains somewhat unclear. Some amino acids can be degraded via the Ehrlich pathway thereby yielding the corresponding higher alcohols, fatty acids and esters (Mendes-ferreira et al. 2011). This process has been reasonably well characterised in *S. cerevisiae*, however strain variation occurs (Henschke and Jiranek 1993; Hazelwood et al. 2008).

Recently, it has also been shown that various non-*Saccharomyces* yeasts can have positive effects on wine quality (Lachance 2016; Andorrà et al. 2012; Benito et al. 2016; Belda et al. 2017). Some species and strains have been shown to produce lower alcohol wine with varying aroma profiles which contribute to wine diversity (Varela et al. 2017). This increases their desirability for use in the wine-making process and thus it is necessary to acquire more knowledge about these yeasts, with particular focus on their nitrogen uptake and preference and the resulting effect on aroma compounds produced. Yeast interactions between various non-*Saccharomyces* and *Saccharomyces* yeast strains must also be researched to determine the effect of yeast-yeast interactions as well as whether competition for nutrients occurs between yeasts, specifically regarding amino acids. Competition for nutrients can lead to stuck or sluggish fermentations and decreased aroma compound production if the non-*Saccharomyces* yeast takes up all the nitrogen sources before *S. cerevisiae* is inoculated.

This review will consider the issue of competition as well as focus on amino acid transport and metabolism and their link with aroma compounds produced in *S. cerevisiae* as well as non-

Saccharomyces species. Known nitrogen source preferences of various yeasts will be discussed as will their effect on fermentation kinetics, growth and aroma compound production.

2.2 Amino acid transport and metabolism

Amino acids account for 30-40% of the total amount of nitrogen within ripe grapes (Ribereau-Gayon et al. 2006), while other sources of nitrogen include ammonium, oligopeptides and polypeptides (Ribereau-Gayon et al. 2006). Yeasts take advantage of this resource through the uptake of these amino acids and the use of their alpha amine group (with the notable exception of proline) via deamination or transamination. The uptake and catabolism of these amino acids are tightly regulated and play a role not only in yeast growth, maintenance and functioning, but also in oenology as some of the so-called fermentative aroma compounds derive from these amino acids. In the following paragraphs, the uptake and fate of amino acids will be further described.

2.2.1 Amino acid uptake

2.2.1.1 Transporters of amino acids and regulation of uptake (for *S. cerevisiae*)

Not all amino acids are assimilated by the yeast simultaneously. They are rather taken up sequentially in order of preference (Jiranek et al. 1995; Crépin et al. 2012; Hirst and Richter 2016). Preferred nitrogen sources are those which are taken up early when all amino acids are present in the medium and can also support strong growth if provided as the sole nitrogen source (Henschke and Jiranek 1993). The time of uptake seems to correlate with particular regulation mechanisms and therefore specific transporters (Crépin et al. 2012). Indeed, certain amino acid transporters display a stronger affinity for specific amino acids (Fig. 1), such as lysine, histidine and methionine which are transported by Lyp1p, Hip1p and Mup1p respectively. Glutamate and aspartate are transported by Dip5p while the Tat1p and Tat2p are responsible for tyrosine, phenylalanine and tryptophan transport. Threonine, glutamine and serine are transported by Gnp1p and Agp1p and isoleucine, leucine and valine transporters are Bap1p and Bap2p. All the aforementioned transporters are under Ssy1-Ptr3-Ssy5 (SPS) control, which regulates amino acid uptake and is induced by extracellular amino acids (Ljungdahl and Daignan-Fornier 2012). Amino acid uptake regulation is also performed by Nitrogen Catabolite Repression (NCR) which functions to ensure that preferred nitrogen sources are first used by the yeast when available and only allows for non-preferred sources to be used once preferred sources are depleted (Ljungdahl and Daignan-Fornier 2012). This leads to the activation of other transporters namely Mep1p, Mep2p and Mep3p which are responsible for ammonium transport, and Gap1p, the general amino acid transporter, which

collectively transports alanine, arginine and glycine. Arginine is also transported by Can1p, while proline is transported by Put4p. The different transporters are depicted on Fig. 1 (Crépin et al. 2012). Amino acid transport is active and is thus coupled with proton or potassium uptake (Bisson et al. 2016).

Until recently, little information was available with regard to the link between regulation of uptake and the order of uptake from the cultivation medium. It has now been elucidated that for early and late consumed amino acids for various *S. cerevisiae* strains, different regulatory mechanisms are activated. For early consumed amino acids, permeases under SPS (Ssy1, Ptr3, and Ssy5) - mediated control which are expressed at the start of amino acid consumption are used for transport (Crépin et al. 2012; Ljungdahl and Daignan-Fornier 2012). In this system, amino acids bind to an amino acid sensor located on the plasma membrane, namely Ssy1 (Fig. 1), which in turn activates the downstream transcription factors Stp1 and Stp2 through Ptr3, Ssy5 (peripheral plasma membrane proteins), and Grr1 (SCF^{Grr1} ubiquitin ligase component) (Liu et al. 2008). Transcription factors Stp1 and Stp2 are latent cytoplasmic proteins which have N-regulation domains which are cleaved when these early assimilated extracellular amino acids lead to the activation of the SPS sensor which then catalyses an endoproteolytic process (Ljungdahl and Daignan-Fornier 2012). Thereafter, these shorter Stp1 and Stp2 factors bind promotors which can activate various amino acid permeases (as described above) (Ljungdahl and Daignan-Fornier 2012; Beltran et al. 2004; Crépin et al. 2012). A shift in regulatory mechanism is observed for later consumed nitrogen sources. Most late consumed amino acids are transported by permeases controlled by NCR. The NCR pathway downregulates the genes responsible for enzymes and permeases which promote the uptake of poor sources of nitrogen (Hirst and Richter 2016; Ljungdahl and Daignan-Fornier 2012). In the absence of preferred nitrogen sources (Fig. 1) , NCR-regulated genes are depressed in order to allow for the uptake of non-preferred nitrogen sources (Ljungdahl and Daignan-Fornier 2012; Crépin et al. 2012). Regulatory components (Ure2 and GATA transcription factors: Gln3, Gat1, Dal80, and Gzf3) control NCR-sensitive genes (Ljungdahl and Daignan-Fornier 2012). Gene expression activators (Gln3 and Gat1) target the nucleus under derepressing NCR-sensitive gene expression, while Dal80 and Gzf3 are repressors that are constitutively confined to the nucleus (Ljungdahl and Daignan-Fornier 2012). Literature regarding uptake of ammonium seems to be contradictory as it is considered a preferred nitrogen source, but is regulated by NCR permeases. Ammonium has been shown to promote good growth when supplied as a sole nitrogen source, and is thus deemed ‘preferred’, however when used in

combination with other nitrogen sources, its preference with regard to order of uptake is low compared to a number of amino acids which have been observed to be taken up before this nitrogen source (Crépin et al. 2012; Kemsawasd et al. 2015). Another nitrogen source which promoted good growth when supplied as a sole nitrogen which is not preferentially taken up when in combination with other nitrogen sources is asparagine (Crépin et al. 2012). Other late consumed amino acids, namely Val, Trp and Tyr, are exceptions and use SPS-regulated low-affinity permeases (Crépin et al. 2012). These permeases are regulated as are the other SPS permeases, however the transporter can only be bound to the amino acid if high concentrations of the amino acid are present (Ashburner et al. 2000). Fig. 1 depicts the order of consumption of nitrogen sources for the 14 *S. cerevisiae* strains tested in Crépin et al. (2012). Poor nitrogen sources include proline, urea and allantoin as growth on these sources is slow, thus they are deemed non-preferred (Crépin et al. 2012). The amino acid proline cannot be degraded by yeast in the absence of oxygen, thus high levels of proline are found in wine after completion of alcoholic fermentation (Ribereau-Gayon et al. 2006). An earlier study by Jones and Pierce (1964) found that, in general, *S.cerevisiae* prefers glutamic acid, aspartic acid, asparagine, glutamine, serine, threonine, lysine, and arginine as amino acid sources and will take these amino acids up before others if they are available. They investigated the sequence of uptake of amino acids by two different *S. cerevisiae* strains in wort fermentation. Asparagine, shown not to be taken up preferentially when in combination with other amino acids, was taken up preferentially in this study, however this could be attributed to a different cultivation medium. Arginine was taken up at different rates dependent on yeast strain within this study and its uptake time is the main difference between this study and that of Crépin et al. (2012). The latter study also used different yeast strains and was conducted in a different medium, which could account for inter-study differences observed. Overall, these results concur with the later results of Crépin et al. (2012).

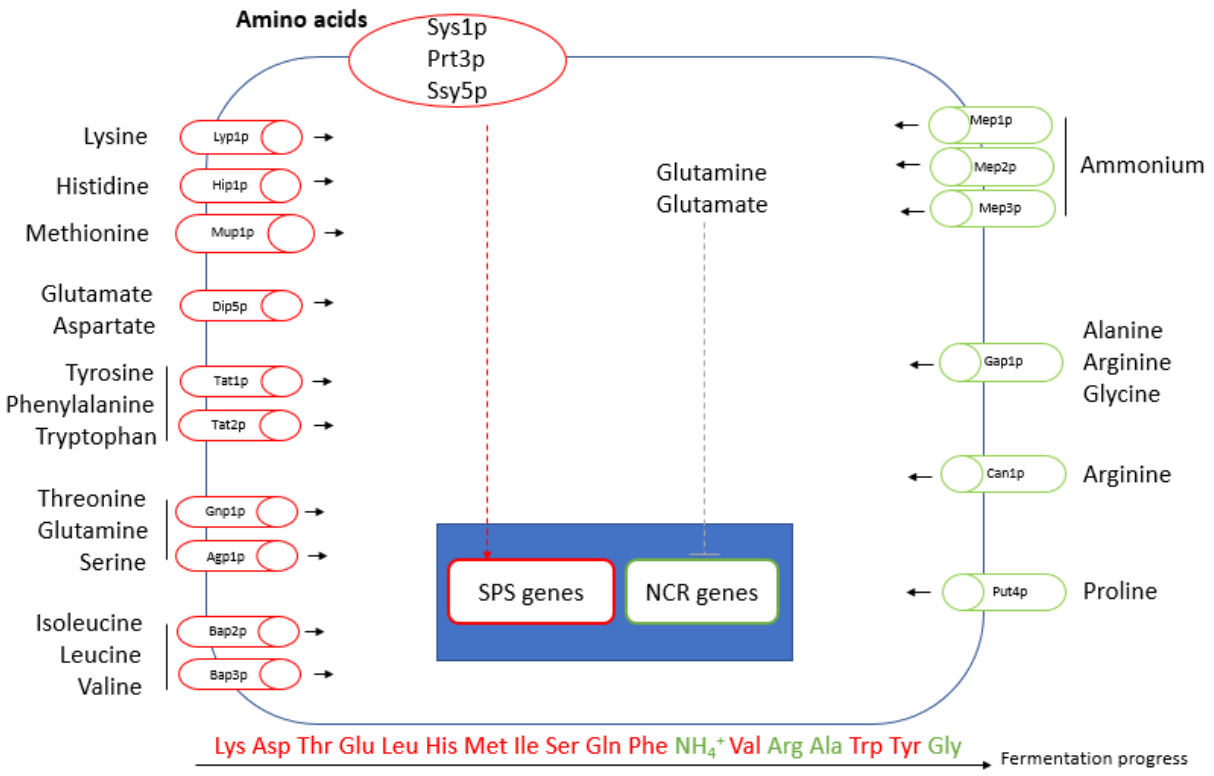

Figure 1 The main nitrogen source transporters and the mechanisms which regulate them. Single or a few specific amino acid carriers include Lyp1p, Hip1p, Mup1, Dip5p, Tat1p, Tat2p, Gnp1, Agp1p, Bap2p, Bap3p, and Can1p. A general amino acid carrier (Gap1p), ammonium permeases (Mep1p, Mep2p, and Mep3) and the arginine transporter (Can1p) are also depicted. Font size of nitrogen sources show their relative abundance in synthetic media containing 200 mg/L YAN. Red: SPS-regulated nitrogen permease genes. Green: NCR-regulated nitrogen permease genes. An order of consumption is depicted at the bottom of the figure (Crépin et al. 2012).

2.2.1.2 Other factors impacting on nitrogen utilisation by *S. cerevisiae*

Various factors have been shown to affect nitrogen utilisation by *S. cerevisiae*. These factors include initial concentration of glucose, supplementation of ammonium, air (and therefore oxygen) presence in the fermentation headspace and yeast strain (Jiranek et al. 1995; Large 1986). Jiranek et al. (1995) tested the effect of strain variation on nitrogen source uptake and these authors found that individual amino acid utilisation differed from strain to strain, however the majority of nitrogen consumed generally consisted of lysine, aspartate, threonine, glutamate, serine and arginine for all strains. All of these amino acids were also early consumed amino acids according to Crepin et al. (2012), except arginine, which although not taken up early, is stored in high levels in the vacuole (Kitamoto et al. 1988). Crepin et al. (2012) investigated the impact of the initial concentration of amino acids on their time of uptake. The order of consumption for these compounds remained the same, regardless of initial concentration, except for ammonium and

arginine. These two nitrogen sources were taken up earlier in fermentations with nitrogen sources present at equal nitrogen levels compared to uptake in a grape must-like representation of nitrogen sources.

2.2.2 Metabolic routes and use of amino acids after uptake

2.2.2.1 Protein formation

Once amino acids have been taken up into the cytoplasm, they can be incorporated into various proteins in the cell. Aminoacyl-tRNA synthetases attach specific amino acids to their connected tRNA molecules via an aminoacylation reaction (Pang et al. 2014). Each aminoacyl-tRNA sythetase is specific to a certain amino acid which it activates via an adenylate intermediate, where after the amino acids are then linked to a ribose hydroxyl which is situated at the 3'-end of tRNA isoacceptor set (Pang et al. 2014). The tRNA molecule carries the amino acid to the mRNA at the ribosome where it is added to the end of a growing polypeptide chain, thus forming a protein (McKinnon 2013).

2.2.2.2 Storage of amino acids in *S. cerevisiae*

Amino acids can also be transported across the vacuolar membrane, as reviewed by Sekito et al. (2008) where they are stored for later use. Some amino acids are transported into the vacuole via active transport, whereas others are found only in the cytosol of the cell. In most cases, amino acid transport is coupled with protons which are produced by vacuolar H1-ATPase. Fig. 2 depicts these transporters.

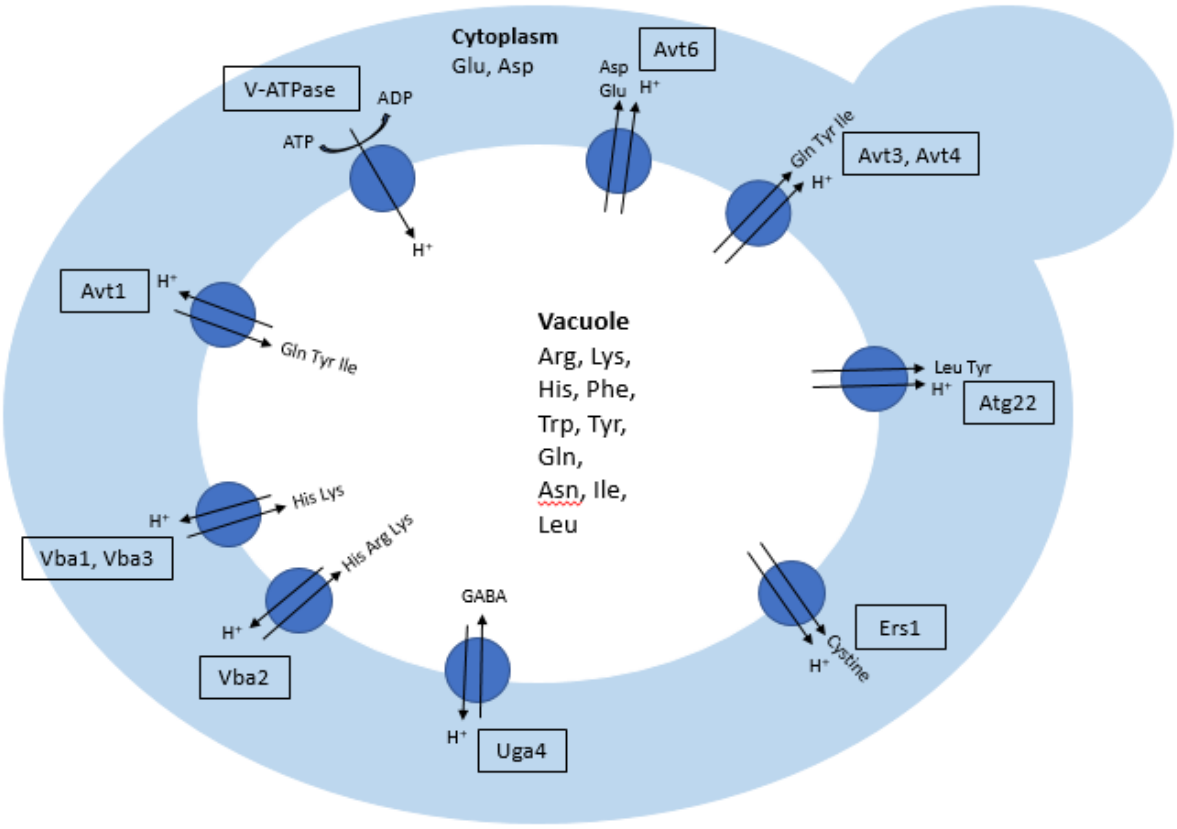

Figure 2 *S. cerevisiae*'s vacuolar amino acid transporters. The ten central amino acids depicted are transported into the vacuole via active transport, while Glu and Asp are found only in the cytosol (Sekito et al. 2008).

Approximately 60% of amino acids in the cell are present in the vacuole (Wiemken & Mathias, 1974). *S. cerevisiae* preferentially accumulates basic amino acids in the vacuoles whereas acidic amino acids are rarely found in the vacuoles making amino acid vacuolar storage the result of a selective process (Wiemken & Mathias, 1974). For example, glutamate and aspartate mostly accumulate in the cytosol, whereas only 11% of aspartate is found in the vacuole along with the majority of arginine (Kitamoto et al. 1988). Crépin et al. (2014) confirmed the latter finding as their data revealed that the *S. cerevisiae* strains tested accumulated mostly arginine in the vacuoles at the start of growth. This suggests that the vacuole plays a role in regulating cytosolic amino acid levels as it is selective regarding the uptake of specific amino acid (Crépin et al. 2014).

2.2.2.3 Deamination reaction

Once the amino acids not taken up into the vacuole have crossed the yeast cell membrane, a deamination reaction takes place which produces ammonium. This ammonium can be utilised directly in biosynthetic processes, for example nucleic acid and protein synthesis (Aranda et al.

2011; Crépin et al. 2017a; Rollero et al. 2017). Amino acids can also be used as substrates for transaminases for glutamate formation (Aranda et al. 2011). Both glutamate and ammonia are important in yeast nitrogen metabolism as nitrogen from ammonia rapidly forms part of biosynthetic pathways and glutamate can be used in the formation of other amino acids as well as aroma compounds (Aranda et al. 2011; Ljungdahl and Daignan-Fornier 2012).

2.2.2.4 Transamination reaction (beginning of the Ehrlich pathway)

As mentioned, amino acids can also be used as substrates for transamination reactions involving the formation of glutamate from α-keto-glutarate, which can be considered as a nitrogen transporter and which is formed in the Krebs cycle (Mendes-ferreira et al. 2011). This reaction denotes the beginning of the Ehrlich pathway in which amino acids are converted to fusel acids and alcohols and ultimately to their corresponding esters. Fusel alcohol production via the Ehrlich pathway directly involves the transamination of certain amino acids, namely leucine, valine, isoleucine, phenylalanine, tyrosine, methionine, threonine, serine, aspartate and tryptophan, therefore concentrations of these amino acids can influence the production of fusel alcohols as well as acetate esters as the former are precursors for the latter (Hazelwood et al. 2008; Hirst and Richter 2016; Belda et al. 2017a). α-keto acids are formed as a product of this transamination step.

2.2.2.5 Decarboxylation step and formation of aroma compounds

The α-keto acids formed from transaminated amino acids are subsequently decarboxylated to form the corresponding aldehydes. The latter are thereafter reduced or oxidised to the corresponding alcohols or acids, respectively (Mendes-ferreira et al. 2011). The full Ehrlich pathway is summarised in Fig. 3.

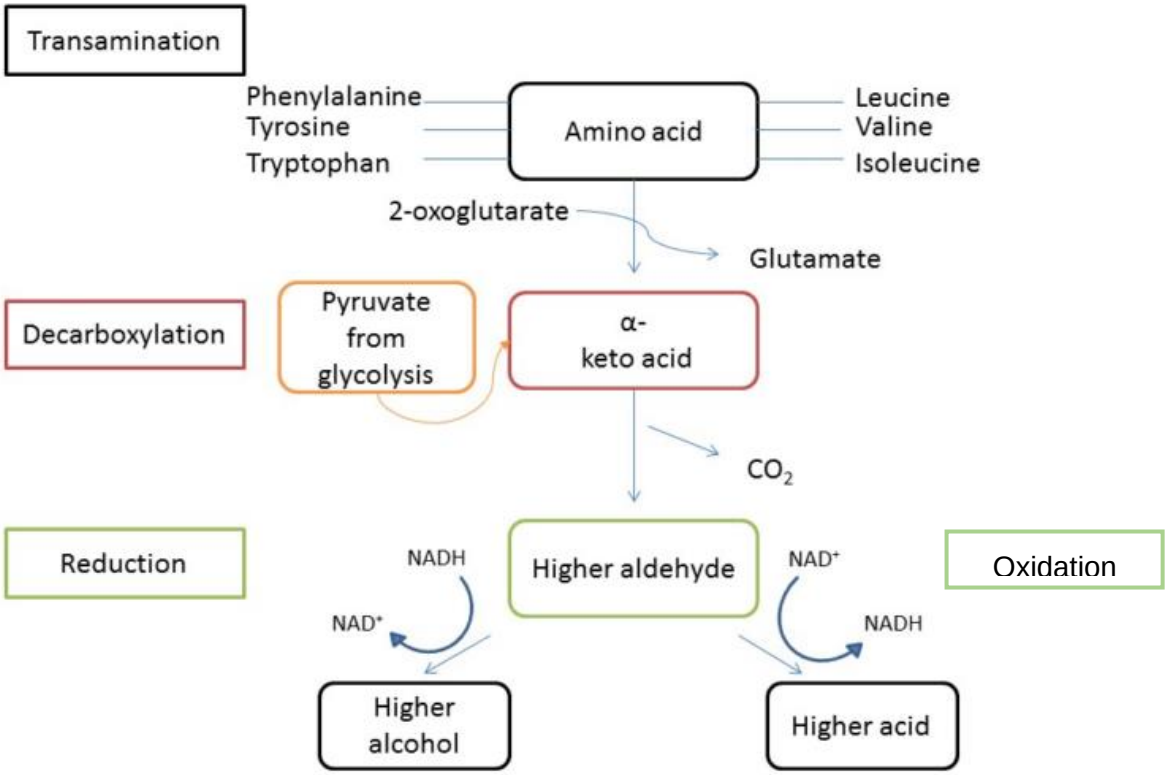

Figure 3 Overview of the Ehrlich Pathway (Adapted by de Koker (2015) from Hazelwood et al. (2008)).

Some examples of amino acids which are catabolised through the Ehrlich pathway and their corresponding products are found in Table 1.

Table 1 Branched chain and aromatic amino acids and their corresponding higher alcohols, fatty acids and esters (aromatic descriptors in brackets) (Burdock 2010; Lambrechts and Pretorius 2000). Aroma descriptors for tyrosine and tryptophan derivatives are not available in literature to the knowledge of the author.

BCAA	Higher alcohol	Fatty acid	Esters
Leucine	Isoamyl alcohol (fusel alchol, whisky)	Isovaleric acid (rancid, cheese-like)	Isoamyl acetate (fruity), ethyl isovalerate (fruity)
Isoleucine	Amyl alcohol (sweet, pleasant)	2-Methylbutanoic acid (apple)	Amyl acetate (apple, banana), ethyl 2-methylbutanoate (fruity)
Valine	Isobutanol (wine-like, penetrating)	Isobutyric acid (rancid butter)	Isobutyl acetate (fruity, floral), ethyl isobutyrate (fruity)
Phenylalanine	Phenylethanol (rose)	Phenylacetic acid (honey) -	Ethyl phenylacetate (honey), phenethyl acetate (floral, honey)
Tyrosine	Tyrosol (honey, bees wax)	p-OH-phenylacetic acid	4-Hydroxyphenylacetate, ethyl 4-hydroxyphenylacetate
Tryptophan	Tryptophol	Indole-3-acetic acid	Tryptophol acetate, Ethyl-3-indoleacetate

2.2.2.6 Distribution of uses for amino acids after uptake

Crépin et al. (2017b) used isotope tracing of labelled nitrogen sources in order to trace the metabolic pathways of amino acids in *S. cerevisiae*. The data suggest that only a small portion of amino acids was used to form proteins and most were instead found to be catabolised where after it was used for *de novo* synthesis of proteinogenic amino acids. This promotes nitrogen redistribution which ensures that the most required nitrogen sources are formed. This could explain why the initial amino acid concentrations do not have such strong influence on the order of uptake of amino acids (Crépin et al. 2012). In addition, only a small portion of amino acids consumed was found to enter into the Ehrlich pathway for aroma compound production as well as be used for α-keto acid precursor formation (Crépin et al. 2017b). For instance, it has been previously found that propanol production is a direct result of intracellular threonine catabolism in *S. cerevisiae* (Mouret et al. 2014), but the origin of the threonine that acted as a substrate was unknown. Crépin et al. (2017) provided labelled threonine in the medium and found that only 19% of propanol derived from this threonine, whereas 81% of propanol derived from threonine synthesised *de novo*. The authors also investigated the fate of labelled valine and leucine. Low incorporation levels of these amino acids in the formation of higher alcohols isobutanol and isoamyl alcohol were observed, therefore the production of these compounds relies largely on central carbon metabolism (Crépin et al. 2017b). Indeed these 2 higher alcohols can also originate from pyruvate. Overall, the central carbon metabolism was found to have a greater impact regarding the above-mentioned processes than did the nitrogen provided in the medium and this will be discussed in more detail in paragraph 2.3.4.

2.3 Factors affecting nitrogen concentration and the production of aroma compounds in *S. cerevisiae*

2.3.1 Influence of viticultural/cellar practices on YAN concentration

Total nitrogen levels in grape must have been shown to vary from one year to the next and this is likely to be due to varying levels of ripeness of the grapes (Ribéreau-Gayon et al. 2006). As the ripening process of the grapes proceeds, the concentration of total free amino acids increases and by harvest, these concentrations are at least twice that of the levels found at the start of colour change (Ribéreau-Gayon et al. 2006). The conclusion could be drawn that as the grapes ripen, the fermentative potential of the must increases. However, analysis of individual amino acids shows a

marked increase in proline approximately two weeks prior to harvest, thus proline can be used as a marker for determining ripeness (Ribéreau-Gayon et al. 2006). Nitrogen concentration is also affected by location of production and the variety of grape used (Ribéreau-Gayon et al. 2006). On average, red wines contain twice the amount of nitrogen as white wines, with Champagne having two to three times the amount of nitrogen as white Bordeaux wines (Ribéreau-Gayon et al. 2006). Red wine's higher nitrogen content is due to the winemaking techniques used, such as maceration at a high temperature, which leads to more dissolving of nitrogenated substances from the seeds and skins, as well as dead yeast cell autolysis (Ribereau-Gayon et al. 2006).

Various other factors have been found to influence the nitrogen concentration of the grape must such as viticultural practices. The timing of nitrogen application as well as the amount of nitrogen applied/supplemented have been shown to affect grape must YAN concentrations (Holzapfel et al. 2015). A study by Holzapfel et al. (2015) showed that YAN concentrations were highest when nitrogen applications were made approximately two weeks after veraison. Differing YAN concentrations translate into differing individual amino acid concentration which can impact on the amount of certain aroma compounds produced, as previously shown by McKinnon, for instance (2013). Nitrogen foliar treatments have also been shown to affect the nitrogen profiles of grape must (Pérez-Álvarez et al. (2016)). Urea application onto the leaves in a vineyard was shown to affect the amino acid profiles of the grape must. This possibly could be used to optimise nitrogen levels thereby reducing the risk of sluggish fermentations as well as influencing the resulting quality of the wine. Furthermore, in the cider formation process, it has been shown that some fungicide usage can contribute to an increased production of off-flavour compounds (Boudreau et al. 2017). When the must contains low levels of YAN, the negative effect of the fungicide tends to be exacerbated. Another factor which can have different effects on grape YAN, nitrogen nutritional status and nitrogen availability is the use of different cover crops (Pérez-álvarez et al. 2015). Using a barley cover crop lead to decreased nitrogen levels and therefore decreased YAN and amino acid levels (Pérez-álvarez et al. 2015). Using a clover cover crop, however, showed nitrogen, YAN and amino acid levels which are similar to that of conventional tillage use (Pérez-álvarez et al. 2015).

2.3.2 Metabolism of amino acids involved in the production of aroma compounds

Some amino acids' catabolism can impact directly on the production of specific aroma compounds. For example, high levels of alanine, glutamic acid, aspartic acid and asparagine were

shown to be required for yeast production of fusel esters and most esters during the fermentation process for cider production, possibly as a result of their interconversion to other amino acids which are catabolised via the Ehrlich pathway, as aspartic acid, glutamic acid and asparagine comprised the majority of amino acids present (Ljungdahl and Daignan-Fornier 2012; dos Santos et al. 2015). However, Mckinnon (2013) observed that branched-chain and aromatic amino acids (BCAAs) had the most pronounced direct effects on production of major volatiles in grape fermentation. It was shown that fusel alcohol and acid production due to BCAA degradation could be predicted from their initial concentrations (McKinnon 2013). The BCAAs Val, Leu, Ile and Phe illustrated the most pronounced and easily predictable aroma compound production from their corresponding BCAA, however these amino acids were provided as sole nitrogen sources and not in excess. It is possible that predictability would decline if a mixture of BCAAs was used (McKinnon 2013). Production of various other secondary metabolites, such as isoamyl acetate and 2-phenylethyl acetate, was also shown to correlate with initial BCAA concentration as these compounds are produced via catabolism of leucine and phenylalanine respectively (McKinnon 2013).

Studies which investigated the effects of various amino acid supplementation were conducted and the effects thereof were observed with regard to fermentation ability of the yeast as well as aroma compounds produced. Table 2 summarises the results of these studies. Slower fermentations were associated with unsupplemented media containing 60 mg N/L YAN, as was higher H_2S production, which is associated with negative, reductive off-flavours and odours and lower post-fermentation amino acid levels (Sturgeon et al. 2013). This result is further confirmed by a study where low nitrogen levels in cider production also lead to sluggish fermentations and a marked decrease in volatile compounds (dos Santos et al. 2015).

The effects of ammonium supplementation were also researched and this nitrogen source was found to be efficient in increasing the fermentation rate, as well as greatly influencing the formation of fermentation compounds, including propanol, isoamyl alcohol and ethyl esters (Clement et al. 2013). An increased formation of these fermentation compounds was hypothesised to be a result of using ammonium for synthesis of other amino acids which were further catabolised to form these compounds via the Ehrlich pathway (Clement et al. 2013). This is further confirmed by Aranda et al. (2011) and Ljungdahl and Daignan-Fornier (2012), which have shown that ammonium is used for synthesis of other amino acids involved in aroma compound production.

Table 2 Amino acid supplementation and effects.

Amino acid(s) supplemented	Effect(s)	Reference
Glutamine	Positive H_2S production results and better fermentation rates	(Sturgeon et al. 2013)
Aspartic acid	Slower fermentations which did not always reach dryness.	(Sturgeon et al. 2013)
Valine	Greatly influenced the formation of fermentation compounds, including propanol, isoamyl alcohol and ethyl esters and greatly increased conversion of valine to isobutyl acetate and isobutanol via the Ehrlich pathway.	(Clement et al. 2013)
Lysine and histidine	Positive effects on flavour volatile formation. Lysine supplementation radically up-regulated SPS-mediated genes compared to NCR-sensitive genes, whereas histidine supplementation slightly activated SPS-mediated genes during exponential phase and strongly repressed NCR-sensitive genes throughout the fermentation process.	(Lei et al. 2013)

2.3.3 Other factors influencing the production of aroma compounds by yeasts and their preservation in wine

The amount of nitrogen present in the grape must is important as it has been shown that nitrogen-repressed conditions during fermentation modify ammonium and amino acid uptake and this difference in uptake may affect important secondary metabolite production (Beltran et al. 2004).

Temperature is another factor shown to play a role in volatile compound production (Rollero et al. 2015; Mouret et al. 2014). Mouret et al. (2014) found lower ester concentrations when high temperature fermentations were conducted and was due mostly to the effect of evaporation. Even though the availability of nitrogen was the main component affecting aroma compound production, temperature determined the degree to which an effect was observed. Furthermore, Rollero et al. (2015), showed that temperature significantly affected isoamyl acetate and ethyl octanoate metabolism, even though the largest effect of temperature was evaporation of these esters, confirming the results found by Mouret et al. (2014). This study also elucidated that lipid content plays a role in volatile compound production and is positively correlated with the synthesis of isobutanol and isoamyl alcohol. Rollero et al. (2017) further confirmed that lipids can affect volatile aroma compound production. Indeed, the latter study showed that low lipid concentrations result in increased acetate ester production.

Building on the effect of lipids, Tesnière et al. (2013) showed that lipid limited conditions coupled with an excess of nitrogen lead to reduced yeast survival. The dying cells present in this environment showed lower storage levels of trehalose and glycogen, which accumulate due to stress response, when compared to nitrogen-limited cells (Tesnière et al. 2013). Amino acid additions (resulting in an excess) of arginine, glutamine, glutamate as well as excess ammonium addition were shown to increase mortality of the yeast, whereas proline and histidine had little or no effect, which is consistent with the yeast using little or none of these amino acids (Tesnière et al. 2013). Casalta et al. (2013) showed that adding lipids into a medium containing 130-250 mg/L assimilable nitrogen lead to improved nitrogen assimilation by yeasts, thereby promoting growth and fermentation and strengthening the argument for a link between lipid and nitrogen levels. Interactions between nitrogen and lipids were also found to have an effect on isobutanol production (Rollero et al. 2017). Phytosterol content changes were found to impact acetyl-CoA availability and were therefore a possible reason as to why isobutyl compounds were more

significantly affected when compared to derivatives of isoamyl alcohol (Rollero et al. 2017). Nevertheless, although temperature and lipid content affected volatile compound production by *S. cerevisiae*, nitrogen concentration was shown to have the largest effect on this parameter.

2.3.4 Connection with the central carbon metabolism

Flavour compounds are not only produced through amino acid catabolism. As already mentioned above, the production of certain secondary metabolites also depends on carbon metabolism (Hirst and Richter 2016). Fig. 4 depicts the relationships between the main carbon and nitrogen metabolism pathways. Some of these will be discussed in the subsequent text.

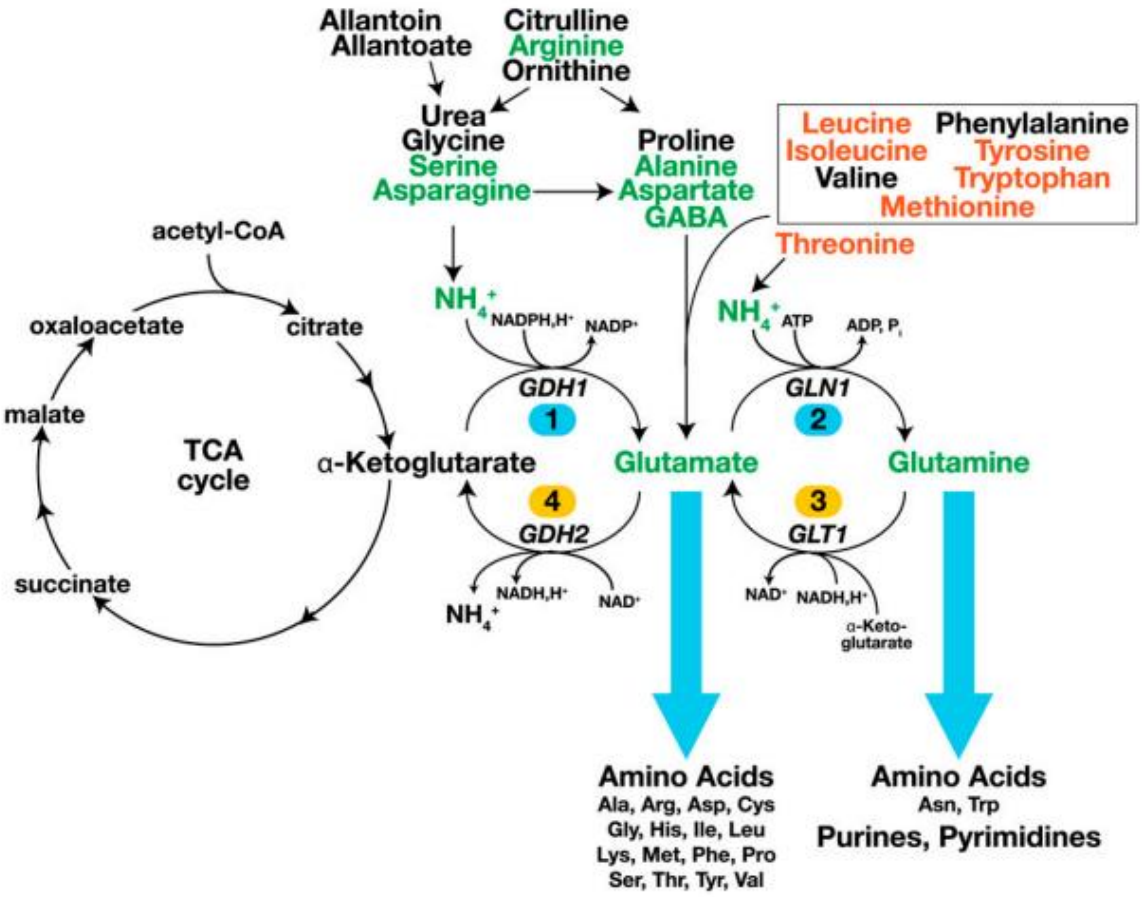

Figure 4 Main nitrogen metabolism pathways. The points of entry of nitrogen sources for various reactions are depicted. Preferred and non-preferred amino acids are shown in green and red respectively. Nitrogen from the amino acids inside the box are transaminated to a-ketoglutarate to form glutamate. The blue arrows show the compounds produced from glutamate and glutamine. 1 and 2 denote central anabolic reactions which are catalysed by NADPH-dependent glutamate dehydrogenase (Gdh1) and glutamine synthetase (Gln1) respectively. 3 and 4 denote central catabolic reactions which are catalysed by NADH-dependent glutamate synthase (Glt1) and NAD+-linked glutamate dehydrogenase (Gdh2) respectively (Ljungdahl and Daignan-Fornier 2012).

It has recently been shown that over 90% of higher alcohols and acids produced during fermentation originate from carbon central metabolism (CCM) intermediates (Rollero et al. 2017). In fact, as mentioned above, amino acid catabolism via the Ehrlich pathway plays a relatively minor role in aroma compound production (Crépin et al. 2017). Indeed, a study by Rollero et al. (2017) investigated the effect of varying nitrogen and lipid sources on the contribution of nitrogen metabolism and CCM to aroma compounds produced. ^{13}C labelling was used to unravel the amounts of valine and leucine used for biomass formation and aroma compound production. At the stage when all nitrogen was consumed, it was found that amino acid isotopic enrichment corresponding to the amount of the original exogenous compound was less than 35%, which suggests that *de novo* synthesis of amino acids via the CCM plays a prominent role at all concentrations of nitrogen tested (70 to 425 mg N l^{-1}). For example, pyruvate formed via the CCM can produce α-keto acids which can be reduced or oxidised to form the corresponding higher alcohols or fatty acids respectively, or the α-keto acids can be used to synthesise amino acids (Rollero et al. 2017). Most of the α-keto precursors for amino acid synthesis originate from sugar catabolism (Crépin et al. 2017). Simplified pathways regarding these processes for valine and leucine are shown in Fig. 5. Moreover, Crépin et al. (2017) demonstrated that only a very small amount of amino acids consumed directly form part of proteins and catabolism of these amino acids largely results in the redistribution of nitrogen, thus promoting *de novo* synthesis of proteinogenic amino acids, confirming the results found by Rollero et al. (2017). Another example is that of 2-phenylethanol as it can be produced via the CCM or the Ehrlich pathway by phenylalanine catabolism (Angelov and Gotcheva 2012). However, other higher alcohols such as propanol have been shown only to be produced via the catabolism of supplied or synthesised amino acids (Mouret et al. 2014). An illustration of how aroma compounds can be produced by nitrogen catabolism as well as by CCM is shown in Fig. 6.

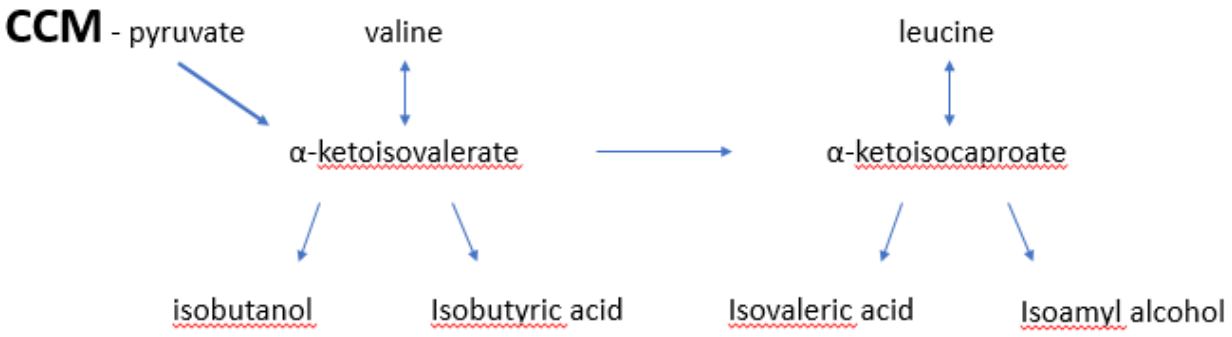

Figure 5 *De novo* synthesis of valine and leucine via CCM and production of aroma compounds from CCM-derived compounds (adapted from Rollero et al. (2017)). α-ketoisovalerate is converted to α-ketoisocaproate via a three-step chain elongation cycle using 2-isopropylmalate synthase, isopropylmalate isomerase complex and 3-isopropylmalate dehydrogenase (Zhang et al. 2008).

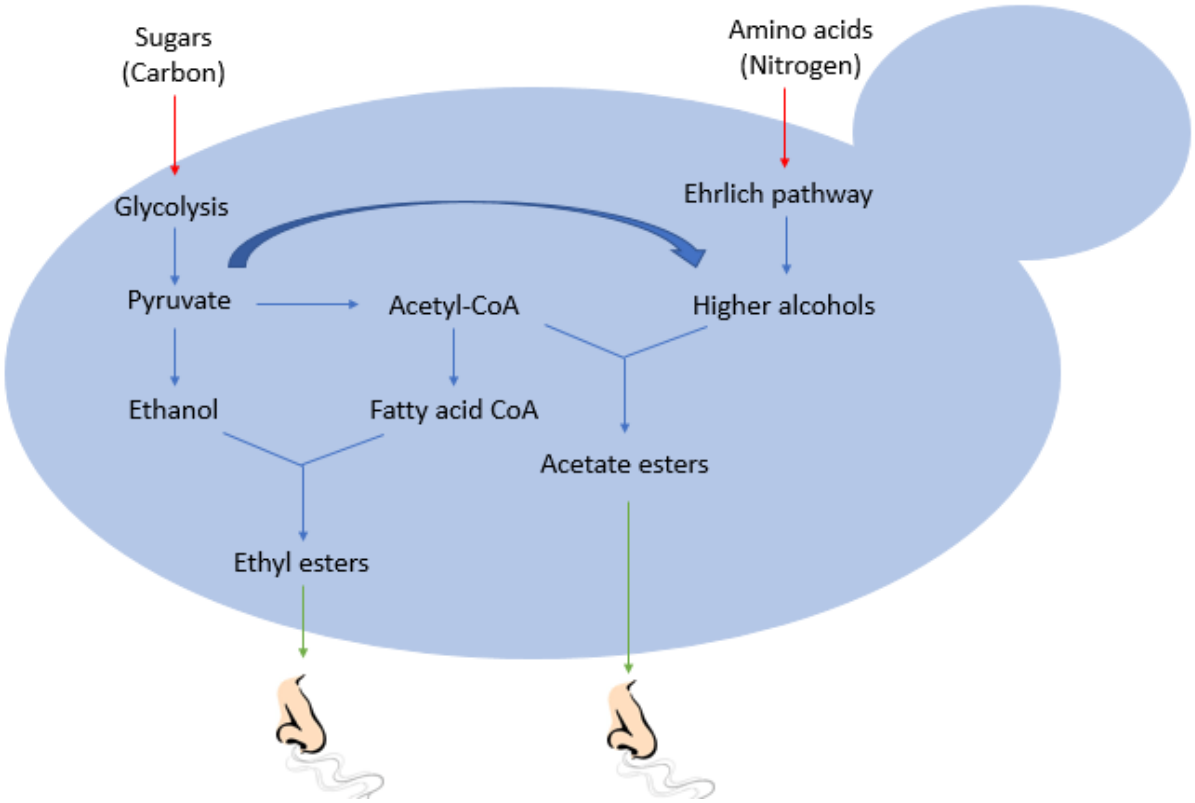

Figure 6 Production of aroma compounds via carbon and nitrogen degradation pathways (adapted from Belda et al. (2017)). The main higher alcohols produced from pyruvate are isobutyl and amyl alcohols (Ribereau-Gayon et al. 2006).

2.4 Non-*Saccharomyces* yeasts' contribution to wine-making

2.4.1 Yeast interactions

Studies have shown that using both *S. cerevisiae* and non-*Saccharomyces* yeast strains can contribute positively to wine complexity and diversity (Belda et al. 2017; Ciani et al. 2010; Liu et al. 2016; Whitener et al. 2015). Non-*Saccharomyces* yeasts tend to have lower ethanol tolerances and be less competitive due to a lack of oxygen when compared to *S. cerevisiae*. However, some of them can still contribute positively to the resulting wine in the early stages of fermentation (Liu et al. 2016; Whitener et al. 2015). Initial inoculation of non-*Saccharomyces* yeasts followed by the sequential inoculation of *S. cerevisiae* can help to ensure that the non-*Saccharomyces*' contribution to wine quality will occur before *S. cerevisiae* is inoculated and competes for nutrients. Non-*Saccharomyces* population levels usually decline rapidly as ethanol levels increase and as *S. cerevisiae* competes for the remaining nutrients. However, this is not always the case as some non-*Saccharomyces* yeasts can survive until the end of fermentation. For example, in a study by Andorrà et al. (2012), *C. zemplinina* was not completely outcompeted by *S. cerevisiae* and survived till the end of fermentation. It is important to note what nitrogen sources are left in the grape must after non-*Saccharomyces* inoculation and before sequential *Saccharomyces* addition in order to ensure that the remaining nitrogen is sufficient for *S. cerevisiae* to complete fermentation as nitrogen consumption profiles of non-*Saccharomyces* yeasts, for example *Hanseniaspora vineae* and *Metschnikowia pulcherrima* have been shown to affect the growth of *Saccharomyces* yeasts (Medina et al. 2012). A study by Lleixà et al. (2016) confirmed that nitrogen consumption by non-*Saccharomyces* yeasts before *Saccharomyces* inoculation can increase the risk of sluggish or stuck fermentations.

Competition for nitrogen sources between multiple *S. cerevisiae* strains has also been observed (Vendramini et al. 2017). Yeast competition was influenced by nitrogen concentrations and was found to be strain dependent. It was shown that a faster ability to consume nitrogen allowed for a higher yeast survival rate.

2.4.2 Amino acid preference and competition

A study by Kemsawasd et al. (2015) tested the effect of 19 single amino acid sources as well as ammonium sulphate as a single nitrogen source on various non-*Saccharomyces* yeast strains, namely *Torulaspora delbrueckii*, *Hanseniaspora uvarum*, *Metschnikowia pulcherrima* and *Lachancea thermotolerans*. It was found that arginine, asparagine, glutamine, isoleucine, and a

mix of the 19 amino acids (for *Torulaspora delbrueckii*), alanine and asparagine (for *Metschnikowia pulcherrima*), alanine (for *Hanseniaspora uvarum*) and serine (for *Lachancea thermotolerans*) were the nitrogen sources that had the most positive effects on performance. When compared to *S. cerevisiae*, it was found that this yeast strain preferred alanine, arginine, asparagine, aspartic acid, glutamine, isoleucine, ammonium sulphate, serine, valine, a mix of 19 amino acids and a mix of 19 amino acids with ammonium sulphate as nitrogen sources. This could pose a potential problem regarding competition of non-*Saccharomyces* yeasts with *S. cerevisiae* as many of these nitrogen sources overlap and this could affect yeast growth, either that of the non-*Saccharomyces* yeasts when both non-*Saccharomyces* and *Saccharomyces* are inoculated together (as *S.cerevisiae* is better developed to grow in a harsh environment and is therefore likely to outcompete the non-*Saccharomyces* yeasts) or that of *S. cerevisiae* when the 2 yeasts are sequentially inoculated, as nitrogen sources can be depleted by the non-*Saccharomyces* yeast by the time *S. cerevisiae* is inoculated. In the latter scenario, this can ultimately lead to stuck fermentations. However, this potential competition problem hypothesised should also be investigated when amino acids are present in combination. Taillandier et al. (2014) investigated the effect of *T. delbrueckii* in mixed and sequential fermentations with *S. cerevisiae*. These authors found that *T. delbrueckii* growth was inhibited in both mixed and sequential fermentations, even when high inoculation levels of these yeast were used, most likely due to a metabolite produced by *S. cerevisiae*. *S. cerevisiae* was not affected negatively at high assimilable nitrogen concentrations (324 mg/L) when inoculated sequentially, but at 176 mg/L, nitrogen depletion by *T. delbrueckii* disabled *S. cerevisiae* development, resulting in stuck fermentations (Taillandier et al. 2014). Thus, nitrogen additions can help alleviate the problem of competition for nutrients between yeasts.

2.4.3 Production of aroma compounds

Non-*Saccharomyces* yeasts species can produce higher or lower levels of different aroma compounds which can make them desirable for use during fermentation. Low acetic acid producers include *Hanseniaspora* and *Zygosaccharomyces* species as well as *S. pombe*, while increased higher alcohol levels are produced by *M. pulcherrima*, *C. zemplinina* and *L. thermotolerans* (Padilla et al. 2016; Belda et al. 2017a). *Candida, Hansenula, Pichia, Hanseniaspora, Rhodotorula, T. delbrueckii* and *K. gamospora* produce high levels of esters (Beckner Whitener et al. 2015; Padilla et al. 2016). At concentrations below 300 mg/L, higher alcohols have a positive effect on wine aroma, however are considered to have a negative effect at levels higher than 400 mg/L (Padilla et al. 2016). *M. pulcherrima, L. thermotolerans* and *C.*

zemplinina produced elevated levels of 2-phenylethyl alcohol, as reviewed by Padilla et al. (2016). Benito et al. (2016) found that sequential fermentations with *L. thermotolerans* followed by *S. cerevisiae* inoculation resulted in the best final wines in terms of general acidity and sensorial properties compared to pure *S. cerevisiae* fermentations and mixed *L. thermotolerans* and *S. cerevisiae* fermentations indicating that non-*Saccharomyces* yeasts can contribute positively to the final wine profile. It also indicates that, in the case of *L. thermotolerans*, it may be best that the non-*Saccharomyces* yeast be first inoculated on its own before *S. cerevisiae* addition. This is possibly due to competition for nutrients. The sequential fermentations also resulted in elevated levels of ethyl lactate, 2-phenylethanol (thus confirming that found by Padilla et al. (2016)) and 2-phenylethyl acetate. *Hanseniaspora* produces higher levels of 2-phenylethyl acetate and isoamyl acetate, which was also produced in high levels by *Pichia* and *Rhodotorula* (Padilla et al. 2016; Belda et al. 2017). *M. pulcherrima* has been shown to increase ethyl acetate and total higher alcohol, ester and sulfur compound levels (Varela et al. 2017).

Some studies have been conducted in order to better understand aroma compound production by non-*Saccharomyces* yeasts. A study by de Koker (2015) in particular, attempted to link YAN concentration and amino acid uptake to the production of certain higher alcohols, volatile fatty acids and esters. This author found that aroma compound production was indeed influenced by the initial YAN concentration. A low YAN concentration induced the most profound differences and yielded higher ester production and lower fatty acid and higher alcohol production. The yeasts *L. thermotolerans, T. delbrueckii, P. kluyveri* and *M. pulcherrima* were researched in pure cultures as well as sequential fermentations with *S. cerevisiae*. *L. thermotolerans* was shown to produce high levels of propanol and butanol, which is confirmatory of previous literature (Gobbi et al. 2013; de Kock 2015). Low nitrogen fermentations were found to increase volatile acidity, while sequential fermentations at low YAN levels lead to diethyl succinate production, which was not produced at higher YAN concentrations (de Koker 2015). Sequential inoculation of *S. cerevisiae* with *M. pulcherrima* and *T. delbrueckii* led to increased fatty acid levels and lower ethyl acetate levels respectively. Furthermore, sequential fermentations of *P. kluyveri* and *S. cerevisiae* produced high levels of esters (de Koker 2015).

2.5 Conclusions and future outlooks

In order for optimal fermentation conditions to be met for the yeast, various nutrient levels for different yeast species need to be optimised. Nitrogen is an important element in this regard as

the total amount of nitrogen as well as amounts of individual nitrogen sources can have major impacts on the resulting wine quality. The nitrogen preferences for *S. cerevisiae,* as well as the production of aroma compounds by this yeast, have been researched more widely than those of non-*Saccharomyces* yeasts. However, even though the general pathways are well known, some of the results for *S. cerevisiae* are very recent and much is still not understood regarding amino acid uptake and catabolism regulation as these are complex processes. In the current context of increasing use of non-*Saccharomyces* yeasts, jointly with *S. cerevisiae* in oenology, it is important to research not only *S. cerevisiae*'s nitrogen preferences, but also the nitrogen preferences of non-*Saccharomyces* yeasts in order to reduce competition for nutrients between yeast species as very little research has been conducted regarding this possible competition. Furthermore, once the nitrogen preferences of non-*Saccharomyces* yeasts are known, the resultant effect on aroma compounds produced can be studied. The optimisation of non-*Saccharomyces* yeast usage will help result in better fermentation management as well as generate more diverse wine styles.

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Chapter 3

Research results

The utilisation of nitrogenous compounds by commercial non-*Saccharomyces* yeasts associated with wine

Chapter 3: The utilisation of nitrogenous compounds by commercial non-*Saccharomyces* yeasts associated with wine

3.1 Introduction

Yeasts are the drivers of the fermentation process through which grape must is converted to wine. The yeast species which is best adapted to the harsh grape juice/wine environment is *Saccharomyces cerevisiae*. Indeed, this yeast survives under the stressful conditions induced by high ethanol concentrations and low oxygen levels amongst other stressful environmental conditions. It is barely detectable in grape juice, however soon dominates the other yeast species present during spontaneous fermentation and is largely responsible for completion of alcoholic fermentation (Aranda et al. 2011). Until recent years, the other yeast species, although dominant in variety and numbers in grape juice, were mostly regarded as spoilage yeasts and therefore undesirable for use in wine fermentation. However, over the past 10 years, it has been reported that many species produce desirable compounds which can improve the flavour and aroma of the final wine or at least contribute to its organoleptic complexity (Liu et al. 2016; Andorrà et al. 2012; Lachance 2016; Whitener et al. 2015). To achieve this improved complexity, it is essential that more research on these yeasts be conducted in order to ensure that they are used optimally in the fermentation process to promote their most positive contributions while minimising their potential for spoilage. Many aspects need to be investigated, one of which is nutrient and nitrogen requirement. Such data will also be useful for improved management of sequential fermentations.

The nitrogenous compounds which the yeast can utilise are referred to as yeast assimilable nitrogen (YAN). Free amino nitrogen (FAN) from all amino acids except proline, as well as ammonium ions contribute the majority of YAN (Mendes-ferreira et al. 2011). Although a proteinogenic amino acid, proline is excluded from the YAN as *S. cerevisiae* is not able to metabolise it (to form other amino acids for instance) under the anaerobic conditions occurring during alcoholic fermentation (Henschke and Jiranek 1993). Low YAN concentrations can lead to stuck fermentations and increased H_2S formation, while too high YAN concentrations can increase turbidity and the possibility of microbial instability (Beltran et al. 2004; Burin et al. 2015; Mendes-ferreira et al. 2011; Sturgeon et al. 2013).

Assimilable nitrogen is taken up within the first few days of alcoholic fermentation (Tesnière et al. 2015). The ammonium ion is an important source of nitrogen for yeasts, as are amino acids with

regard to consumption by yeast during the first stages of fermentation for promoting growth and fermentation ability and their catabolism can lead to diverse aroma compound production (Ribéreau-Gayon et al. 2006; Crépin et al. 2014). Some amino acids are preferentially assimilated over others and support better yeast growth (for example Glu and Gln). They can also lead to improved aroma compound production, for example branched chain and aromatic amino acids which produce aroma compounds via the Ehrlich pathway (Henschke and Jiranek 1993; Sturgeon et al. 2013; dos Santos et al. 2015; Mendes-ferreira et al. 2011). Preferred amino acids regarding better growth and faster uptake have been researched for *S. cerevisiae*, but little research has been conducted for amino acid uptake and utilisation regarding non-*Saccharomyces* yeasts. This knowledge is nevertheless critical if wine-makers wish to increase non-*Saccharomyces* yeast use for organoleptic profile diversification. This is particularly important in the context of co- or sequential inoculation of the non-*Saccharomyces* yeasts with *S. cerevisiae* imposed by the fact that non-*Saccharomyces* are not able to complete alcoholic fermentation on their own.

The effect of nitrogen on aroma compound production for non-*Saccharomyces* yeasts has not been researched extensively. Nevertheless, the limited number of reports demonstrate that initial concentration of assimilable nitrogen has an effect on the concentrations of various aroma compounds produced by non-*Saccharomyces* yeasts. A low initial YAN showed the most profound effect on aroma compound production where higher concentrations of esters and lower concentrations of fatty acids and higher alcohols were observed (de Koker 2015). Low YAN levels also resulted in higher acetic acid production and in general, for sequential fermentations, more organic acids and their corresponding esters. This study also confirmed higher propanol and butanol production for *L. thermotolerans* than for *S. cerevisiae* (Gobbi et al. 2013; de Kock 2015). Moreover, *M. pulcherrima* in sequential fermentation with *S. cerevisiae* promoted volatile fatty acid production. Sequential *P. kluyveri* and *S. cerevisiae* fermentations promoted high ester levels. However, definite links to nitrogen consumption could not be made in these regards.

This study investigated the nitrogen preferences and order of uptake of nitrogen sources for various commercial non-*Saccharomyces* yeast strains as well as potential competition for nitrogen between *S. cerevisiae* and non-*Saccharomyces* yeasts. *S. cerevisiae* was used as a reference species and the results found for this strain were compared with previous findings in literature. The effects of nitrogen uptake on fermentation and growth kinetics were also investigated as well as the link between nitrogen source and aroma compounds produced. Different amino acid ratios

were tested as well as the effect of the presence of ammonium in the fermentation media. Total YAN was kept constant for all fermentations.

3.2 Materials and methods

3.2.1 Yeast species used

Five commercial wine yeast strains were used in this study as described in Table 1. *Saccharomyces cerevisiae* Lalvin® EC1118 (Lallemand, Blagnac, France) was used as a control in pure culture fermentations and sequentially inoculated with non-*Saccharomyces* yeast strains, namely *Pichia kluyveri* Viniflora® FootZen™ (Chr. Hansen, Hørsholm, Denmark), *Torulaspora delbrueckii* Biodiva™ TD291 (Lallemand), *Metschnikowia pulcherrima* Flavia® MP346 (Lallemand) and *Lachancea thermotolerans* Viniflora® Concerto™ (Chr. Hansen).

Table 1 Yeast species and strains used in this study.

Yeast species	Strain/Commercial name	Region of isolation/institution	Manufacturer
Saccharomyces cerevisiae	Lalvin® EC1118	Champagne, France	Lallemand
Pichia kluyveri	Viniflora® FootZen™	Auckland University, New Zealand	Chr. Hansen
Torulaspora delbrueckii	Biodiva™ TD291	North America	Lallemand
Metschnikowia pulcherrima	Flavia® MP346	Universitad de Santiago de Chile	Lallemand
Lachancea thermotolerans	Viniflora® Concerto™	Greece	Chr. Hansen

3.2.2 Preculture and fermentation conditions

Precultures were carried out initially by inoculating a single colony of each yeast into 5 mL Yeast Extract Peptone Dextrose (YPD) broth (Merck (Pty) Ltd, Modderfontein, South Africa) and growing

the cells at 30°C on a test tube rotator for approximately 24 h. 50 µL of this preculture was then re-inoculated into 5 mL fresh YPD broth and grown for approximately 24 h at 30°C on the test tube rotator. Cells were then inoculated from this preculture into 200 mL YPD broth at $OD_{600\ nm}$ 0.1 and grown at 30°C with shaking at 120 rpm until the cells reach mid-exponential phase (ca. 9 h). A nitrogen starvation preculture was then conducted in a Yeast Nitrogen Base medium (20 g/L glucose, 1.7 g/L YNB base without amino acids and ammonium (Difco Laboratories)). The whole 200 mL YPD broth preculture was centrifuged at 2,795 *g* for 5min and the supernatant was discarded. The cells were resuspended in 0.9% NaCl solution and centrifuged at 2,795 *g* for 5 min. The supernatant was removed and the cells were resuspended in the YNB medium. The cells were left in the YNB medium at 25°C with shaking at 120 rpm until growth stopped as determined by optical density readings. Once growth plateaued, the yeast were deemed starved of nitrogen and were then inoculated into synthetic grape must medium in which the fermentations were performed.

Three different synthetic grape must media were prepared in order to test the effects of different nitrogen sources and concentrations. Table 2 shows the differing nitrogen source concentrations for each treatment. Pure cultures of each yeast strain were inoculated alone, or sequentially with *S. cerevisiae*, into three synthetic musts with nitrogen source levels as shown in Table 2. In the sequential fermentations, *S. cerevisiae* was inoculated 48 h after the non-*Saccharomyces* yeast (in order to replicate typical wine-making practices) and another treatment was added. The latter entailed filtering out the non-*Saccharomyces* yeast before *S.cerevisiae* inoculation. All fermentations were carried out in triplicate and at 25°C with shaking at 120 rpm.

Table 2 Individual nitrogen source concentrations per treatment. Total YAN for each treatment was 200 mg/L. Treatment 1: Amino acids present at equal assimilable nitrogen levels (calculated in terms of nitrogen equivalents considering the nitrogen content of each amino acid), with ammonium. Treatment 2: Amino acids present at equal assimilable nitrogen levels, without ammonium. Treatment 3: grape-must like nitrogen source concentrations.

Nitrogen source	Treatment 1 pure cultures (mg/L)	Treatment 2 pure cultures (mg/L)	Treatment 3 pure cultures, sequential cultures, sequential cultures + filtering (mg/L) (Adapted from Bely et al. (1990))
Alanine	47,77	63,69	96.87
Arginine	23,29	31,06	249.58
Asparagine	35,38	47,17	35.33
Aspartic acid	71,43	95,24	29.67
Cysteine	64,94	86,58	8.73
Glutamine	39,06	52,08	336.85
Glutamic acid	78,95	105,26	80.29
Glycine	40,32	53,76	12.22
Histidine	27,68	36,90	21.82
Isoleucine	70,09	93,46	21.82
Leucine	70,09	93,46	32.29
Lysine	39,06	52,08	11.34
Methionine	79,79	106,38	20.94
Phenylalanine	88,24	117,65	25.31
Proline	61,48	81,97	408.41
Serine	56,39	75,19	52.36
Threonine	63,56	84,75	50.61
Tryptophan	54,74	72,99	119.56
Tyrosine	97,40	129,87	12.22
Valine	62,50	83,33	29.67
Ammonium chloride	189	0	306.67

Table 3 indicates the concentrations of other components present in the synthetic grape must medium. These concentrations were constant over treatments. Concentrations are adapted from Henschke & Jiranek (1993).

Table 3 Components of synthetic grape must medium**.**

		Amount/L
Carbon Sources	Glucose	115 g
	Fructose	115 g
Acids	KH Tartrate	2.5 g
	L-Malic acid	3 g
	Citric acid	0.2 g
Salts	Potassium hydrogen phosphate	1.14 g
	Magnesium sulfate heptahydrate	1.23 g
	Calcium chloride dehydrate	0.44 g
Trace elements	Manganese(II) chloride tetrahydrate	200 µg
	Zinc chloride	135 µg
	Iron(II) chloride	30 µg
	Copper(II) chloride	15 µg
	Boric acid	5 µg
	Cobalt(II) nitrate hexahydrate	30 µg
	Sodium molybdate dehydrate	25 µg
	Potassium iodate	10 µg
Vitamins	Myo-inositol	100 mg
	Pyridoxine hydrochloride	2 mg
	Nicotinic acid	2 mg
	Calcium pantothenate	1 mg
	Thiamine hydrochloride	0.5 mg
	PABA.K	0.2 mg
	Riboflavin	0.2 mg
	Biotin	0.125 mg
	Folic acid	0.2 mg

Anaerobic factors	Ergosterol	10 mg
	Tween 80	0.5 mL
Nitrogen Sources	As described in Table 2	

3.2.3 Sampling and fermentation kinetics monitoring

Sampling was conducted at the time points of 0 h, 2 h, 6 h, 18 h, 24 h, 48 h and endpoint (which is when fermentation ceased as no more weight loss was quantified, regardless of residual sugar level) for treatments 1 and 2, while only time points 0 and 48 h where sampled for treatment 3. Amino acid concentration analysis was performed at all time points until 48 h. Samples were centrifuged at 2516 g for 5 min and the supernatant was filtered through a 0.22-µm membrane (Starlab Scientific, Cape Town, South Africa). The samples were frozen at -20°C until amino acid, glucose, fructose and ammonium analysis could be performed. Glucose, fructose and ammonium concentrations as well as accumulated weight loss were monitored throughout fermentation in order to determine the fermentation kinetics and analysis was performed using Enzytec Fluid (Roche, R-biopharm) kits E5140, E5120 and E5390 respectively. Fermentations were stopped when weight losses became negligibly low. Plate counts on YPD agar (Merck (Pty) Ltd, Modderfontein, South Africa) and $OD_{600\ nm}$ measurements were made throughout fermentation in order to monitor growth kinetics for treatments 1 and 2. Plates were incubated for 3-4 days at 30°C after which colonies were counted. For treatment 3, GC-FID was performed in addition to the other analyses at 48 h and endpoint. In the sequential inoculations, *S. cerevisiae* (also nitrogen-starved) was inoculated 48 h after non-*Saccharomyces* yeast inoculation. Sequential inoculations were carried out with both yeasts present after 48 h, as well as first filtering out the non-*Saccharomyces* yeast before inoculating *S.cerevisiae* at 48 h.

3.2.4 Major volatile compound analysis (Adapted from Louw et al. (2009))

The concentrations of various major volatile compounds (Isoamyl alcohol, isobutanol, phenylethanol, propanol, 2-phenylethyl acetate and isoamyl acetate) were measured using gas chromatography fitted with a flame ionisation detector. A J&W DB-FFAP capillary GC column (Agilent, Little Falls, Wilmington, DE) with dimensions 60 m length × 0.32 mm internal diameter × 0.5 µm was used for analysis. Sample preparation was performed as follows: 5 mL of each sample (samples were filtered and centrifuged to ensure that no yeast cells were present for analysis) were placed into glass vials after which 100 µL of the internal standard (4-methyl- 2-pentanol) was

added to each sample. 1 mL diethyl ether was then added to the vials and the vials were placed in an ultrasonic bath for 5 minutes (for high sugar samples, magnetic stirrer bars were used for agitation instead of the ultrasonic bath in order to prevent an emulsion due to sugar and solvent reaction). The vials were centrifuged at 1789 g for 3 minutes (high sugar samples were spun at 447 g). GC-FID vials were prepared with sodium sulfate to absorb water. The top layers (separation visible) of samples were placed into inserts which were in turn placed into the GC-FID vial. These samples were then analysed.

3.2.5 Amino acid analysis using HPLC

High performance liquid chromatography (HPLC) was used for individual amino acid analysis of samples (based on a method described in Henderson and Brooks (2010) with some injection and derivatisation modifications). Derivatization used iodoacetic acid (Sigma Aldrich) for cysteine, o-phthaldialdehyde (Sigma-Aldrich) for primary amino acids and fluorenylmethyloxycarbonyl chloride (Sigma Aldrich) for secondary amino acids. Sarcosine (Sigma Aldrich) and norvaline (Sigma Aldrich) as internal standards were spiked before derivatisation for each sample. A Poroshell HPH-C18 (4.6 mm, 150 mm, 2.7 µm) column (Agilent) was used for analysis. Samples were filtered and centrifuged to ensure that no yeast cells were present for analysis.

3.2.6 Statistics

Tukey test analyses were performed on RStudio (https://www.rstudio.com/products/rstudio/download/) to determine statistical significance between treatments and strains.

3.3 Results

3.3.1 Pure culture fermentations with amino acids at the same assimilable nitrogen levels

3.3.1.1 Ensuring nitrogen starvation of yeast before final inoculation

In order to accurately monitor the uptake and release of nitrogenous compounds in the final fermentation medium, it was required that the yeast be starved of nitrogen before inoculation.

In an attempt to achieve such starvation, the various yeasts studied were inoculated into YNB medium containing glucose but devoid of nitrogen sources after an initial preculture in the nutrient rich medium YPD until mid-exponential phase. Results indicated that *P. kluyveri* and *M. pulcherrima* population growth stopped after approximately 4h, whereas *L. thermotolerans*, *T. delbrueckii* and *S. cerevisiae* population growth only stopped after approximately 8h (Fig. 1). At this time point (i.e. 4h or 8h depending on the yeast species), the yeasts were deemed starved of nitrogen, because the only limiting factor was nitrogen (all other nutrients were in excess). They were then inoculated into the final medium. An average of 1.3 generations were achieved for the yeast, except for *L. thermotolerans* which achieved 0.7.

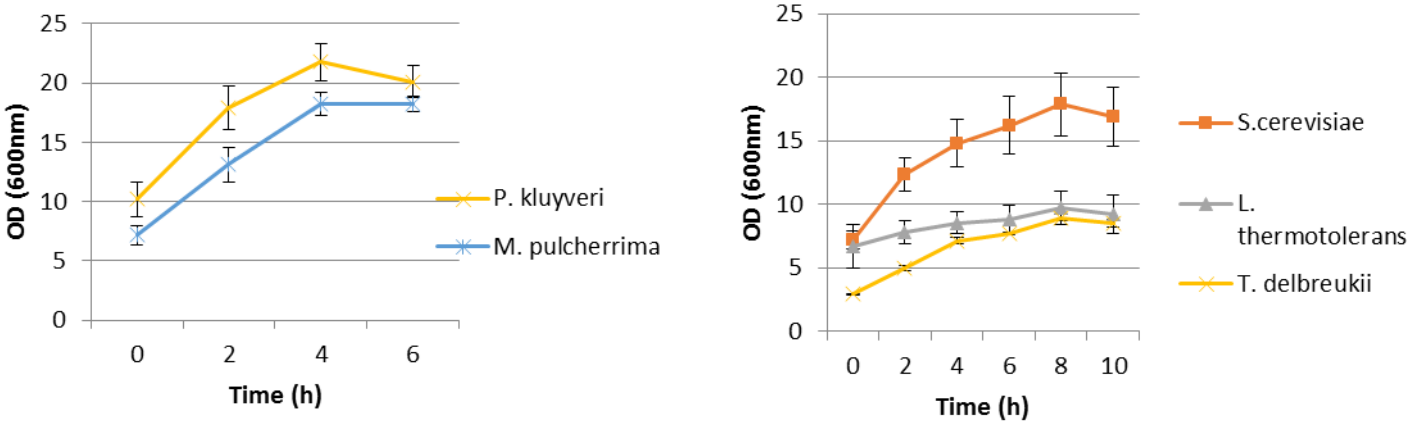


Figure 1 Growth under nitrogen-deprived conditions.

3.3.1.2 Fermentation kinetics, yeast population dynamics and consumption of nitrogen in pure culture fermentations

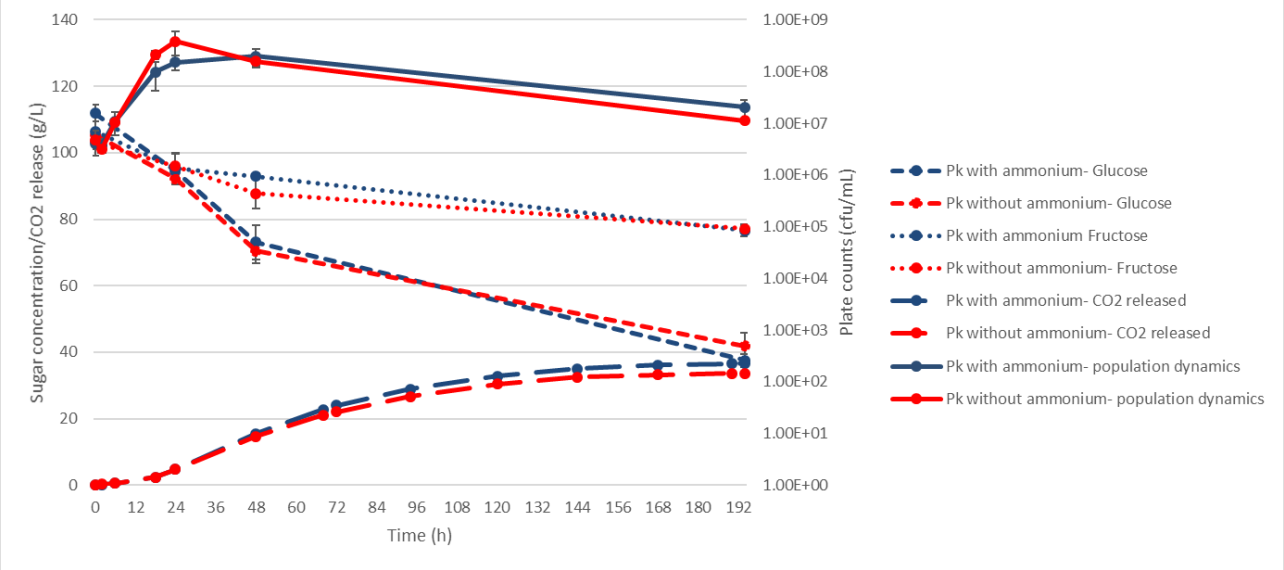
Pure culture fermentations were conducted by inoculation from the nitrogen starvation medium into synthetic grape must-like medium with nitrogen concentrations as described in Table 2 (treatments 1 and 2).

Fermentation kinetics

While *S. cerevisiae* fermented the media to dryness, none of the fermentations carried out by the non-*Saccharomyces* yeasts could achieve a similar outcome (Fig. 2). No significant differences were observed for residual sugar concentration at the end of fermentation between treatments with and without ammonium for each species. The presence of ammonium also seemed to have

no major impact on rate and level of CO_2 release (except for *T. delbrueckii*). The known glucophilic nature of all yeasts studied was also confirmed as glucose was always consumed faster than fructose (Fig. 2). Similar sugar consumption rates were observed for *S. cerevisiae*, *L. thermotolerans* and *T. delbrueckii* within the first 48 h. *P. kluyveri* and *M. pulcherrima*, however, showed slower consumption rates. The total CO_2 release was highest for *S. cerevisiae*, followed by *L. thermotolerans* and *T. delbrueckii* showing almost identical CO_2 release over time, except for *T. delbrueckii* in the presence of ammonium which showed a higher CO_2 release (approximately 27 g/L higher compared to when no ammonium was present) (Fig. 2). *P. kluyveri* showed a marked lower CO_2 release, followed by *M. pulcherrima* with the lowest CO_2 release. CO_2 release was proportional to sugar consumption for the different yeasts tested.

A



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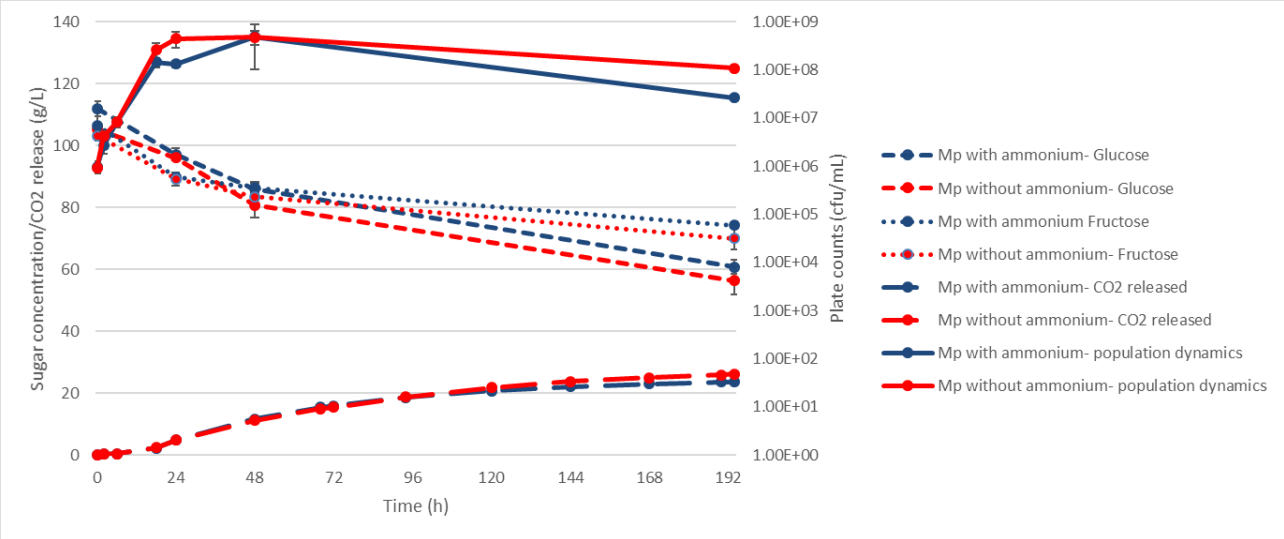

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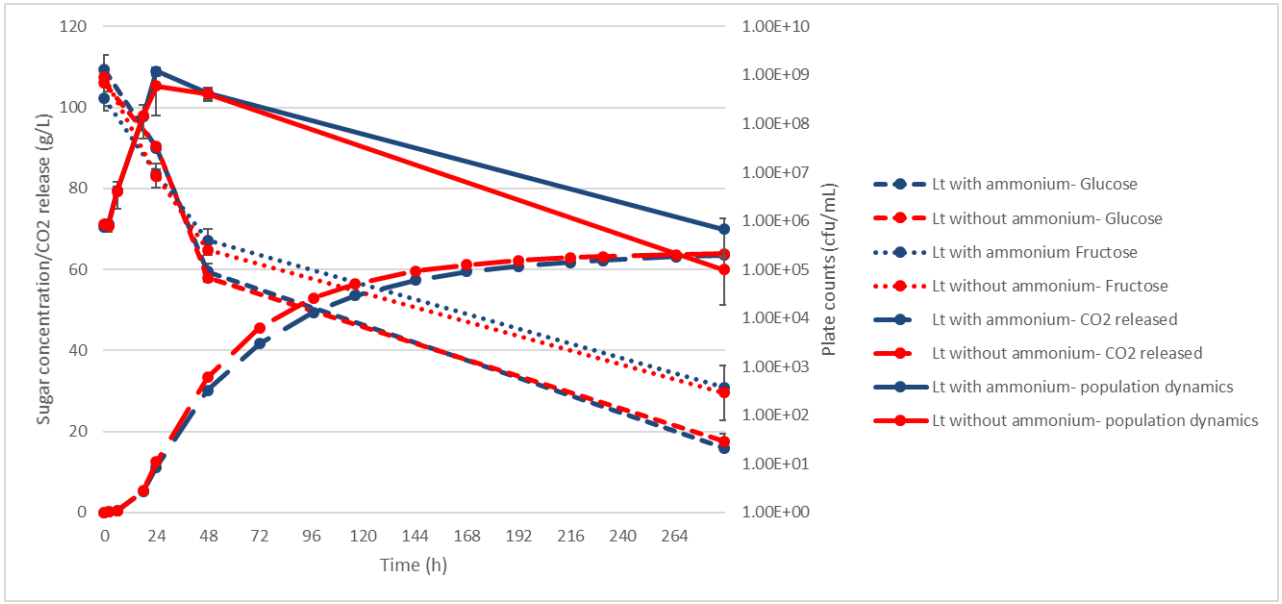
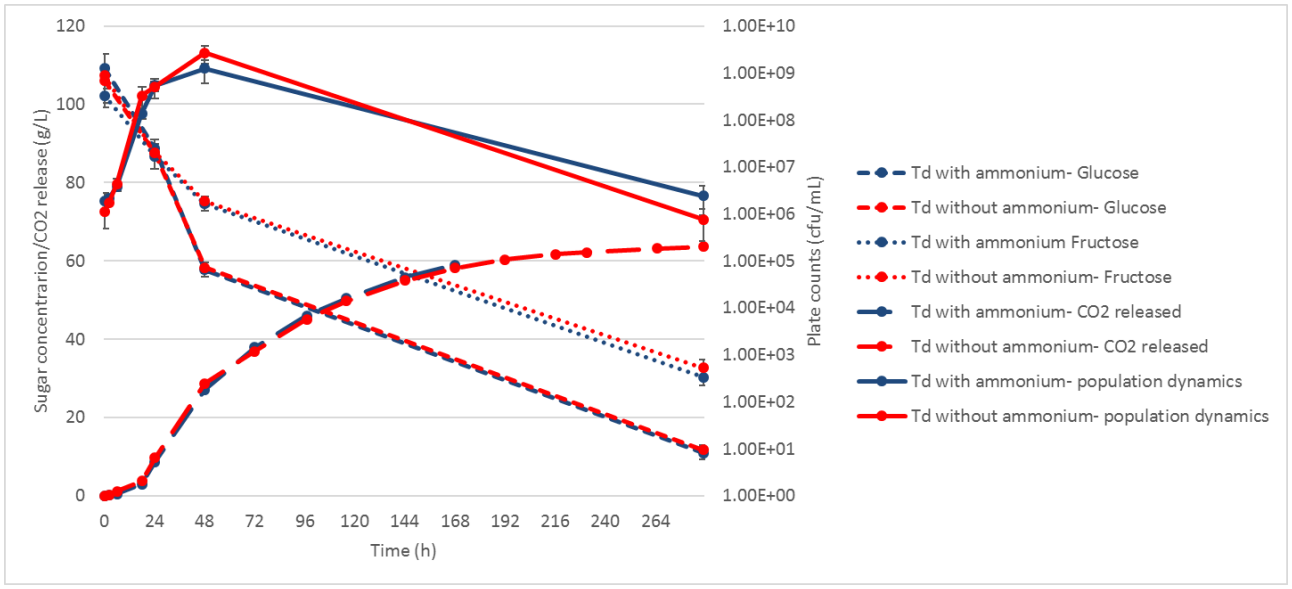

Sugar concentration/CO2 release (g/L)
Plate counts (cfu/mL)
Time (h)
Lt with ammonium- Glucose
Lt without ammonium- Glucose
Lt with ammonium Fructose
Lt without ammonium- Fructose
Lt with ammonium- CO2 released
Lt without ammonium- CO2 released
Lt with ammonium- population dynamics
Lt without ammonium- population dynamics

D


Sugar concentrarion/CO2 release (g/L)
Plate counts (cfu/mL)
Time (h)
Td with ammonium- Glucose
Td without ammonium- Glucose
Td with ammonium Fructose
Td without ammonium- Fructose
Td with ammonium- CO2 released
Td without ammonium- CO2 released
Td with ammonium- population dynamics
Td without ammonium- population dynamics

E

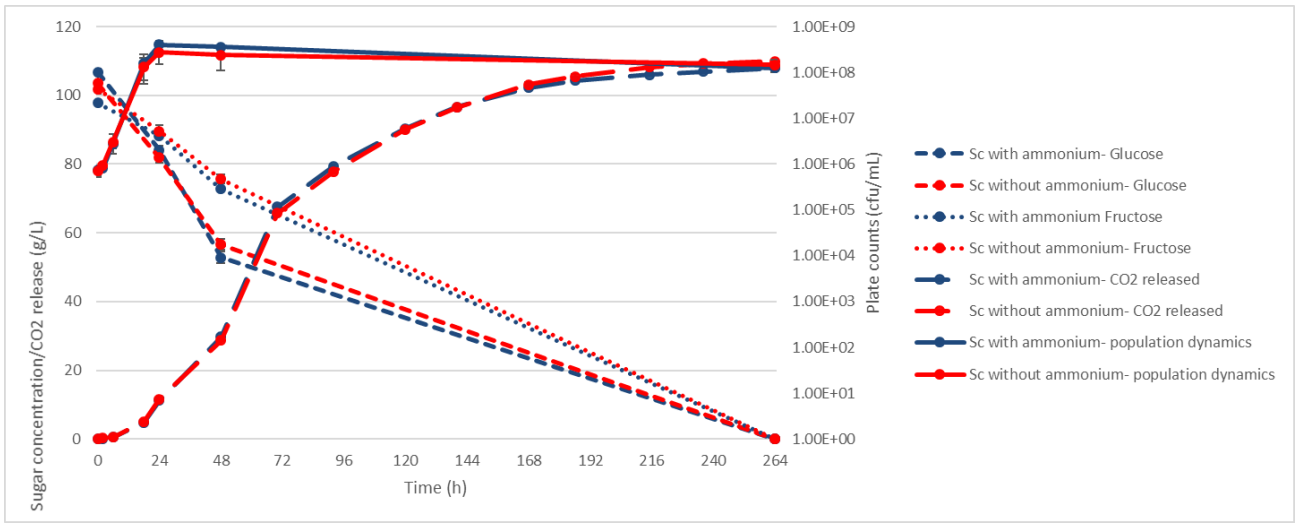

Sugar concentration/CO2 release (g/L)
Plate counts (cfu/mL)
Time (h)
Sc with ammonium- Glucose
Sc without ammonium- Glucose
Sc with ammonium Fructose
Sc without ammonium- Fructose
Sc with ammonium- CO2 released
Sc without ammonium- CO2 released
Sc with ammonium- population dynamics
Sc without ammonium- population dynamics

Figure 2 Fermentation and growth kinetics of pure culture fermentations, with and without the presence of ammonium. A: *P. kluyveri*; B: *M. pulcherrima*; C: *L. thermotolerans*; D: *T. delbrueckii*; E: *S. cerevisiae*.

Population dynamics

Yeast growth was monitored regularly during the first 48 h and again at the end of fermentation. Maximum cell counts and the time at which these were attained are shown in Table 5. No differences for time of maximum cell counts reached between treatments within species were observed, however *P. kluyveri* reached its maximum cell counts earlier when ammonium was not present in the medium. Plate counts showed that some yeast species behaved similarly over the course of the fermentations. *L. thermotolerans* and *T. delbrueckii* showed the highest cell counts while *P. kluyveri* and *M. pulcherrima* showed similar cell counts to *S. cerevisiae* (Fig. 2).

Table 5 Maximum cell count and time point at which it was attained. Pk: *P.kluyveri*; Mp: *M. pulcherrima*; Lt: *L. thermotolerans*; Td: *T. delbrueckii*; Sc: *S. cerevisiae*.

Yeast strain and treatment	Maximum cell count	Standard deviation	Time attained
Pk with ammonium	1.99E+08	7.00E+07	48 h
Pk without ammonium	3.82E+08	2.07E+08	24 h
Mp with ammonium	4.80E+08	3.79E+08	48 h
Mp without ammonium	4.73E+08	1.53E+08	48 h
Lt with ammonium	1.20E+09	2.50E+08	24 h
Lt without ammonium	5.91E+08	4.45E+08	24 h
Td with ammonium	1.24E+09	6.30E+08	48 h
Td without ammonium	2.69E+09	1.10E+09	48 h
Sc with ammonium	4.03E+08	9.53E+07	24 h
Sc without ammnonium	2.73E+08	1.24E+08	24 h

Nitrogen consumption

Full ammonium consumption occurred at different rates depending on yeast species. *L. thermotolerans*, *T. delbrueckii* and *S. cerevisiae* consumed all ammonium (below 5 mg/L remaining) by 18 h. *P. kluyveri* ammonium consumption was complete at 24 h and the slowest consumption of ammonium was by *M. pulcherrima* which took longer than 48 h (Fig. 3).

The uptake of individual amino acids was monitored over time. The onset of uptake as well as the time of uptake completion are shown in Tables 6 and 7, respectively.

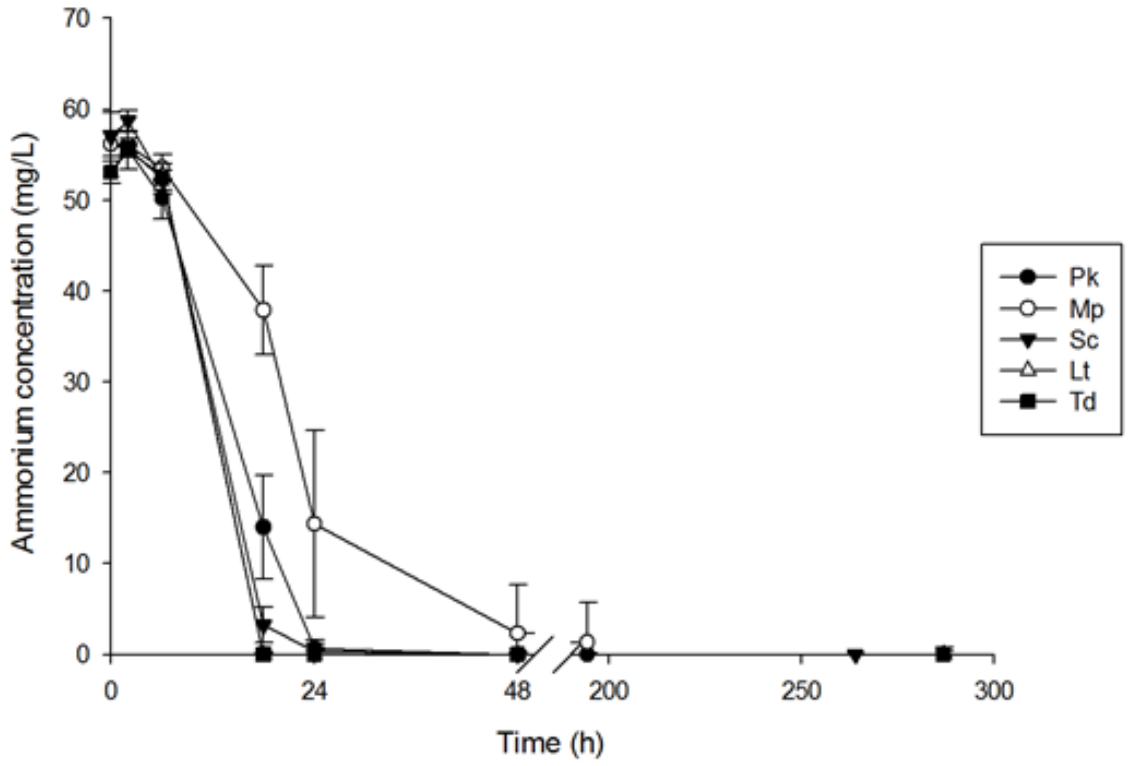


Figure 3 Ammonium levels of pure culture fermentations. Pk: *P.kluyveri*; Mp: *M. pulcherrima*; Lt: *L. thermotolerans*; Td: *T. delbrueckii*; Sc: *S. cerevisiae*.

The uptake of a large portion of amino acid began within 6 h after inoculation and the majority of amino acids started being taken up within 18 h. Strain as well as treatment (i.e. presence or absence of ammonium) differences were observed with regard to time of initial uptake of amino acids (Table 6). *M. pulcherrima* and *P. kluyveri* showed a more delayed initial uptake of a large portion amino acids over time, while *S. cerevisiae*, *L. thermotolerans* and *T. delbrueckii* showed that for most amino acids, initial uptake occurred early on. *T. delbrueckii* showed the most profound differences for onset of initial uptake between treatments with and without ammonium. This yeast showed delayed initial uptake of most amino acids when ammonium was not present compared to when ammonium was present. *S. cerevisiae* showed the most similarity in initial uptake to *L. thermotolerans*. Interestingly, glycine was released during fermentation by *P. kluyveri*

only. When *M. pulcherrima* fermented with ammonium, no statistically significant uptake of glycine and threonine was detected.

Table 6 Onset of individual amino acid uptake. Pk: *P.kluyveri*; Mp: *M. pulcherrima*; Lt: *L. thermotolerans*; Td: *T. delbrueckii*; Sc: *S. cerevisiae*. amm: with ammonium; w/o: without ammonium. Standard deviations were considered.

Time (h)	Pk amm	Pk w/o	Mp amm	Mp w/o	Sc amm	Sc w/o	Lt amm	Lt w/o	Td amm	Td w/o
>0≥6	Asp, Glu, Asn, His, Thr, Arg, Ala, Tyr, Trp, Phe, Ile, Lys, Gln	Asp, Glu, His, Thr, Arg, Ile, Lys, Pro	Asp, Glu, Asn, Thr, Lys, His, Arg,	Asp, Glu, Gln, His, Thr, Arg, Lys,	Asp, Glu, Asn, Ser, Thr, Arg, Ala, Tyr, Val, Trp, Phe, Ile, Leu, Lys	Asp, Glu, Asn, Ser, Thr, Arg, Ala, Tyr, Val, Met, Trp, Phe, Ile, Leu, Lys	Asp, Glu, Asn, Ser, Gln, His, Gly, Thr, Arg, Ala, Tyr, Val, Met, Trp, Phe, Ile, Leu, Pro, Lys	Asp, Glu, Asn, Ser, Gln, His, Gly, Thr, Arg, Ala, Tyr, Val, Met, Trp, Phe, Ile, Leu, Lys	Asp, Glu, Asn, Ser, Thr, Arg, Ala, Tyr, Val, Met, Trp, Phe, Ile, Leu, Pro, Lys	Asp, Gln, Val, Met, Trp,
>6≥18	Met, Pro	Gln, Ala, Met, Trp, Phe, Leu	Ser, Met, Ile, Pro	Gln, Trp, Leu,	Asn, Ser, Met, Trp, Ile, Pro	Gln, His, Met, Pro	Gln, His, Gly, Pro		Pro	His,	Glu, Asn, Ser, His, Gly, Thr, Arg, Ala, Tyr, Phe, Ile, Leu, Lys, Pro
>18≥24	Leu	Tyr, Val	Ala, Phe	Val,	Ala, Tyr, Val, Phe, Leu	Gly				Gln, Gly
>24≥48	Ser, Val	Asn, Ser			Gly					
Later than 48 or not taken up	Gly-released	Gly-released	Gly, Thr-no statistically significant uptake detected							

L. thermotolerans and *T. delbrueckii* showed similar consumption patterns to each other and took up most of the amino acids fully within 48 h, whereas *P. kluyveri* and *M. pulcherrima*, also showing similar consumption to each other (however different to that of *L. thermotolerans* and *T. delbrueckii*), did not take up the majority of amino acids fully within the same time period (Table 7). *M. pulcherrima* and *P. kluyveri* released a large number of amino acids before subsequent uptake occurred.

S. cerevisiae took up all amino acids fully in the presence of ammonium with the exception of Pro. The amino acids that were consumed earliest for this treatment (within 18 h) were Asp, Glu, Arg and Lys. All other fully consumed amino acids were fully taken up within 24 h, with the exceptions of Asn and Gly which were fully consumed by 48 h. In the absence of ammonium, the same amino acids were consumed within 18 h as for the ammonium treatment. All other amino acids were fully taken up by 24 h except for Asn which was not consumed fully even at 48 h. For this treatment however, a release of the following amino acids was observed after being fully consumed: Glu, Gln, His, Thr, Arg, Trp, Phe and Ile.

For fermentations with the presence of ammonium, amino acids fully taken up by both *P. kluyveri* and *M. pulcherrima* were Arg, Met, Leu and Lys. In addition to these, *P. kluyveri* fully consumed His and Ala, while *M. pulcherrima* also fully consumed Glu, Ser, Gln and Ile. For fermentations without the presence of ammonium, common fully taken up amino acids for these two yeast species were as for with ammonium, with the addition of His, Val and Ile. For this treatment, *P. kluyveri* also fully consumed Ala and Phe, while *M. pulcherrima* also fully consumed Glu, Ser and Gln.

For *L. thermotolerans*, the presence or absence of ammonium resulted in no major differences with regard to amino acids which were fully consumed except for Asn, which was fully consumed when ammonium was not present, but not with the presence of ammonium. In the latter case, Asn was only partically (48%) taken up and then released. All other amino acids were taken up fully in both treatments for this yeast, with most amino acids being fully consumed after 18 h from yeast inoculation.

T. delbrueckii took up all amino acids fully for both treatments. Most amino acids were fully consumed after 24 h from yeast inoculation, however for the treatment with ammonium, Arg, Met and Lys were fully consumed in 18 h and for the treatment without ammonium, these amino acids with the additions of Asp and Glu were also fully taken up in 18 h.

Table 7 Kinetics of amino acid % uptake. % uptake of 90% and above was considered fully taken up. Negative values indicate amino acid release.

With ammonium																		
Pk	ASP	GLU	ASN	SER	GLN	HIS	GLY	THR	ARG	ALA	TYR	VAL	MET	TRP	PHE	ILE	LEU	LYS	PRO
<6-6h	7	-10	-40	-69	100	28	-161	-14	17	-109	-36	-118	-59	-44	-38	-79	-80	100	-24
>6-18h	25	14	-22	-31	100	64	-136	13	100	-41	-16	-50	22	15	11	-14	-2	100	10
>18-24h	32	25	-21	-18	100	88	-183	18	100	12	-7	-15	30	13	29	12	23	100	15
>24-48h	49	66	4	45	100	100	-38	63	100	100	50	77	100	70	84	85	100	100	56
Mp	ASP	GLU	ASN	SER	GLN	HIS	GLY	THR	ARG	ALA	TYR	VAL	MET	TRP	PHE	ILE	LEU	LYS	PRO
<6-6h	11	11	-33	-52	-52	31	-152	3	8	-101	-34	-107	-61	-37	-38	-74	-78	100	-18
>6-18h	39	100	20	68	100	68	-53	62	100	-8	-15	4	27	36	36	36	28	100	22
>18-24h	58	100	34	85	100	76	-36	77	100	29	-8	46	45	18	9	78	72	100	19
>24-48h	78	100	50	100	100	89	19	86	100	59	13	74	100	45	40	100	100	100	46
Sc	ASP	GLU	ASN	SER	GLN	HIS	GLY	THR	ARG	ALA	TYR	VAL	MET	TRP	PHE	ILE	LEU	LYS	PRO
<6-6h	11	6	3	6	-4	-10	-7	10	6	2	2	2	2	22	4	4	5	100	-9
>6-18h	100	100	41	87	45	54	1	55	100	27	24	39	69	59	58	60	71	100	2
>18-24h	100	100	70	100	100	100	88	100	100	100	100	100	100	100	100	100	100	100	13
>24-48h	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	38

Lt	ASP	GLU	ASN	SER	GLN	HIS	GLY	THR	ARG	ALA	TYR	VAL	MET	TRP	PHE	ILE	LEU	LYS	PRO
<6-6h	11	6	2	7	2	3	4	10	29	3	3	3	7	23	4	4	5	100	7
>6-18h	100	100	46	100	100	100	76	100	100	100	73	80	100	76	100	100	100	100	37
>18-24h	100	100	48	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
>24-48h	100	100	0	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Td	ASP	GLU	ASN	SER	GLN	HIS	GLY	THR	ARG	ALA	TYR	VAL	MET	TRP	PHE	ILE	LEU	LYS	PRO
<6-6h	10	5	5	5	0	3	1	9	30	2	2	3	3	19	4	4	5	100	16
>6-18h	82	59	39	79	-42	34	24	80	100	51	46	49	100	44	69	77	89	100	15
>18-24h	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
>24-48h	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Without ammonium

Pk	ASP	GLU	ASN	SER	GLN	HIS	GLY	THR	ARG	ALA	TYR	VAL	MET	TRP	PHE	ILE	LEU	LYS	PRO
<6-6h	11	-7	-37	-66	-23	24	-169	-15	21	-104	-32	-108	-58	-41	-35	-75	-78	64	-13
>6-18h	29	22	-17	-25	4	71	-101	14	100	33	-1	-19	30	20	35	10	27	100	57
>18-24h	43	40	-6	-2	15	100	-106	30	100	77	16	20	50	32	58	40	55	100	64
>24-48h	55	71	13	45	54	100	-17	67	100	100	57	100	100	79	100	100	100	100	100

Mp	ASP	GLU	ASN	SER	GLN	HIS	GLY	THR	ARG	ALA	TYR	VAL	MET	TRP	PHE	ILE	LEU	LYS	PRO
<6-6h	26	25	-3	-19	26	53	-83	24	32	-58	-6	-65	-18	0	-9	-40	-39	100	14
>6-18h	39	100	24	69	100	66	-41	62	100	2	-17	-2	27	25	-9	25	10	100	28
>18-24h	34	34	-7	-2	19	100	-74	32	100	76	14	18	52	30	57	37	56	100	64
>24-48h	82	100	66	100	100	100	54	88	100	79	11	100	100	34	42	100	100	100	63

Sc	ASP	GLU	ASN	SER	GLN	HIS	GLY	THR	ARG	ALA	TYR	VAL	MET	TRP	PHE	ILE	LEU	LYS	PRO
<6-6h	9	6	4	6	-7	-4	-2	10	1	3	4	3	2	23	5	4	5	100	-7
>6-18h	100	100	42	84	68	52	37	82	100	62	49	66	81	71	75	77	79	100	9
>18-24h	100	100	84	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	26
>24-48h	100	82	66	100	68	-6	100	43	30	100	100	100	100	76	86	59	100	100	100

Lt	ASP	GLU	ASN	SER	GLN	HIS	GLY	THR	ARG	ALA	TYR	VAL	MET	TRP	PHE	ILE	LEU	LYS	PRO
<6-6h	5	3	3	3	6	2	0	6	24	1	1	1	2	18	2	2	3	6	-6
>6-18h	100	100	46	100	87	100	89	100	100	100	87	100	100	83	100	100	100	100	100
>18-24h	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
>24-48h	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Td	ASP	GLU	ASN	SER	GLN	HIS	GLY	THR	ARG	ALA	TYR	VAL	MET	TRP	PHE	ILE	LEU	LYS	PRO
<6-6h	3	0	-1	-1	-1	-17	-10	1	0	-1	-2	-3	0	7	-2	-2	-1	1	-8
>6-18h	100	100	43	78	66	66	47	73	100	78	55	55	100	60	81	75	89	100	26
>18-24h	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
>24-48h	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

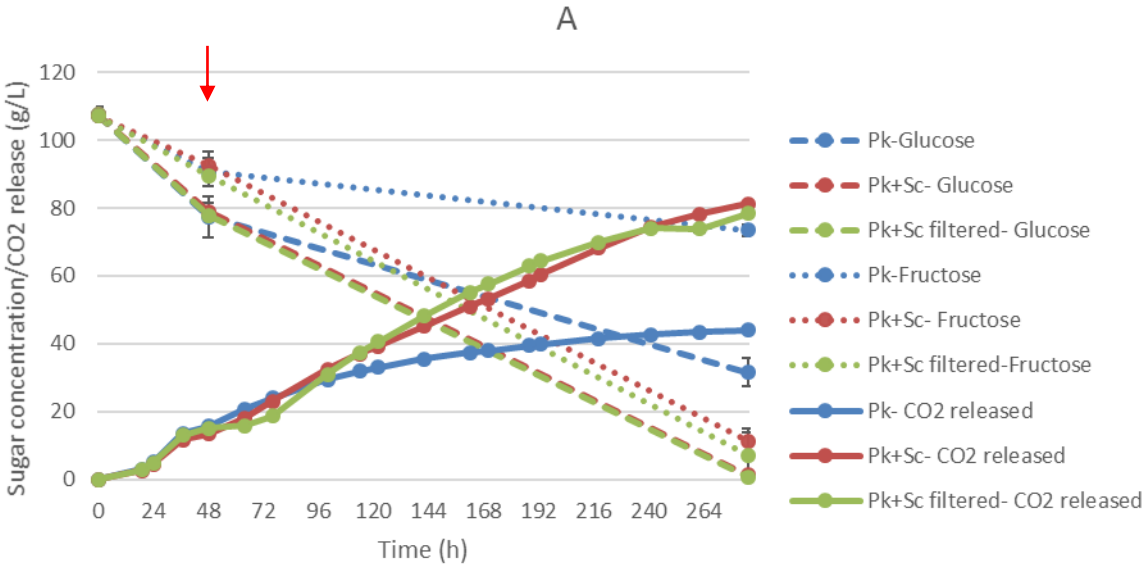
3.3.2 Fermentation kinetics, consumption of nitrogen and aroma compounds production

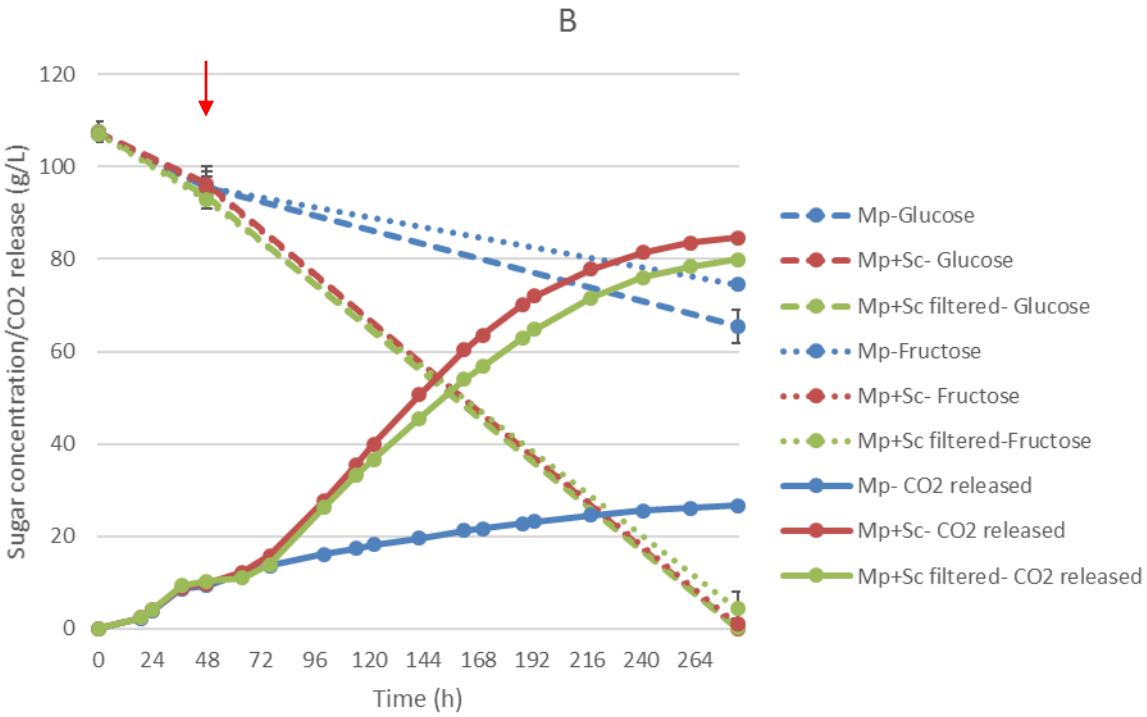
Fermentations in synthetic grape must medium were carried out with varying concentrations of nitrogenous compounds in order to mimic the composition of an average grape must. The medium was inoculated with pure cultures of non-*Saccharomyces* yeasts or sequentially with the non-*Saccharomyces* yeasts followed 48 h later by *S. cerevisiae*. Furthermore, two different sequential inoculations for each non-*Saccharomyces* strain were carried out. In the first, *S. cerevisiae* was inoculated 48 h after non-*Saccharomyces* inoculation and were thereafter both present in the medium. In the second, the non-*Saccharomyces* yeast was filtered out at 48 h before inoculating *S. cerevisiae*.

Fermentation kinetics

The *S. cerevisiae* fermentation was used as a reference. This yeast fermented to dryness and released almost 100 g/L CO_2 (Fig. 4E).

P. kluyveri and *M. pulcherrima* pure culture fermentations showed the highest residual glucose levels compared to both filtered and unfiltered sequential fermentations which showed no residual glucose at the end of fermentation. *L. thermotolerans* and *T. delbrueckii* however showed that the filtered sequential fermentations had the highest residual glucose concentrations, followed by the pure culture and the lowest residual glucose levels were found in the unfiltered sequential fermentations. None of the fermentations with *L. thermotolerans* were able to consume all the glucose present and only the unfiltered sequential *T. delbrueckii* fermentation was able to consume all glucose. Fructose consumption showed similar trends, however with higher residual levels (Fig. 4). Therefore, all the fermentations containing non-*Saccharomyces* yeasts ceased to ferment before the depletion of sugars.





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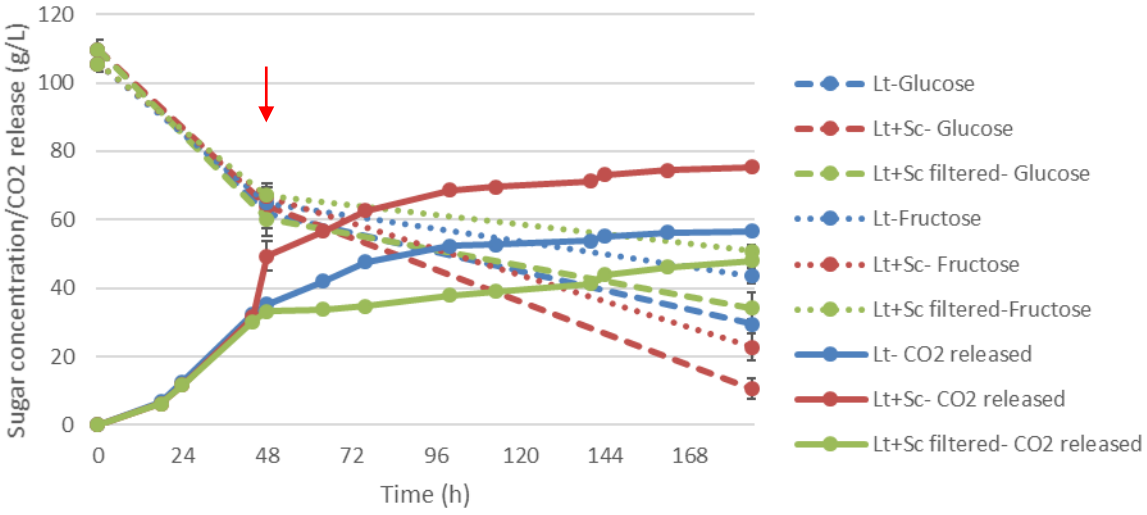

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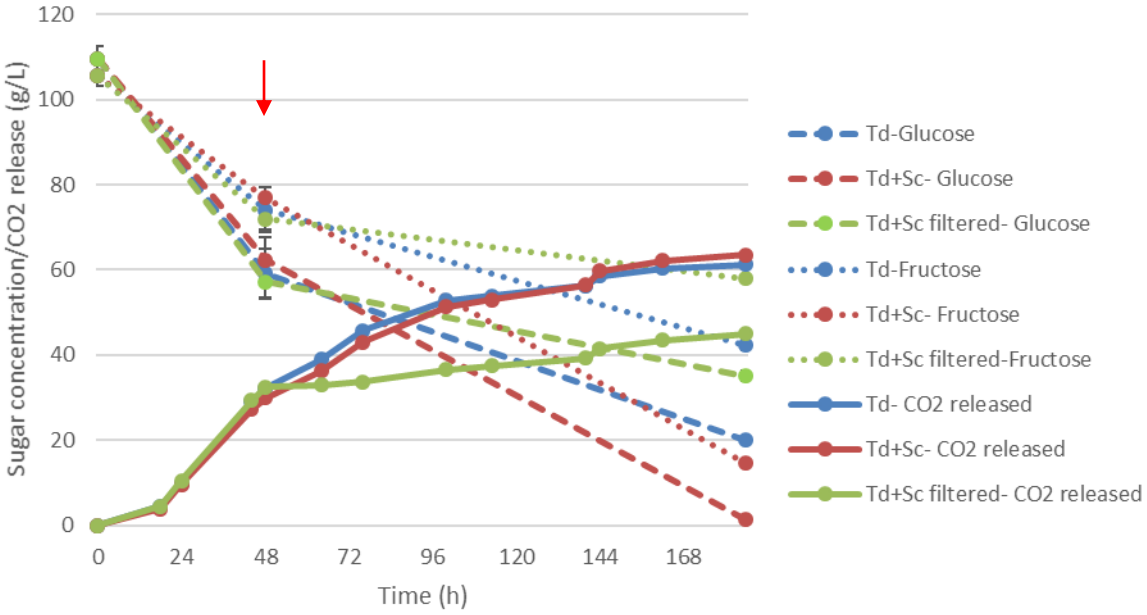


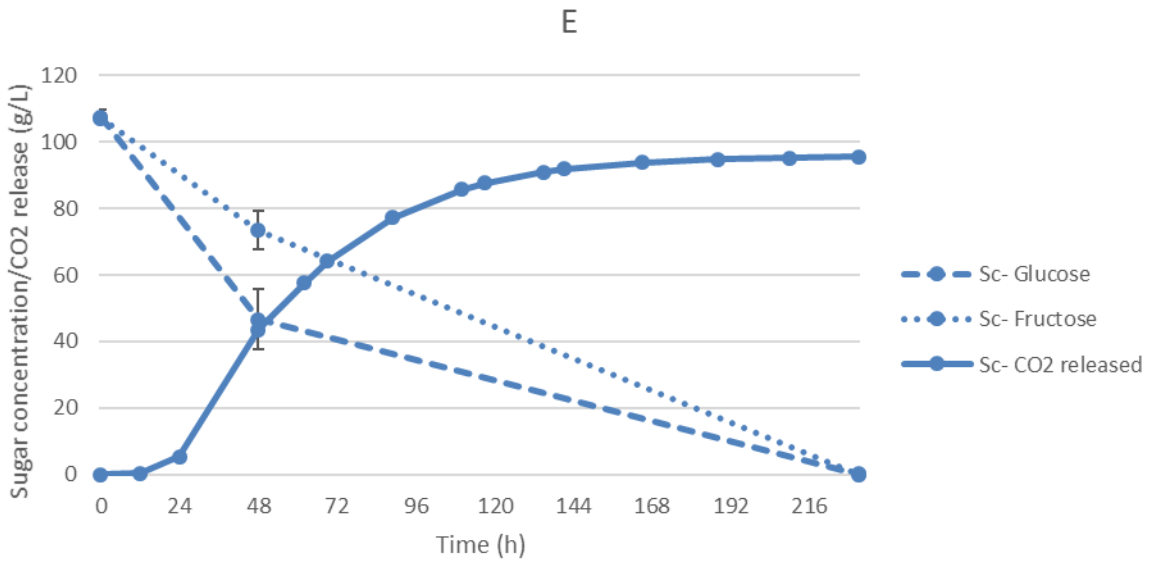


Figure 4 Fermentation kinetics of pure culture and sequential fermentations. A: *P. kluyveri*; B: *M. pulcherrima*; C: *L. thermotolerans*; D: *T. delbrueckii*; E: *S. cerevisiae*. Pk: *P.kluyveri*; Mp: *M. pulcherrima*; Lt: *L. thermotolerans*; Td: *T. delbrueckii*; Sc: *S. cerevisiae*. Pk+Sc, Mp+Sc, Lt+Sc, Td+Sc: Sequential fermentations. Pk+Sc filtered, Mp+Sc filtered, Lt+Sc filtered, Td+Sc filtered: Sequential fermentations, however non-*Saccharomyces* yeast were filtered out before *S. cerevisiae* inoculation. The red arrows indicate time of *S. cerevisiae* inoculation in sequential fermentations.

Nitrogen consumption and aroma compounds produced

A comparison of the percentage uptake of amino acids at 48 h between treatments with and without ammonium, as well as with grape-must like nitrogen concentrations is displayed in Table 8. *S. cerevisiae*, *L. thermotolerans* and *T. delbrueckii* behaved similarly to each other with regard to amino acid uptake and had taken up the majority of amino acids fully by 48 h. These yeasts behaved very differently to *P. kluyveri* and *M. pulcherrima* however as these yeasts displayed much lower amino acids consumption profiles, similarly to the previous observations.

For *S. cerevisiae*, fermentations with grape must-like amino acid concentrations had similar percentage amino acid uptake levels at 48 h to the amino acids all at the same assimilable nitrogen concentration with the presence of ammonium. Some exceptions can however be noted, where the former treatment showed lower percentage uptake of the amino acids Asn, Gly, Arg and Pro. With regard to *L. thermotolerans* fermentations, all three treatments showed similar percentage uptake of amino acids at 48 h except for Glu, Asn (compared to

treatment without ammonium), Gly, Ala, Val, Trp and Pro for the treatment with grape must-like amino acid concentrations for which lower percentage uptake was observed. For *T. delbrueckii*, lower uptakes for the grape must-like nitrogen treatment was found for the amino acids Asn, Gly and Pro.

For *P. kluyveri* fermentations, the most marked differences between treatments with amino acids at the same assimilable nitrogen level and grape must-like nitrogen concentrations are the following: For grape must-like nitrogen concentrations, His was taken up half of the observed percentage uptake for the treatments with amino acids at the same assimilable nitrogen levels, regardless of the presence of ammonium. Gly was taken up in the grape-must like nitrogen treatment whereas a release was observed for the other treatments. Arg, Ala and Pro percentage uptake were however less for this treatment compared to the other two treatments. *M. pulcherrima* fermentations had lower percentage uptakes for the grape must-like nitrogen concentration treatment compared to the other treatments for the amino acids Asp, Glu, Asn, Ser, Gln, Thr, Arg, Ala and Pro.

Table 8 % uptake at 48 h. 1: Treatment with ammonium with amino acids at the same assimilable level. 2: Treatment without ammonium with amino acids at the same assimilable level. 3: Grape-must like nitrogen source concentrations.

	ASP	GLU	ASN	SER	GLN	HIS	GLY	THR	ARG	ALA	TYR	VAL	MET	TRP	PHE	ILE	LEU	LYS	PRO
Pk																			
1	49	66	4	45	100	100	-38	63	100	100	50	77	100	70	84	85	100	100	70
2	55	71	100	45	54	100	-17	67	100	100	57	100	100	79	100	100	100	100	100
3	39	80	24	59	63	50	24	77	61	82	60	87	100	74	100	100	100	100	19
Mp																			
1	78	100	50	100	100	89	19	86	100	59	13	74	100	45	40	100	100	100	57
2	82	100	66	100	100	100	54	88	100	79	11	100	100	34	42	100	100	100	70
3	24	68	23	50	87	80	14	59	29	35	19	100	100	36	35	100	100	100	19
Sc																			
1	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	52
2	51	82	67	100	68	-6	100	43	30	100	100	100	100	76	86	59	100	100	100
3	100	100	79	100	100	100	74	100	82	100	100	100	100	100	100	100	100	100	2
Lt																			
1	100	100	0	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
2	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
3	100	88	48	100	100	100	-20	100	100	81	100	87	100	79	100	100	100	100	-35
Td																			
1	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
2	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
3	100	100	62	100	100	100	40	100	100	100	100	100	100	100	100	100	100	100	-15

Ammonium consumption is shown in Table 9. *P. kluyveri* and *M. pulcherrima* showed high levels of ammonium at 48 h, whereas for *L. thermotolerans, T. delbrueckii* and *S. cerevisiae*, ammonium is mostly or all consumed at 48 h, therefore there was no ammonium left in the medium at *S. cerevisiae* inoculation. At the end of fermentation, pure cultures of *P. kluyveri* and *M. pulcherrima* showed residual ammonium, however all ammonium was consumed for all other fermentations. The average initial ammonium concentration for fermentations was 77 mg/L, which was close to the theoretical addition of 80.25 mg/L.

Table 9 Residual ammonium concentrations.

Yeast strain and treatment	Residual ammonium at 48 h (mg/L)	Residual ammonium at endpoint (mg/L)
Pk	47.0 ± 1.6	43.2 ± 2.3
Pk + Sc	49.0 ± 5.5	0 ± 0.1
Pk + Sc filtered	47.9 ± 5.5	0 ± 0.3
Mp	59.8 ± 4.8	56.6 ± 5.5
Mp + Sc	59.6 ± 6.5	0 ± 0.4
Mp + Sc filtered	55.0 ± 3.2	0 ± 0.3
Lt	0 ± 0.3	0 ± 0.3
Lt + Sc	0 ± 0.6	0 ± 0.2
Lt +Sc filtered	4.7 ± 7.7	0 ± 0.1
Td	0 ± 0.5	0 ± 0.3
Td + Sc	0 ± 0.3	0 ± 0.1
Td + Sc filtered	0 ± 0.3	0 ± 0.5
Sc	0 ± 0.1	0 ± 0.2

Uptake of amino acids catabolised through the Ehrlich pathway and their corresponding higher alcohol production

A general higher percentage uptake for the amino acids Leu, Val, Phe, Ser and Thr (some of the amino acids involved in the Ehrlich pathway) was observed for *S. cerevisiae*, *L. thermotolerans* and *T. delbrueckii* when compared with *P. kluyveri* and *M. pulcherrima* (Table 10). Leucine was fully consumed by all yeasts by 48 h. *S. cerevisiae*, *L. thermotolerans* and *T. delbrueckii* consumed all of these amino acids with the exception of valine for *L. thermotolerans*. *P. kluyveri* consumed only leucine and phenylalanine fully, while *M. pulcherrima* consumed only leucine and valine fully.

Table 10 Percentage uptake of amino acids involved in the Ehrlich pathway at 48h (before sequential inoculation). An uptake of 90% or higher was recorded as 100% taken up. Pk: *P.kluyveri*; Mp: *M. pulcherrima*; Lt: *L. thermotolerans*; Td: *T. delbrueckii*; Sc: *S. cerevisiae*.

Amino acid → Yeast ↓	Leu	Val	Phe	Ser	Thr
Pk	100	87	100	59	77
Mp	100	100	35	50	59
Sc	100	100	100	100	100
Lt	100	87	100	100	100
Td	100	100	100	100	100

Fig. 5 displays the production of some of the higher alcohols which can be produced via the Ehrlich pathway at 48 h. Higher propanol concentrations were observed for the yeast strains which took up 100% of Threonine and Serine, namely *S. cerevisiae*, *L. thermotolerans* and *T. delbrueckii*. Statistical analysis shows that for isoamyl alcohol, the concentrations at 48 h were statistically significant for *P. kluyveri* compared to *S. cerevisiae*, *L. thermotolerans* and *T. delbrueckii*, which in turn grouped as not significantly different to each other, whereas *M. pulcherrima* showed no significant differences when compared to any of the other yeast strains. For isobutanol, the only significant differences were between *M. pulcherrima* and *S. cerevisiae*. Phenylethanol concentrations were statistically different for *M. pulcherrima* when compared to *P. kluyveri* and *S. cerevisiae*, whereas propanol showed statistically significantly

higher levels produced by *S. cerevisiae*, followed by *L. thermotolerans* and *T. delbrueckii*. *P. kluyveri* only produced isobutanol at 48 h.

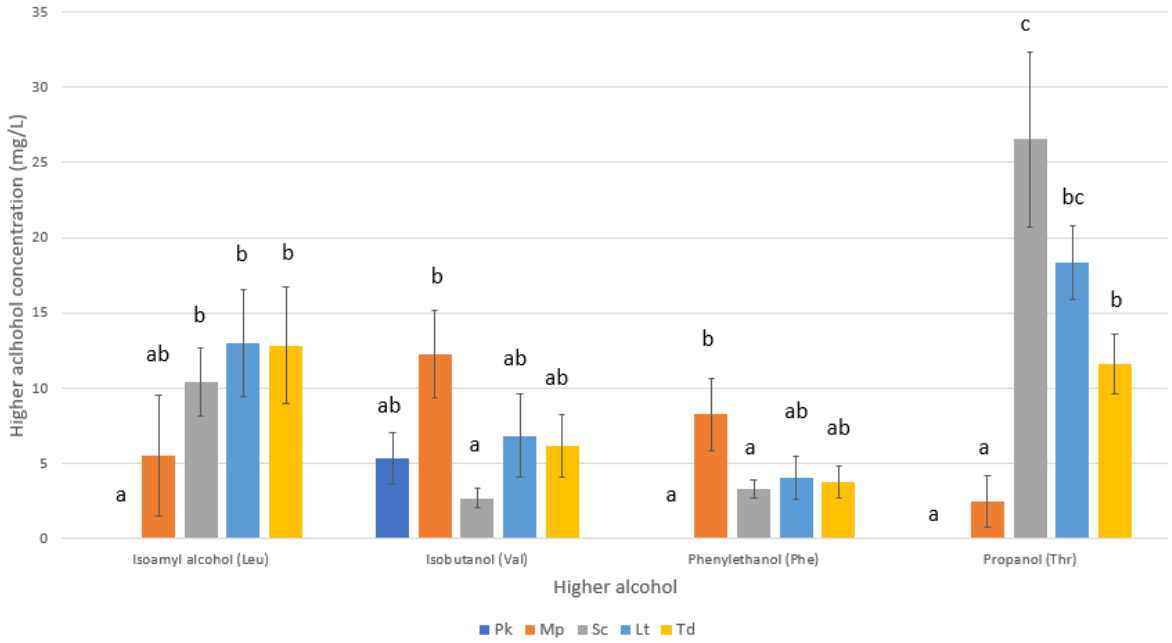
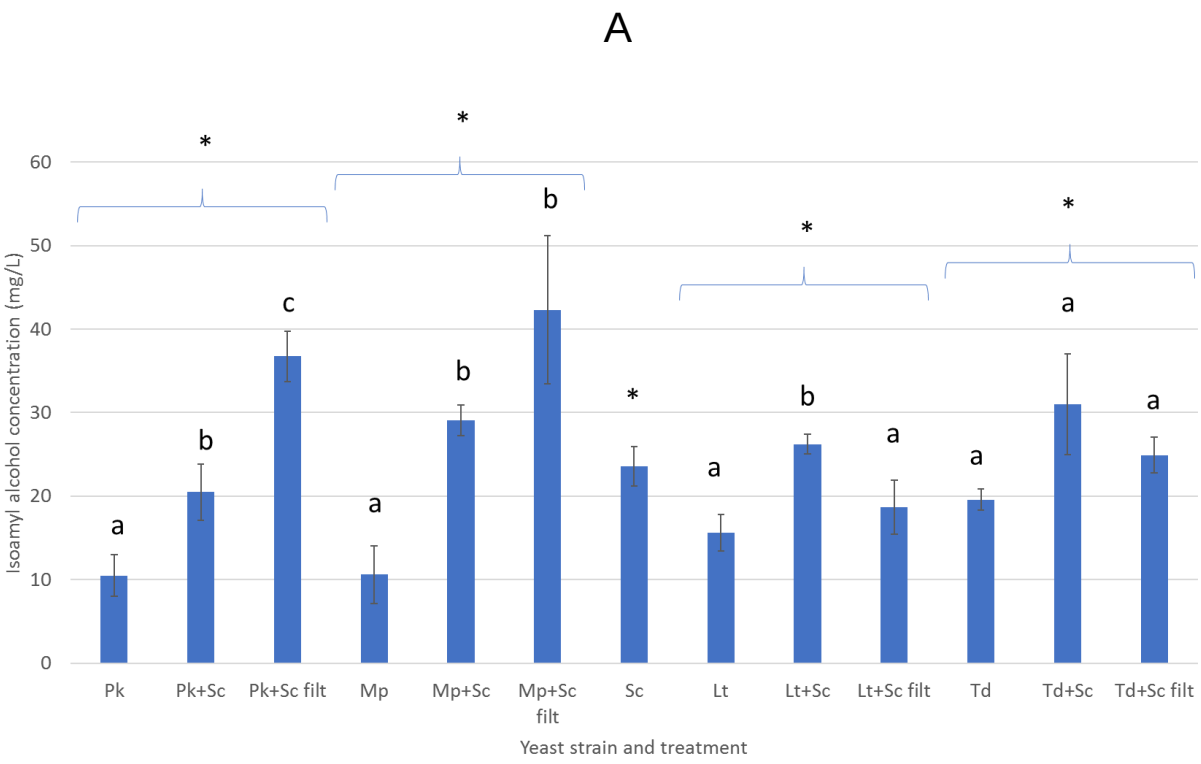


Figure 5 Higher alcohol production at 48 h from corresponding amino acids which can be catabolised via the Ehrlich pathway. Pk: *P.kluyveri*; Mp: *M. pulcherrima*; Lt: *L. thermotolerans*; Td: *T. delbrueckii*; Sc: *S. cerevisiae*. a,b,c: show statistical significance within a compound over strain. Amino acids which are catabolised during the Ehrlich pathway are in brackets next to their corresponding higher alcohols.

At the end of fermentation, it could be seen that sequential fermentations with *P. kluyveri* and *M. pulcherrima* generally showed higher production of higher alcohols when compared to fermentations with *L. thermotolerans*, *T. delbrueckii* and *S. cerevisiae* (Fig. 6). The only statistically significant differences regarding strain (regardless of treatment) were found for *M. pulcherrima* for isobutanol and phenylethanol. Statistically significant differences between treatments were found for a number of compounds within a yeast strain. In general, sequential fermentations resulted in higher higher alcohol concentrations compared to pure culture fermentation. For *P. kluyveri* and *M. pulcherrima*, the filtered sequential fermentations yielded the highest higher alcohol concentrations except for isobutanol, however differences were not statistically significant. For *L. thermotolerans* and *T. delbrueckii*, the non-filtered sequential fermentations resulted in the highest production of higher alcohols. For isoamyl alcohol, isobutanol and phenylethanol, higher alcohol concentrations produced by fermentation with pure *S. cerevisiae* showed similar levels to *L. thermotolerans* and *T. delbrueckii*, whereas for propanol, levels were more similar to sequential fermentations with *P. kluyveri* and *M.*

pulcherrima. Some corresponding acetate esters were also measured. One of the most noteworthy acetate ester productions was that of 2-phenylethyl acetate, formed from phenylethanol by *P. kluyveri*, which produced 2.1 mg/L, 2.2 mg/L and 0.9 mg/L of this compound in pure, sequential and filtered sequential fermentations, respectively. No other yeast produced this compound above detectable levels. Isoamyl acetate was produced in fermentations containing *P. kluyveri* and pure culture *M. pulcherrima* fermentations only. Concentrations produced were 1.0 mg/L, 1.6 mg/L, 0.6 g/L and 0.06 mg/L for pure *P. kluyveri*, sequential *P. kluyveri*, filtered sequential *P. kluyveri* and pure *M. pulcherrima* fermentations respectively.



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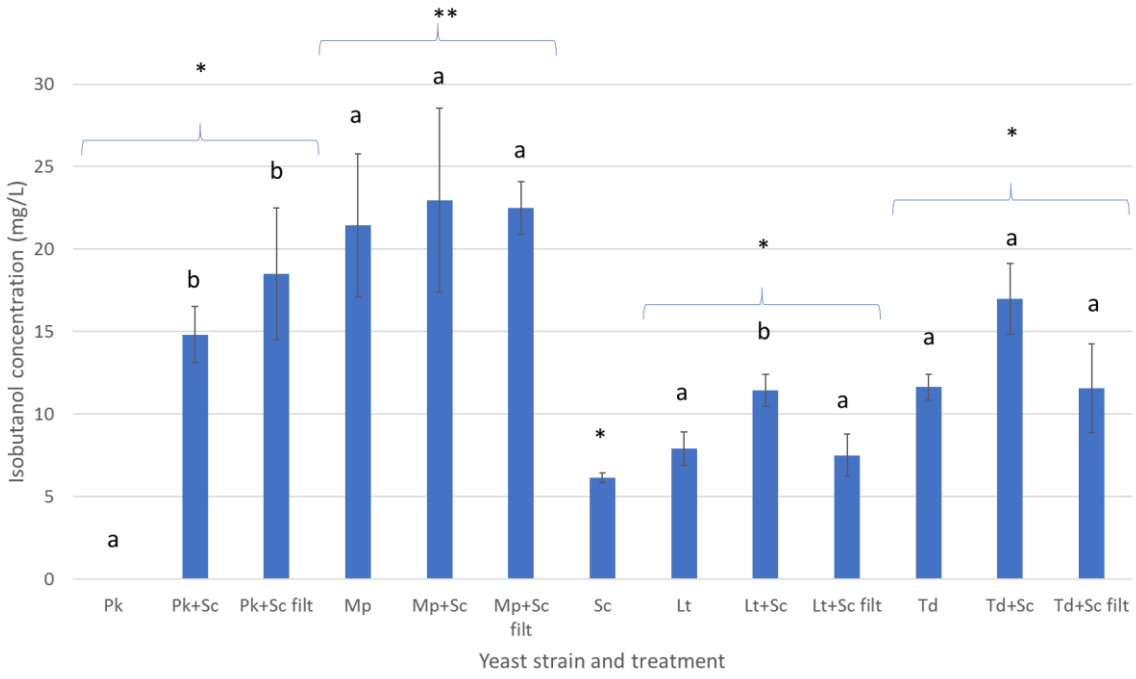

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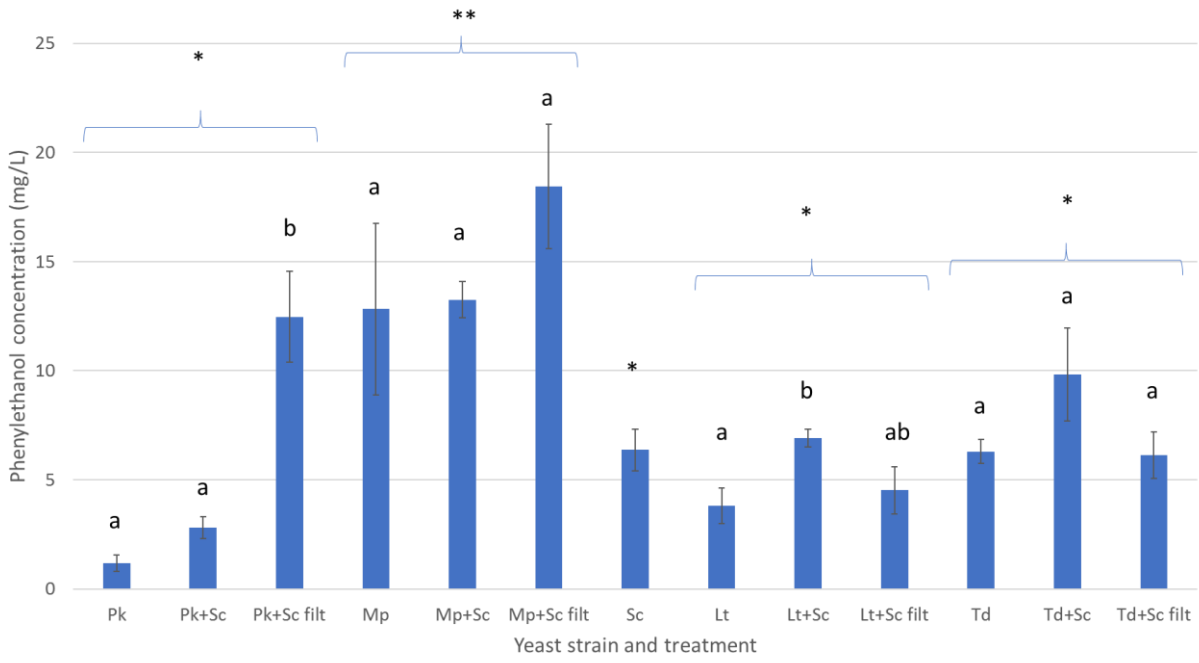

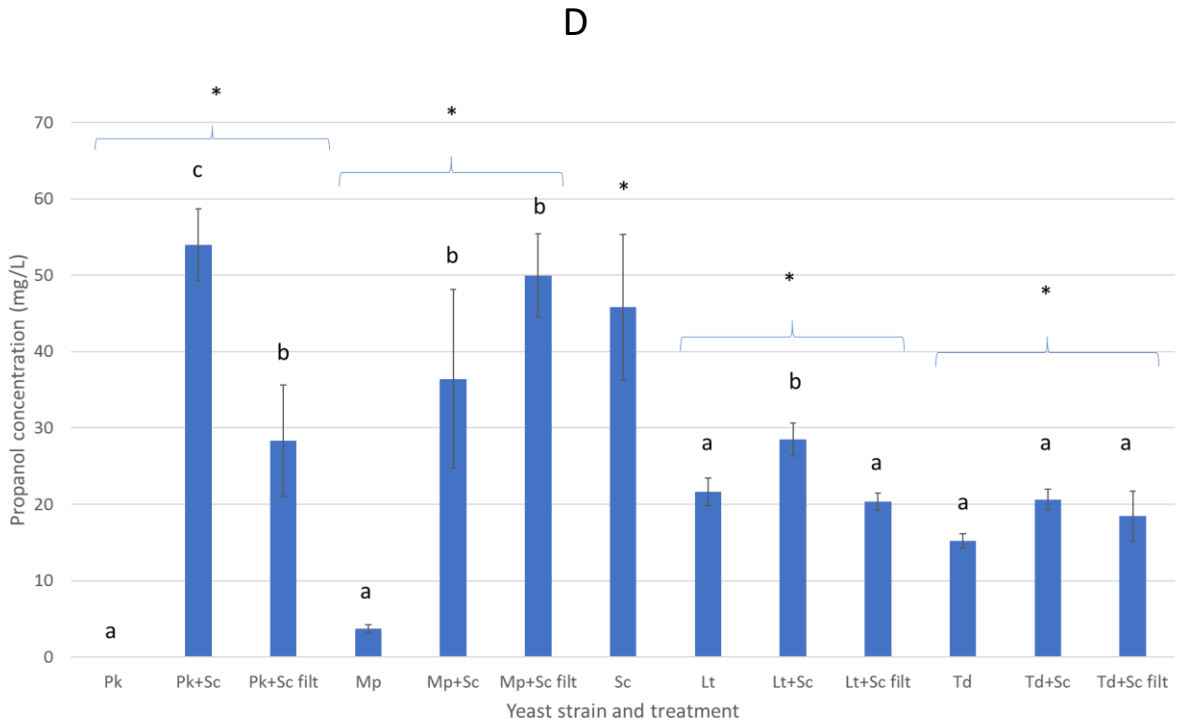

Figure 6 Production of higher alcohols at endpoint which could possibly be as a result of specific amino acid catabolism via the Ehrlich pathway. Pk: *P.kluyveri*; Mp: *M. pulcherrima*; Lt: *L. thermotolerans*; Td: *T. delbrueckii*; Sc: *S. cerevisiae*. Pk+Sc, Mp+Sc, Lt+Sc, Td+Sc: Sequential fermentations. Pk+Sc filtered, Mp+Sc filtered, Lt+Sc filtered, Td+Sc filtered: Sequential fermentations, however non-*Saccharomyces* yeast were filtered out before *S. cerevisiae* inoculation. A: Isoamyl alcohol; B: Isobutanol; C: Phenylethanol; D: Propanol. a, b, c: show statistical significance within each strain between treatments. *, **: show statistical significance between strains (not taking into account treatment).

3.4 Discussion

The main aims of this study were to determine amino acid preferences of various commercial non-*Saccharomyces* yeasts, to identify any potential for competition between *S. cerevisiae* and any of the non-*Saccharomyces* yeasts investigated in this study as well as to establish a tentative correlation between amino acid uptake and aroma compounds produced.

3.4.1 General fermentation kinetics and links with nitrogen uptake

L. thermotolerans and *T. delbrueckii* showed higher cell counts than *M. pulcherrima* and *P. kluyveri*; Wang et al. (2016) also showed that *T. debrueckii* indeed produces higher biomass than *M. pulcherrima.* The better growth observed for these yeasts compared to *P. kluyveri* and *M. pulcherrima* correlates with their higher uptake of nitrogen sources. Gutiérrez et al. (2016)

also found correlations between level of amino acid uptake and growth. The latter study also demonstrated that nitrogen source uptake began before the onset of growth, which is confirmed in the current study. Fermentation performance (CO_2 release and sugar consumption) and yeast growth was also reflected in the uptake of nitrogen compounds. Pure cultures of *L. thermotolerans* and *T. delbrueckii* were observed to be the faster non-*Saccharomyces* fermenters tested in this study with regard to CO_2 release, as found by du Plessis et al. (2017), and rate of sugar consumption and residual sugar concentrations as found by de Koker (2015). Overall, the faster fermenters assimilated more nitrogen compounds than the slower fermenters in this study, as previously observed by de Koker (2015). However, *S. cerevisiae* was the only yeast able to ferment to dryness. Ammonium was fully consumed by all yeast species but its uptake was much faster in *L. thermotolerans, T. delbrueckii* and *S. cerevisiae* than in *P. kluyveri* and *M. pulcherrima*. Furthermore, most nitrogen source uptake began earlier in the stronger fermenters than in *M. pulcherrima* and *P. kluyveri*, thus possibly confirming the link between nitrogen uptake and fermentation ability previously reported in literature (Ribéreau-Gayon et al. 2006). However, the latter two yeast species may just take longer to adapt to the grape must medium.

3.4.2 Nitrogen source preference

3.4.2.1 Response of yeast to the presence of ammonium

The presence or absence of ammonium did not impact on the onset of amino acid uptake overall, with only one exception: *T. delbrueckii* began its uptake of most amino acids later without the presence of ammonium than the other pure culture fermentations conducted using the strong fermenters. This result seems to correlate with fermentation kinetics as a lower CO_2 release was observed for *T. delbrueckii* fermented without ammonium, compared to with ammonium.

A lack of ammonium promoted the uptake of some amino acids for example, Pro for *S. cerevisiae*; Val, Ile and Phe for *P.* kluyveri; His and Val for *M. pulcherrima*; Asn for *L. thermotolerans* and Asp and Glu uptake for *T. delbrueckii*, most likely to compensate for the lack of ammonium. Val and Asn are usually taken up later by low-affinity SPS permeases and thus the absence of ammonium possibly led to their earlier activation (Schreve et al. 1998;

Crépin et al. 2012). A lack of ammonium for *S. cerevisiae* also led to a release of a number of amino acids after being taken up fully. It is possible that in order to compensate for the lack of ammonium, the yeast synthesised a number of amino acids *de novo*, stored or used what was needed and released the excess.

3.4.2.2 Order of uptake of amino acids

The earliest consumed amino acids for *S. cerevisiae* (within 18 h) were Asp, Glu, Arg and Lys. These were also early consumed amino acids in Crépin et al. (2012) under equal assimilable nitrogen source levels. Differences in rate of uptake were observed for Arg and ammonium in Crépin et al. (2012) when varying nitrogen sources levels were used. These early consumed amino acids were also deemed preferred sources in the study carried out by Jones and Pierce (1964), however arginine uptake rates were found to be dependent on yeast strain. Lys was the most rapidly consumed amino acid for both treatments for *S. cerevisiae* which is confirmatory of literature (Crépin et al. 2012).

The most rapid uptakes observed for *P. kluyveri* were those of Lys and Arg, and for *M. pulcherrima* were those of Lys, Arg, Glu and Gln. This rapid uptake of Lys is similar to that observed for *S. cerevisiae*. As a sole nitrogen source, lysine does not promote the growth of *S. cerevisiae*, however it has been able to promote the growth of various non-*Saccharomyces* yeasts when provided as a single nitrogen source, therefore, while *S. cerevisiae* cannot synthesise other amino acids with Lys as a sole nitrogen source, some non-*Saccharomyces* yeasts, including *Pichia* species and *M. pulcherrima*, show the potential for this to occur (Walters et al. 1953). It can therefore be hypothesised that *de novo* synthesis of amino acids occurred within the cell, as discussed in Crépin et al. (2017), possibly by using catabolised lysine in order to form proteinogenic amino acids which were then released extracellularly. The yeast then later took up the desired amino acids. However, this cannot account for the total level of amino acids released. Other possibilities include that the yeasts were not completely starved of nitrogen during the preculture in YNB medium and the amino acids still in the cell were released into the medium at inoculation or that protein degradation led to amino acid release. Arg was provided in much lower concentrations than are usually found in grape must-like media, therefore this could explain its rapid uptake for these yeasts. Rapid Glu and Gln uptake for *M. pulcherrima* could be attributed to this yeast's possible increased need for these amino

acids due to their central role in the *de novo* synthesis of other amino acids (Ljungdahl and Daignan-Fornier 2012). *M. pulcherrima* and *P. kluyveri* also released a great deal of amino acids before uptake began which suggests that the response to the stresses of inoculation of these yeasts led to a different pattern compared to the other yeasts tested.

All amino acids were fully taken up for both treatments for *L. thermotolerans* (except Asn as mentioned above), with the majority of amino acids being taken up within 18 h. Thus, this yeast has an especially fast uptake rate, even compared to *S. cerevisiae*. This was contrary to that observed in de Koker (2015), as it was found that *S. cerevisiae* showed the fastest uptake of alpha amino nitrogen, however in the current study, agitation of fermentations took place, but not in de Koker (2015)'s study, thus possibly accounting for this difference. *T. delbrueckii* also fully consumed all amino acids for both treatments, but the majority of amino acids were fully taken up by 24 h. It could be hypothesised that *L. thermotolerans* and *T. delbrueckii* could pose problems with regard to competition for nitrogen with *S. cerevisiae* due to their high uptake levels and rates, as demonstrated (for other yeast species) in Medina et al. (2012). However, this hypothesis needs to be confirmed when amino acids are present at grape must-like concentrations.

3.4.3 Competition for nitrogen sources and correlation with aroma compound production (grape must-like nitrogen source concentrations)

3.4.3.1 Competition for nitrogen sources and its effect on fermentation ability

M. pulcherrima and *P. kluyveri* took up the lowest amount of nitrogen sources under grape must-like concentrations compared to the other yeasts tested (as seen previously when nitrogen sources were present at equal assimilable nitrogen levels), leaving 54 and 41 mg/L YAN respectively in the must at the time of *S. cerevisiae* inoculation. These YAN levels were found to be adequate for fermentation after the inoculation of *S. cerevisiae*. *L. thermotolerans* and *T. delbrueckii* took up the majority of nitrogen sources (leaving only 6 and 2 mg/L YAN respectively) before *S. cerevisiae* inoculation. Sequential inoculations (no filtering) showed improved sugar consumption and CO_2 release compared to pure cultures (although they still had higher residual sugar concentrations compared to sequential fermentations with *M. pulcherrima* and *P. klyuveri*), however filtered sequential fermentations showed the poorest

fermentation kinetics. It can be hypothesised that the non-*Saccharomyces* yeast stored the assimilated nitrogen sources and when these yeasts died, they released these nitrogen sources back into the medium through autolysis, thus enabling *S. cerevisiae* to use these nitrogen sources to continue the fermentation process in the sequential fermentations (no filtering). Therefore, when these yeasts were filtered out of the medium before *S. cerevisiae* inoculation, these stored nutrients were also removed before they could be released into the medium for use by *S. cerevisiae*. Thus *T. delbrueckii* and *L. thermotolerans* show the highest potential for competition with *S. cerevisiae* due to their poorer fermentation kinetics observed.

3.4.3.2 Comparison of nitrogen uptake for different initial nitrogen source concentrations at 48 h

Differences regarding percentage uptake at 48 h between treatments with amino acids present at the same assimilable nitrogen levels and at grape must-like concentrations were observed for some amino acids per yeast strain. These could be due to the sometimes vastly different amounts of these amino acids (and ammonium) initially present in the must for the different treatments, for example Arg. The majority of this amino acid was taken up regardless of initial concentration (as it is an important nitrogen source shown to be stored in the vacuole (Kitamoto et al. 1988)) for *S. cerevisiae*, *L. thermotolerans* and *T. delbrueckii*. However, for *P. kluyveri* and *M. pulcherrima,* a saturation of uptake was observed for grape must-like Arg levels which indicated a lower requirement of this amino acid for these yeasts. The majority of Glu was taken up by all yeast strains tested. This amino acid plays a key role in the *de novo* synthesis of other amino acids (Ljungdahl and Daignan-Fornier 2012). *M. pulcherrima*, however, showed a lower uptake of Glu, as well as Ser at grape-must like concentrations, possibly due to higher concentration present of other preferred amino acids such as Arg. This is also possibly the case for *P. kluyveri*'s low uptake of His at grape must-like nitrogen concentrations. Gln can also lead to the production of other amino acids and is present in the grape must medium in high concentrations (compared to when amino acids are provided at equal assimilable nitrogen levels) and high uptake levels are observed for this amino acid in this study for all yeasts as this amino acid plays a critical role in nitrogen turn-over. Asn was generally taken up weakly or slowly for all yeasts and all treatments except for *T. delbrueckii* for nitrogen sources at equal assimilable nitrogen levels, possibly due to the compensation for another more preferred amino acid being present at lower levels.

3.4.3.3 Aroma compound production and the link with amino acid uptake and competition

At 48 h, the only higher alcohol that can be produced via the Ehrlich pathway to show a correlation with the uptake of its corresponding amino acid was propanol. *S. cerevisiae*, *L. thermotolerans* and *T. delbrueckii*, assimilated the highest levels of threonine and produced the highest levels of propanol. De Koker (2015) also found high propanol levels produced by *L. theromotolerans.* The correlation between amino acid uptake and propanol produced is further strengthened by Mouret et al. (2014) which found that propanol was not connected to the central carbon metabolism and its production rather directly correlated with the phase of nitrogen consumption, initial nitrogen concentration and intracellular threonine catabolism. The other higher alcohols tested, however, can be produced either via the CCM or the Ehrlich pathway (Crépin et al. 2017; Rollero et al. 2017; Belda et al. 2017). For instance, *M. pulcherrima*'s high production of phenylethanol compared to the other yeasts is consistent with results reported by Padilla et al. (2016), however this yeast's low uptake of phenylalanine suggests that this compound's production largely originates from the central carbon metabolism.

At end point, *M. pulcherrima* and *P. kluyveri* filtered sequential fermentations generally produced the highest higher alcohol and acetate ester levels, which can possibly be attributed to the fact that the non-*Saccharomyces* yeasts were no longer available to consume nitrogen, thus promoting the increased aroma compound production by *S. cerevisiae* (as competition for nitrogen sources was not particularly evident) and/or that their production originated from carbon metabolism. In most cases for *P. kluyveri* and *M. pulcherrima* sequential fermentations, more higher alcohols were produced compared to the pure culture of *S. cerevisiae* indicating that these yeasts remained active producing most of the higher alcohols. This is further suggested by propanol production where there is similar production for sequential fermentations and pure *S. cerevisiae. P. kluyveri* was the only yeast to produce 2-phenylethyl acetate in this study and literature confirms this yeast's high capacity for the production of esters (Padilla et al. 2016). *P. kluyveri* also showed the highest production of isoamyl acetate, which is a known attribute of this yeast, as reviewed in Padilla et al. (2016). Fermentations with *L. thermotolerans* and *T. delbrueckii* showed low aroma compound production in general compared to the other yeasts tested, possibly due to the competition for nitrogen sources as

previously determined, however when comparing fermentations treatments within these yeast species, non-filtered sequential fermentation showed the most favourable results, which

correlated with their previously determined more favourable fermentation kinetics. Benito et al. (2016) found that *L. thermotolerans* contributed to elevated 2-phenylethanol concentration while in sequential fermentation with *S. cerevisiae*, however the concentrations of this compound found in this study for the same treatment were low compared to the other yeast strains. *L. thermotolerans'*s characteristically high 2-phenylethyl acetate production levels were also not confirmed for the yeast strain in the current study compared to that found in Benito et al. (2016). These contradictions could possibly be a result of different strains being used. *T. delbrueckii* has also been noted as a high ester producer, however no acetate ester production was noted in this study for the esters tested (Padilla et al. 2016). This is possibly also due to strain variation.

3.5 Conclusion

This study confirmed the correlation between strong non-*Saccharomyces* fermenters and their higher nitrogen source uptake which can be detrimental to aroma compound production and lead to stuck or sluggish fermentations when *S. cerevisiae* is inoculated sequentially into the already nitrogen-depleted medium. Weaker non-*Saccharomyces* yeasts showed better fermentation kinetics and aroma compound production when *S. cerevisiae* was inoculated sequentially, therefore, nitrogen supplementation will be most required for sequential inoculations with *S. cerevisiae* and strong non-*Saccharomyces* fermenters. Medina et al. (2012), however, found that sequential inoculation of *S. cerevisiae* into *M. pulcherrima* fermentations resulted in more sluggish fermentations than co-inoculation of these yeasts due to nitrogen uptake by *M. pulcherrima* before *S. cerevisiae* inoculation, indicating that even slow fermenting non-*Saccharomyces* yeasts can pose a problem of competition. It could therefore be suggested that more nitrogen be added to the must for sequential fermentations, particularly for strong fermenting species and to a lesser extent for sequential fermentations with *M. pulcherrima* and *P. kluyveri.* Preferred nitrogen sources for the non-*Saccharomyces* yeasts should be supplied in higher concentrations to ensure that enough nitrogen sources are left for use by *S. cerevisiae* for successful fermentation.

Generally, the sequential fermentations which performed best for each yeast strain showed better aroma compound production compared to the pure culture of *S. cerevisiae*, indicating that non-*Saccharomyces* yeasts can indeed improve aroma compound production and therefore wine quality (Andorrà et al. 2012; Benito et al. 2016; Lachance 2016; Belda et al. 2017). The data also suggest that some higher alcohols are mostly produced from sugar metabolism rather than nitrogen metabolism in a species dependent manner. This study improved our general knowledge regarding uptake order and preference of nitrogen sources for various commercial non-*Saccharomyces* yeast strains, however the regulation of this uptake is not yet fully understood for these yeasts and more research should be conducted in this regard.

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Chapter 4

General discussion and conclusions

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4.1 General discussion

This study aimed to determine amino acid preferences of various commercial non-*Saccharomyces* yeasts, to investigate the effect of nitrogen source uptake on aroma compound production and fermentation ability and to determine whether competition for nitrogen occurs between *S. cerevisiae* and non-*Saccharomyces* yeasts in sequential fermentations. The various yeasts tested had different nitrogen source preferences (tested in synthetic grape must media with nitrogen sources present at equal assimilable nitrogen levels), however commonly preferred amino acids for all yeasts were Lys, Arg, Gln, Leu and Met. *L. thermotolerans* showed the fastest uptake of the majority of amino acids compared to the other yeasts, including *S. cerevisiae*. The effect of ammonium on the onset of amino acid uptake was negligible, except for *T. delbrueckii*, where the treatment without ammonium resulted in a later start of uptake for the majority of the amino acids, as well as slower and lower CO_2 release. A lack of ammonium also increased the % uptake of some amino acids for the various yeast strains, most likely to meet the overall nitrogen requirement. Non-*Saccharomyces* yeasts showed variation with regard to growth and fermentation kinetics in pure culture fermentations. Indeed, *L. thermotolerans* and *T. delbrueckii* were confirmed better fermenters than *P. kluyveri* and *M. pulcherrima* which translated into higher biomass formation and better fermentation efficiency as well as higher consumption of total sugars. These groupings confirm those of de Koker (2015). The stronger fermenters in the current study also showed higher levels of nitrogen source uptake compared to the weaker fermenters, as also found by de Koker (2015), which translated to a higher level of competition for nitrogen sources between the better fermenters and *S. cerevisiae* during sequential fermentations with grape must-like nitrogen source concentrations. It has previously been shown that nitrogen consumption by non-*Saccharomyces* yeasts before *S. cerevisiae* inoculation can lead to more sluggish fermentations as a result of nitrogen competition (Medina et al. 2012; Lleixà et al. 2016). In this study, this competition was confirmed and it further translated to aroma compound production. Indeed, *M. pulcherrima* and *P. kluyveri* produced the most positive aroma profiles in sequential inoculation as there was enough nitrogen left in the medium for further fermentation by *S. cerevisiae* as well as further aroma compound production following its inoculation. No such positive metabolic outcome could be identified for the other non-*Saccharomyces* yeasts tested.

However, the number of metabolites quantified in this study was very limited and their positive footprint could originate from the production of other metabolites. Indeed, *L. thermotolerans* in sequential inoculation with *S. cerevisiae* has been shown to produce elevated levels of other desirable compounds not tested in this study, for example ethyl lactate, therefore it is still a desirable yeast for winemaking (Benito et al. 2016). Other strains have also been shown to be high 2-phenylethanol and 2-phenylethyl acetate producers (Benito et al. 2016). *T. delbrueckii* has been shown to produce low levels of ethyl acetate (de Koker 2015) and high levels of esters (Padilla et al. 2016), many of which were not tested in this study. Overall, this study confirmed that non-*Saccharomyces* yeasts can indeed improve aroma compound production, particularly when *S. cerevisiae* is inoculated sequentially (Beckner Whitener et al. 2015; Benito et al. 2016; Padilla et al. 2016; Belda et al. 2017). In sequential fermentations, the exclusion of the non-*Saccharomyces* strong fermenters before *S. cerevisiae* inoculations led to poor aroma results as well as fermentation kinetics, thus it is likely that when these yeasts were removed, they took with them the nitrogen sources they had assimilated. It can therefore be hypothesised that when the non-*Saccharomyces* yeasts die, they release the nitrogen sources back into the medium for use by *S. cerevisiae*.

Considering these results, wine makers can be advised accordingly. It has been shown that sequential fermentations with non-*Saccharomyces* and *S. cerevisiae* yeasts can lead to desirable aroma compound production (Benito et al. 2016). Thus, non-*Saccharomyces* yeasts should be inoculated at the beginning of fermentation to ensure that they can produce desirable compounds without being outcompeted by *S. cerevisiae*. *S. cerevisiae* should then be inoculated sequentially. Minimal nitrogen supplementation is required for sequential inoculation with slow fermenting non-*Saccharomyces* yeasts as they do not take up most of the nitrogen sources, thus enough are left in the medium for use by *S. cerevisiae* to carry on the fermentation and for aroma compound production. Sequential fermentations with strong fermenting non-*Saccharomyces* yeasts may however require nitrogen source supplementation. This supplementation should mainly consist of branched-chain amino acids, as these not only promote growth, but also promote aroma compound production (McKinnon 2013), however these amino acids are not all preferred sources for the different yeast species, therefore amino acids deemed preferred by each yeast tested in this study should also be supplemented in order to ensure fermentation completion. Too high supplementation of branched-chain amino acids can lead to undesirable wine character, therefore the most optimal concentrations to add should be determined.

This study has certain limitations which should be highlighted. The sampling points used limit the amount of information which can be gathered. It is possible that amino acid uptake continues (to a lesser extent than observed in the first 48 h) beyond 48 h, however this was not investigated in this study. A large portion of amino acids was taken up between 6 and 18 h, thus time points in between these two should be added in order for a more accurate determination of order of uptake. This research was conducted using synthetic grape must media, which may not accurately reflect a real grape must situation. Only one strain per species was tested, thus the results found in this study may not be applicable for the entire species.

4.2 Future work

Further research should be conducted in order to strengthen these results. Sampling for determining amino acid uptake should occur more often within the first 18 h and also at time points after 48 h, especially for the weaker fermenters (*P. kluyveri* and *M. pulcherrima*) in order to determine whether uptake continues after this time point for these yeasts and whether full consumption occurs for other amino acids. Isotopic filiation could also be used in order to determine the fate of amino acids after uptake, as described in Rollero et al. (2017) and Crépin et al. (2017), for these yeast strains in order to determine which pathways each yeast favours, as well as confirm hypotheses suggested in this study with regard to the origin of higher alcohols (either from central carbon metabolism or nitrogen metabolism). These results could be used to determine the best nitrogen source supplementation regime per yeast species to ensure the best fermentation kinetics and aroma profiles of the resulting wine. More strains per species should be tested in order to determine the impact of strain variation of nitrogen source uptake and aroma compound production. For strong fermenting non-*Saccharomyces* yeasts, fully taken up amino acids (as determined in this study) could also be tested as sole nitrogen sources; however, these sources should be provided in abundance in order to determine the concentration at which uptake saturation takes place for each amino acid per yeast species. The concentration of each amino acid that needs to be supplemented in order to negate the effect of competition between yeasts could therefore be elucidated. Experiments in real grape must should be performed in order to determine the industrial accuracy of the results found in this study. Direct amino acid supplementation into the grape must is prohibited, however amino acids additions can be incorporated into yeast-derived products. Some of the preferred amino acids are the same across species (Lys, Arg, Gln, Leu and Met), thus perhaps a standard mix of amino acids for incorporation into yeast-derived products could

be developed for the majority of yeasts. If most of the preferred amino acids are included in this mix, yeast growth and fermentation performance could be improved as well as help to alleviate the concern for competition between yeast species. Additionally, if legislation regarding direct amino acid supplementations changed, adding preferred amino acids directly into the must would be beneficial.

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