The Impact of Amino Acids on Growth Performance and Major Volatile Compound Formation by Industrial Wine Yeast

By

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Declaration

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Summary

Nitrogen composition of grape must is highly variable and impacts on the health of the fermenting yeast population as well as the formation of aroma and flavour compounds in wine. Insufficient yeast assimilable nitrogen (YAN), mostly consisting of amino acids and ammonium, can lead to stuck or sluggish fermentations as well as the formation of undesirable compounds such as H_2S. Furthermore, it is well established that the total concentration of YAN and the specific amino acid composition have a significant impact on the final aroma and flavour of wines. However, the impact of individual amino acids and of specific amino acid compositions on fermentation kinetics and on the production of aroma and flavour impact compounds under winemaking conditions is not well understood.

The first goal of this study was to evaluate the effect of single amino acids on growth kinetics and major volatile production of two industrial wine yeast strains under conditions resembling wine fermentations. To facilitate these fermentation conditions while also allowing for easy reproducibility and manipulation of the initial components, a synthetic grape media was utilized. Biomass formation, exponential growth rate, lag phase, and fermentation duration were utilized to evaluate the efficiency of single amino acids.

The data show that previously observed trends in laboratory strains mostly apply to these conditions and strains. In general, the efficiency of amino acids to be used as nitrogen sources and the production of major volatiles due to their presence followed the same patterns for both industrial yeast strains. However, the production of the secondary metabolites butanol, propanol, acetic acid, and ethyl acetate were found to be produced in different final concentrations dependent upon the yeast strain.

The branched-chained and aromatic amino acids (BCAAs) treatments were observed to have the most dramatic effects on major volatile production. Investigating the relationships between the initial concentration of the BCAAs and the final concentrations of major volatile compounds, it was found that the production of fusel alcohols and fusel acids due to the degradation of BCAAs by *S. cerevisiae* could be predicted from the initial concentration of BCAAs. While under simple nitrogen conditions the production of several other secondary

metabolites such as butanol, propionic acid, valeric acid, decanoic acid and 2-phenylethyl acetate were found to be correlated to the initial concentration of BCAAs in the media.

Future studies should focus on the validation of these trends in aroma production in real grape musts under various fermentation temperatures for a number of industrial wine yeast strains.

Opsomming

Die stikstof samestelling van druiwemos is hoogs veranderlik en impakteer op die kerngesondheid van die fermenterende gis populasie asook die produksie van aroma- en geurverbindings in wyn. Onvoldoende assimileerbare stikstof (ook genoem “yeast assimilable nitrogen” (YAN)), wat meestal bestaan uit aminosure en ammonium, kan aanleiding gee tot steek- of slepende fermentasies, asook die vorming van ongewensde verbindings soos H_2S. Dit is alombekend dat die totale konsentrasie van YAN en dan ook die spesifieke aminosuur samestelling ‘n noemenswaardige impak op die finale aroma en smaak van wyn het. Die invloed van individuele am inosure en spesifieke aminosuur samestellings op die fermentasie kinetika, asook die produksie van verbindings met ‘n impak op die wyn aroma en smaak, word egter nie deeglik verstaan nie.

Die eerste doelwit van die studie was om twee industriële gisrasse te gebruik om die effek van enkel aminosure op die groei-kinetika en produksie van die vername vlugtige verbindings onder wynmaak toestande te bepaal. Kunsmatige, gedefinieerde druiwermosmedium is gebruik om wynmaak toestande te simuleer en ook herhaalbaarheid en manipulering van die aanvanklike samestelling van die medium te verseker.

Die studie het vorige tendense wat opgemerk is in die evaluasie van laboratorium rasse onder soortgelyke toestande bevestig. Die doeltreffendheid waarmee aminosure oor die algemeen gebruik word as stikstofbron, asook die produksie van die vernaamste vlugtige verbindings wat gekoppel is aan hulle teenwoordigheid, het ‘n vergelykbare patroon vir beide rasse gevolg. Die sekondêre metaboliete butanol, propanol, asetaat en etiel-asetaat is egter wel in verskillende eindkonsentrasies geproduseer deur die verskillende gisrasse.

Die vertakte-ketting en aromatiese aminosuur (“branched-chained and aromatic amino acids” (BCAAs)) behandelings het die mees dramatiese effek op die produksie van die vernaamste vlugtige komponente gehad. Ondersoek na die verwantskap tussen die aanvanklike konsentraies van die BCAAs en die finale konsentrasies van dié verbindings het aangedui dat die produksie van hoër alkohole en sure, as gevolg van die afbraak van BCAAs deur *S. cerevisiae,* met behulp van die aanvanklike konsentrasie van die BCAAs voorspel kon word. Terselfdertyd is gevind dat onder eenvoudige stikstoftoestande, verskeie ander

sekondêre metaboliete soos butanol, propionaat, valeriaat, dekanoësuur en 2-fenieletiel-asetaat, gekorreleer kan word met die aanvanklike BCAAs in die media.

Verdere studies moet poog om hierdie tendense ten opsigte van aromaproduksie te bevestig en wel deur gebruik te maak van ware druiwemos, verskeie fermentasietemperature, asook ’n verskeidenheid van wyngisrasse.

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Preface

This thesis is presented as a compilation of 4 chapters. Chapter 1 introduces the background and aims of this study. Chapter 2 provides an overview of the literature related to the topic of study. Chapters 3 will be submitted for publication and is written according to a general style. Chapter 4 overviews the main findings of the study and concludes the work.

Chapter 1	**General Introduction and project aims**
Chapter 2	**Literature review** *Saccharomyces cerevisiae* Amino Acid Catabolism
Chapter 3	**Research results** The Effect of Amino Acids on the Growth Kinetics and Major Volatile Formation of Industrial *S. cerevisiae* yeast.
Chapter 4	**General discussion and conclusions**

Contents

Chapter 1

General Introduction and Project Aims

Chapter 1 - General Introduction and Project Aims

1.1 Introduction

Wine is a complex chemical matrix and is the result of numerous metabolic and chemical reactions. The quality of wine is mostly judged based on its flavour and aroma profile. The conversion of grape must into wine is largely the result of the metabolic activity of the yeast *Saccharomyces cerevisiae*. The yeast utilizes grape must compounds for the production of many flavour and aroma impact compounds. These compounds are often divided into two categories; primary and secondary metabolites (Smit, 2013). The primary metabolites (ethanol, acetic acid, acetaldehyde, glycerol, and CO_2) are the direct result of the utilization of simple sugars (glucose, fructose, and sucrose) by fermentative metabolism. The secondary metabolites (higher alcohols, volatile acids, esters, and fatty acids) are as their name suggests the result of secondary metabolic functions. Both these groups of compounds have an impact on the final flavour and aroma of wine however, their production is largely unpredictable. Nitrogen compounds, specifically amino acids, have been observed to have a significant impact on the final concentration of both primary and secondary metabolite flavour and aroma impact compounds in wine (Hernández-Orte *et al.*, 2002; Hernández-Orte *et al.*, 2006; Miller *et al.*, 2007; Albers *et al.*, 1996; Vilanova *et al.*, 2007; Carrau *et al.*, 2008; Barbosa *et al.*, 2009).

Amino acids represent the largest group and most diverse group of yeast assimilable nitrogen (YAN) compounds found in grape must (Bell & Henschke, 2005). The composition of amino acids in wine is dependent upon vintage, cultivar, climate, and agricultural practices (Huang and Ough, 1991; Spayd & Andersen-Bagge, 1996; Hernández-Orte *et al.,* 1999). During the conversion of grape must to wine, the metabolism of amino acids by *S. cerevisiae* has been shown to be directly related to the production of secondary metabolites (Lambrechts & Pretorius, 2000; Styger *et al.*, 2011). The best studied of these are the branched-chain and aromatic amino acids (BCAAs); the degradation of these amino acids has been observed to result in the production of specific higher alcohols and volatile acids (Ough, 1964; Thomas & Ingledew, 1990; Garcia *et al.*, 1993 Hazelwood *et al.*, 2008) as well as the esters associated with the degradation of those higher alcohols (Saerens *et al.*, 2010; Verstrepen *et al.*, 2003).

Unfortunately, these studies concerning the impact of amino acids on the production of aroma compounds are often under conditions which are not reproducible (real grape must) or utilizing very complex nitrogen treatments. Due to these factors, it is problematic to interpret the results from a metabolic perspective.

Along with impacting on the final concentration of flavour and aroma compounds as the largest group of YAN compounds the amino acid composition of grape must is an important factor affecting the fermentation performance of *S. cerevisiae*. It has been found that the YAN concentration of grape musts is frequently the limiting factor for the completion of wine fermentations (Jiranek *et al.*, 1995; Varela *et al.*, 2004; Bell & Henschke, 2005). Amino acids are utilized by the cell as substrate for the production of structural and functional compounds required for the maintenance of a healthy yeast population (Ljungdahl & Daignan-Fornier, 2012). Insufficient YAN can result incomplete fermentation as well as the production undesirable compounds such as H_2S and an increased production of acetic acid (Bely *et al.*, 2003). Conversely, over supplementation of nitrogen in musts may increase microbial instability as the surplus of nitrogen can be utilized as a nutrient for the growth of spoilage organisms (Jiranek *et al.*, 1995). To prevent stuck or sluggish fermentation nitrogen is often added in the form of di-ammonium phosphate (DAP) to fermenting musts with little consideration for the impact on the final wine aroma.

Numerous studies have shown that not all amino acids are equally efficient in supporting *S. cerevisiae* growth. The evaluation of amino acids has been based on; (i) the generation time of yeast grown in minimal media containing a single amino acid as the source of nitrogen and (ii), the ability of some amino acids to repress the utilization of other nitrogen sources when supplied as mixture (Cooper, 1982; Godard *et al.,* 2007; Magasanik & Kaiser, 2002). The current consensus regarding the effectiveness of nitrogen sources is that NH_4^+, glutamate, glutamine, alanine, arginine, asparagine, aspartate, and serine are ‘good’ nitrogen sources, while the other amino acids are classified into different levels of inefficiency (Ljungdahl & Daignan-Fornier, 2012). These studies have been essential to our understanding of the metabolic impact of single amino acids, however, these studies are carried out utilizing laboratory yeast strains under conditions that are not observed under winemaking conditions.

To our knowledge there has yet to be a confirmational study to verify that the findings of these studies are valid under winemaking conditions for industrial wine yeast.

This study is part of a larger project at the Institute for Wine Biotechnology, Stellenbosch University investigating the effects of wine must constituents on the growth characteristics and aroma production by *S. cerevisiae*. It focused on the effect of initial amino acid concentration on the growth characteristics and major volatile compound formation under conditions that resembling wine fermentations utilizing industrial wine yeast strains.

1.2 Project Aims

The goal of this project was to investigate the effect of amino acids on the production of major volatile compounds in fermentations resembling wine conditions. The mains aims of the project were as follows:

1. To assess the effect of single amino acids on the growth kinetics of industrial wine yeast strains under conditions resembling wine fermentations.

2. To investigate the effect of yeast genetic background and single amino acids as the sole source of nitrogen on the final concentration of major volatile compounds.

3. To examine the robustness of correlations between final major volatile compound concentration and initial amino acid concentration in simple and complex nitrogen treatments.

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Chapter 2

Literature Review

Saccharomyces cerevisiae Amino Acid Catabolism

Chapter 2 - Literature Review

Saccharomyces cerevisiae Amino Acid Catabolism

2.1 Introduction

Nitrogen is one of the essential macronutrients required for the growth of *Saccharomyces cerevisiae* and is used by the cell for the synthesis of proteins, enzymes, and nucleic acids (Henschke & Jiranek, 1993). *S. cerevisiae* is able to synthesize all amino acids and nitrogenous compounds needed for growth provided sufficient YAN is present within the cell (Cooper, 1982). However, yeasts can also obtain amino acids from the external environment, making it more efficient at meeting its nitrogen requirements by reducing the amount of energy required for amino acid biosynthesis.

Though the total YAN concentration is important for fermentation performance and, all amino acids are required by the cell for the biosynthesis of nitrogenous compounds, not all nitrogen sources are equally efficient (Table 1) (Cooper 1982; Godard *et al.*, 2007; Ljungdahl & Daignan-Fornier, 2012). Not all amino acids can be used as the sole source of nitrogen as is the case for glycine, lysine, and histidine (Cooper, 1982). Furthermore, proline can only be utilized when sufficient oxygen is present for the function of degradation enzymes (Ingledew *et al.*, 1987). Currently, amino acids are classified as either ‘good’ or efficient nitrogen sources and ‘poor’ or less efficient nitrogen sources (Table 2.1) (Cooper, 1982). This classification is currently based upon the performance of yeast growth when a single amino acid is the sole nitrogen source, and secondly, on the ability of the amino acid to transcriptionally repress the genes related to the uptake and catabolism of other amino acids when present in a mixture of amino acids (Ljungdahl & Daignan-Fornier, 2012). Amino acids which inhibit the production of permeases and enzymes required for the assimilation and catabolism of other amino acids are considered preferred by the cell and therefore a more efficient source of nitrogen (Magasanik & Kaiser, 2002).

Table 2.1. Amino acids categorized in terms of preference based on the generation time when provided as the sole source of nitrogen and the ability to affect the nitrogen catabolite repression (NCR) system. Adapted from Ljundahl & Daignan-Fornier (2012)

Good	Intermediate	Poor	Non-Utilized
Alanine	Proline	Isoleucine	Glycine
Arginine	Valine	Leucine	Histidine
Asparagine	Phenylalanine	Methionine	Lysine
Aspartate		Threonine	
Glutamate		Tryptophan	
Glutamine		Tyrosine	
$NH4^+$			
Serine			

In the context of wine, amino acids make up the largest group of YAN compounds (Spayd & Andersen-Bagge, 1996; Bell & Henschke, 2005) and their metabolism results in the production of many flavour compounds important for the final sensory quality of wine (Styger *et al.,* 2011). This makes amino acids some of the most important nitrogenous compounds in wine fermentations. The amino acid content of grape must varies significantly depending upon vineyard location, agricultural practices and year with a general trend of the amino acids arginine and proline being in the highest concentrations (Ough & Bell, 1980; Huang & Ough, 1989; Huang & Ough, 1991; Spayd & Andersen-Bagge, 1996). Insufficient YAN can result in stuck or sluggish fermentations which do not complete or take an undesirable long time to complete (Bely *et al.*, 1990; Jiranek *et al.*, 1995; Vilanova *et al.,* 2007) resulting in financial losses. Standard practice for winemakers for the prevention of poor fermentation performance involves the analysis of grape must for the total YAN concentration, ignoring that not all sources of YAN are equal. Based on this measurement, if the musts are lacking in sufficient total YAN from this rudimentary measurement they are then supplemented with DAP (Gutiérrez *et al.,* 2012).

Advances in analytical techniques allow winemakers to rapidly obtain detailed analysis of the contents of their grape must. This technology, combined with a greater understanding of the effects of amino acids on the fermentation performance, would provide winemakers with another tool to help in the optimization of supplementation for the prevention of stuck or sluggish fermentations and aide in the prediction of the final composition of wines.

This review will summarize the molecular framework of amino acid utilization by the yeast *Saccharomyces cerevisiae* exploring how these compounds are taken up into the cell and utilized. The function and mode of action of the regulatory mechanisms will be discussed for uptake and catabolism. Additionally, the current amino acid classification system and possible improvements to this classification will also be discussed.

2.2 Amino Acid Permeases

Due to their size and chemical properties amino acids are unable to diffuse across the plasma membrane and therefore must be transported inside the cell. This is accomplished by a number of cell membrane transport proteins called amino acid permeases. These amino acid permeases make use of a H^+ gradient between the cell and the external environment to transport amino acids into the cell (Horak, 1997).

Amino acid permeases are divided into two categories, specific and general (Figure 2.1). The specific permeases are those which select for a single amino acid or a specific group of amino acids for transport. The possibility of a general permease for the uptake of neutral and basic nitrogen compounds to compliment the known specific permeases for some of the amino acids was first suggested by Grenson *et al.* (1970). Since then it has been confirmed that this general permease (Gap1p) is the sole general amino acid transporter and the only permease known to transport the amino acids alanine, and glycine (Crépin *et al.*, 2012). The specific amino acid permeases that are specific to a single amino acid are Lyp1p, Hip1p, Mup1p, Can1p, and Put4p for lysine, histidine, methionine, arginine, and proline respectively (Marini *et al.*, 1997). The other specific permeases transport groups of amino acids such as Tat1p and Tat2p for the aromatic amino acids, Bap2p and Bap3p for the branched-chain amino acids, Dip5p for the acidic amino acids, and Agp1p and Gnp1p for threonine, glutamine, and serine (Marini *et al.*, 1997).

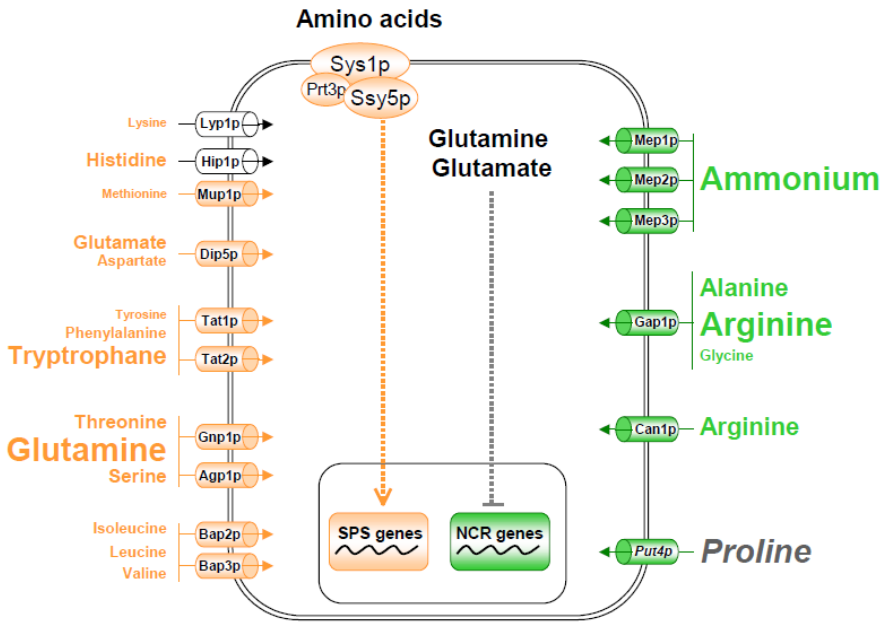

Figure 2.1. Amino acid permeases and their associated amino acids. Permeases shown in green are regulated by NCR and those shown in orange are regulated by the SPS adapted from Crépin *et al.,* (2012).

2.3 Permease Regulation:

The regulation of amino acid permeases is controlled by two unique and separate regulatory systems: the Ssy1p-Ptr3p-Ssy5p (SPS) and the nitrogen catabolite repression (NCR). The SPS is a plasma membrane sensing system which in addition to regulating genes associated with amino acid uptake is also responsible for the cell's ability to sense amino acids in the external environment (Forsberg *et al.,* 2001). NCR is also responsible for gene regulation but, in contrast to the SPS which is only involved in the regulation uptake, the NCR regulates uptake, utilization, and biosynthesis of amino acids. The NCR and SPS sensing system represent the main known regulatory systems for amino acid uptake. This section will summarize literature on these two regulatory systems.

2.3.1 Sensing Amino Acids in the External Environment

The SPS is a plasma membrane associated nutrient sensor system specifically for the sensing of extracellular amino acids and is involved in the regulation of the expression of several specific permeases and catabolic enzymes (Forsberg & Ljungdahl, 2001). This sensing system is made up of three unique plasma membrane proteins Ssy1p, Ptr3d, and Ssy5p. Ptr3d

and Ssy5p are located within the cell while the Ssy1p permease-like protein extends across the plasma membrane and into the external environment (Klasson *et al.*, 1999). This allows for the SPS system to interact with amino acids which are present in the external environment (Forsberg & Ljungdahl, 2001). In the presence of an environment containing amino acids, this sensor modulates the genetic expression of permeases related to the amino acids present. It does this by the activation of Stp1 and Stp2, two transcriptional factors which promote the expression of specific amino acid permeases (Ljungdahl & Daignan-Fornier, 2012).

The functioning and structure of this complex sensing system still remains to be fully understood and requires further study. Each component of the SPS sensing system undergoes conformational changes in response to the availability of amino acids in the external environment. When one of these components is dysfunctional the entire SPS becomes inactivated, thus supporting the hypothesis that cooperation between the three components of the SPS complex is required for it to function (Ljungdahl & Daignan-Fornier, 2012).

The regulation of the SPS system is part of a complex interaction network. It has been hypothesized that Ssy1p may function to regulate gene expression by detecting the intracellular and extracellular amino acid ratio (Ljungdahl & Daignan-Fornier, 2012).

It is thought that the major mechanism of regulation of the SPS system is via unidentified posttranslational mechanisms. These mechanisms are proposed to be similar to those involved in the regulation of other metabolite membrane transporters (Forsberg & Ljungdahl, 2001).

It is understood that the NCR and SPS are two separate regulatory systems as they are involved in the regulation of two separate groups of amino acid permeases (Crépin *et al.,* 2012). The specific permeases are under the regulation of SPS with the exception of the proline, arginine, and ammonium transporters. Therefore the cell is still able to assimilate amino acids even when the NCR is repressing the catabolism of less preferred amino acids and the functioning of the NCR regulated premeases. As a result the amino acid concentration of media is often exhausted well before the completion of fermentation (Crépin *et al.,* 2012). This suggests that the scavenging of various amino acids from the external environment regardless of the nitrogen demand of the cell or the quantity of good amino acids available is still desirable

for the cell perhaps to ensure sufficient nitrogen reserves are available for the cell when nutrients become scarce.

Finally, it has been observed that the general order of uptake is consistent for industrial and laboratory yeasts regardless of the concentration of amino acids (Jiranek *et al.* 1995; Crépin *et al.*, 2012). This suggests that the order of amino acid uptake is highly conserved and that the nitrogen content plays a very minor role in the regulation of amino acid uptake.

2.3.2 Nitrogen Catabolite Repression Regulation:

In contrast to SPS regulation which is involved with the sensing of external amino acids and regulation of several specific permeases, NCR is involved with the regulation of amino acid utilization and uptake (Cooper & Sumrada, 1983). The permeases which are under NCR control include: Gap1p general permease, Agp1p arginine permease, Put4p proline permease, and the ammonium permeases (Mep1p, Mep2p, and Mep3p) (Crépin *et al.*, 2012). Interestingly there is no overlap between NCR and SPS permease control and no hypotheses exist to explan this lack in overlap.

NCR is the most extensively studied of the transcriptional nitrogen regulatory systems (Cooper, 1982; Coffman *et al.*, 1997; Godard *et al.,* 2007; Magasanik & Kaiser, 2002; Georis *et al.*, 2009). As amino acids enter the cell they are incorporated into the cell's nitrogen pool where they can be used for the biosynthesis of larger polypeptides or catabolized (Ljungdahl & Daignan-Fornier). At this stage the NCR regulation of amino acid uptake and utilization becomes active. The current accepted model for NCR is that it functions to repress and de-repress the transcription of catabolic genes depending on the nitrogen requirements of the cell (Magasanik & Kaiser, 2002). In the presence of sufficient 'good' nitrogen sources the transcription of genes encoding amino acid transport and degradation proteins is repressed while at the same time the amino acid biosynthetic pathways are also repressed (Godard *et al.,* 2007). Conversely, when 'good' nitrogen sources become scarce the catabolic genes become de-repressed by the same system. NCR regulation is accomplished by the GATA transcriptional factors including Gln3p and Gat1p/Nil1p and the regulatory protein Ure2p (Coffman *et al.*, 1997). When preferred amino acids such as glutamine or glutamate are in abundance the

activators Gat1p and Gln3p are phosphorylated by a pair of Tor proteins (Tor1 and Tor2). After phosphorylation these activators form complexes with the gene Ure2p in the cytoplasm, resulting in an absence of transcription of amino acid transport and, degradation genes, and the repression of permeases under NCR control (Gap1p, Apg1p, Put4p, Mep1p, Mep2p, and Mep3p)(Magasanik & Kaiser, 2002). In the absence of glutamine or glutamate these genes become derepressed as Gln3p and Gat1p transcriptional activators are dephosphorylated and move to the nucleus, which prevents the formation of complexes between these proteins and Ure2p; thus, transcription of genes encoding for uptake and degradation proteins occurs (Magasanik & Kaiser, 2002).

NCR is known to be regulated by the presence of glutamine, glutamate, proline, urea, arginine, gamma-Aminobutryate, and allantoine within the cell (Hofman-Bang, 1999). There is also evidence that some BCAAs (leucine, phenylalanine, & methionine) have an effect on NCR mediated repression (Boer *et al.*, 2007). It is hypothesized that NCR may be regulated by one or many regulatory loops, accounting for the cell's capability to finely manage its response to changes in environmental conditions and cellular nitrogen demands (Coffman *et al.*, 1997). Like SPS, a solid foundation of knowledge exists for the NCR, however, we only have a partial understanding of the underlying mechanisms of this regulatory system and further investigation is required.

2.4 Amino Acid Utilization:

The current classification of amino acids is dependent upon the ability of the yeast to grow when an amino acid is used as the sole nitrogen source. This section will investigate the literature on the understanding of how amino acids are degraded and incorporated in metabolic processes.

2.4.1 Preferred Amino Acid Utilization

The preferred amino acids have been identified as glutamate, glutamine, arginine, asparagine, aspartate, alanine, and serine (Table 2.1) (Cooper, 1982; Godard *et al.,* 2007; Crépin *et al.*, 2012). Once inside the cell these amino acids can be converted into glutamate via

transamination of the amino group to cellular alpha-ketoglutarate or after being first deaminated to form ammonium (Horak, 1997) (Figure 2.2). Ammonium may also be transaminated with alpha-ketoglutarate or glutamate to form glutamate and glutamine, respectively. Conversely these reactions can be reversed to supply the TCA cycle with alpha-ketoglutarate and glutamate in the case of glutamine and ammonium and alpha-ketoglutarate in the case of glutamate (Cooper, 1982). Glutamate is used for the synthesis of all other amino acids while glutamine is largely required to for the synthesis of purines and pyrimidines, but may also be used as a precursor for the synthesis of asparagine and tryptophan (Cooper, 1982). One of the defining characteristics of preferred amino acids is that after their initial transamination or deamination reaction, their resulting carbon skeletons can be immediately incorporated into cellular metabolic pathways such as the TCA cycle (Godard *et al.,* 2007).

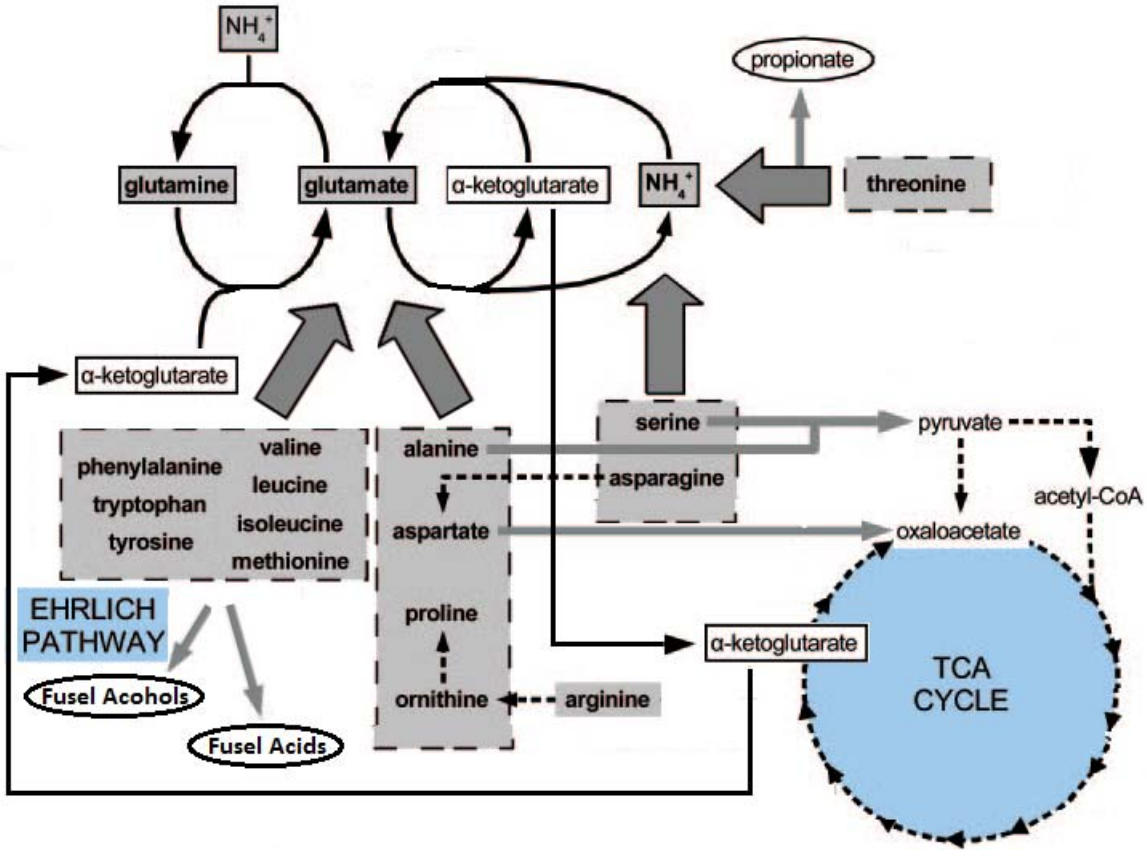

Figure 2.2 Simplified diagram of amino acid utilization by *Saccharomyces cerevisiae* adapted from Godard *et al.* (2007).

Arginine and asparagine are the exceptions to this rule. Unlike the other amino acids in this group which require a single step to complete degradation and be incorporated into metabolic pathways, these amino acids require a multistep degradation pathway.

Asparagine degradation has been shown to first require the breakdown of asparagine into aspartate and NH_4^+ by an asparaginase (Dunlop & Roon, 1975). The cell can then degrade aspartate via a transamination reaction to form α-ketoglutarate and glutamate as described above or, NH_4^+ can be converted into glutamate or glutamine by NADPH-dependent glutamate dehydrogenase and glutamine synthetase, respectively.

The degradation of arginine also requires a multistep reaction to complete. The accepted arginine degradation pathway by Brandriss & Magasanik (1980) is a five-step pathway where arginine is converted to ornithine and then to pyrroline-5-carboxylate (P5C). P5C is then converted to proline by a P5C reductase, proline is then converted to P5C by a proline oxidase, after which the reaction follows proline degradation to glutamate by P5C decarboxylase (Ljungdahl & Daignan-Fornier, 2012). Considering that degradation of proline is an integral step in the arginine catabolism pathway, and that proline is often reported as an intermediate nitrogen source, it is unclear why arginine is better source of nitrogen than proline.

These differences in degradation pathways between these amino acids suggest that the classification of amino acids is potentially more complex than the binary ‘good’ and ‘poor’ sources and the classification should include a greater number of criteria to give a better understanding of amino acid utilization.

2.4.2 Utilization of Non-Preferred Amino Acids

While much literature is present on the subject of preferred amino acids metabolism, there has been little literature to-date on less preferred amino acids. For the comparison of the effects of non-preferred amino acids on yeast metabolism, proline is almost exclusively used as a model for all non-preferred amino acids (Coffman, 1997; Cooper, 2002; Courchesne & Magasanik, 1988; Stanborough *et al.*, 1995). This is problematic as the non-preferred amino acids appear to be grouped together because of their exclusion from the preferred amino acid group; however, unlike the preferred group who share highly similar degradation pathways, these amino acids do not. Furthermore, yeast are capable of good growth when provided with proline as the sole nitrogen source when supplied with sufficient oxygen (Ingledew *et al.*, 1987). Considering these factors, the use of proline as a model for the growth of *S. cerevisiae* on non-

preferred amino acids is a poor choice. The most complete research on non-preferred amino acid degradation focuses solely on the production fusel alcohol from BCAA catabolism via the Ehrlich pathway. This research will be discussed in more detail in a later section in this review.

Excluding the BCAAs, the degradation pathways of the non-preferred amino acids appear to be unrelated. The first step in the catabolism of these amino acids appears to be a transamination reaction resulting in the formation of glutamate (Cooper, 1982; Godard *et al.*, 2007). However, unlike the preferred amino acids the resulting carbon skeleton are either unable to enter the TCA cycle as is the case for the preferred amino acids, or require further modification before entering the TCA cycle.

The threonine degradation pathway is linked to the biosynthesis of glycine (Liu *et al.*, 1997). Although glycine is not a viable single nitrogen source for yeast growth, like all amino acids it is important for the biosynthesis of larger polypeptides and is utilized by the cell. In addition to serving as a precursor for glycine biosynthesis, threonine is deaminated to form $NH4^+$ as part of its degradation pathway (Cooper, 1982).

Finally, histidine, glycine, and lysine degradation pathways have not been well characterized in *S. cerevisiae* as they do not support growth as the sole nitrogen source. Lysine degradation has been partially characterized in *S. cerevisiae* and completely characterized in several related fungi. It is hypothesized that lysine requires multiple enzymes for the completion of degradation to glutarate (Rothstein & Hart, 1964). These amino acids are unable to support growth individually as the sole nitrogen sources for *Saccharomyces cerevisiae* (Cooper, 1982; Godard *et al.*, 2007). Since these amino acids either do not support growth or give rise to stuck fermentations when supplied as the sole nitrogen source it is understandable why they have been neglected from research. Nevertheless, these compounds are taken up by the cell and utilized, thus knowledge of their role in the cell's metabolism would help to better understand the global amino acid metabolic network and how it is regulated.

2.4.3 Branched-Chain and Aromatic Amino Acid Utilization

The Ehrlich pathway proposes that fusel alcohol production is a result of amino acid catabolism (Ehrlich, 1907). This model makes up the bulk of research on non-preferred amino

acid metabolism and focuses almost exclusively on the formation of aroma impact compounds while neglecting the effect of these amino acids on yeast growth. The Ehrlich pathway has been validated as the source of fusel alcohol production due to BCAA degradation through the *in vivo* tracking of ^{13}C labelled amino acids (Dickinson *et al.,* 1997; Dickinson *et al.,* 1998; Dickinson *et al.*, 2000; Dickinson *et al.,* 2003; Lopez-Rituerto *et al.*, 2010). This section will give an overview of the pathway, including a detailed explanation of the intermediate steps.

2.4.3.2 The Ehrlich Degradation Pathway

The amino acids which are most often associated with the Ehrlich pathway are the branched-chain amino acids (isoleucine, leucine, & valine), the aromatic amino acids (phenylalanine, tyrosine, & tryptophan) as well as the sulphur containing amino acid methionine. The catabolism of each of these amino acids leads to the production of a fusel alcohol and a fusel acid unique to each amino acid (Hazelwood *et al.* 2008) (Figure 2.3). After which these fusel alcohols and acids are either excreted into the extracellular environment or first utilized for ester synthesis (Hazelwood *et al.* 2008). The presence of these alcohols, acids, and esters will then have an important impact on the final aroma and flavour of the wine (Lambrechts & Pretorius, 2000).

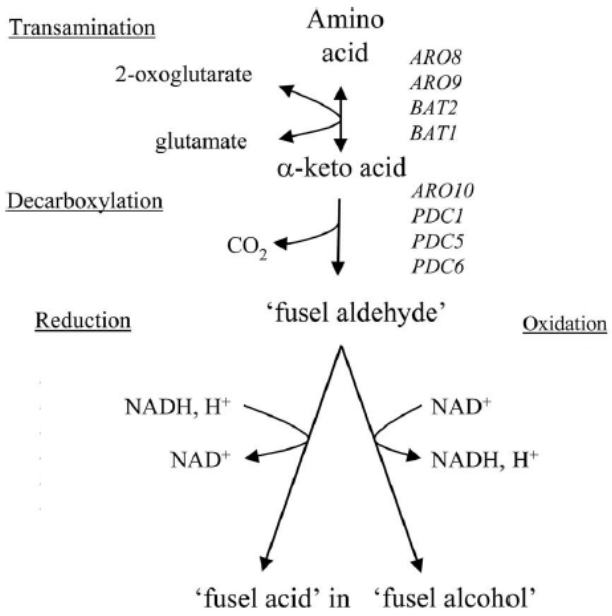

Figure 2.3 Degradation pathway of branched-chain and aromatic amino acid with associated transaminases and decarboxylases adapted from Hazelwood *et al. (*2008).

The first step in this reaction is the reversible transamination of the amino acid into the corresponding alpha-ketoacid. There are two aminotransferases associated with each of the amino acid groups. Regardless of the enzyme or amino acid the reaction is generally the same; the amino group from the amino acid is transferred to 2-oxoglutarate to form glutamate resulting in the production of each amino acid's associated alpha-keto acid (Table 2.2) (Sentheshanmuganathan *et al.*, 1960; Dickinson *et al.,* 2003).

The aromatic amino acids (phenylalanine, tyrosine, & tryptophan) are associated with two aminotransferases named aromatic aminotransferase I & II (also known as Aro8p & Aro9p respectively) (Kradofler *et al.*, 1982). It is unclear how these two aromatic aminotransferases interact; however, it may be that they act complimentary to one another. Aro9p has been shown to be responsible in the first step of amino acid catabolism (Kardofler *et al.,* 1982; Iraqui *et al.*, 1999). Conversely, Aro8p is the sole aminotransferase linked with aromatic amino acid biosynthesis, yet appears to have no role in the catabolism of these amino acids (Urrestarazu *et al.*, 1998).

For the initial step of the degradation of branched chain amino acids is the result of two associated branched-chain aminotransferases (BCAATs) which are respectively located in the mitochondria (Bat1p) and cytosol (Bat2p) (Dickinson *et al.*, 1997; Dickinson *et al.*, 1998). These BCAATs have been identified as encoded for by the genes ECA39 and ECA40 (now known as BAT1 and BAT2) for Bat1p and Bat2p respectively (Eden *et al.*, 2001). Unlike with the aromatic aminotransferases it appears that both BCAATs are involved in catabolism but at different stages of growth. There is evidence to show that Bat1p is more active during logarithmic growth, while Bat2p is more active during the stationary phase (Eden *et al.*, 2001). No theories exist as to why these aminotransferases appear in different regions of the cell or the link between their location and activity at different stages of growth.

The reversible transamination reaction is followed by the irreversible decarboxylation reaction the alpha-keto acid is converted into the corresponding fusel aldehyde releasing carbon dioxide (Table 2.2). There are 5 decarboxylases which have been shown to be associated with this step in the Ehrlich pathway (Hazelwood *et al.*, 2008). Unlike the

aminotransferases the majority of these decarboxylases are not unique for branched-chain or aromatic amino acids. Rather, there are three pyruvate decarboxylases encoding genes (PDC1, PDC5, & PDC6) and a single indol-3-pyruvate decarboxylase encoding gene (ARO10) which are general for all BCAAs in this pathway (Hazelwood *et al.*, 2008; Iraqui *et al.*, 1998). The 5th decarboxylase encoded by THI3 appears to be only specific decarboxylase and is associated with the decarboxylation of branched-chain amino acids. THI3 has been observed to be responsible for the catabolism of valine, isoleucine, and leucine, but it is not involved in decarboxylation of phenylalanine or tyrosine (Dickinson *et al.*, 2003).

Table 2.2 Branched-chain and aromatic amino acids and their associated α-keto acids, fusel aldehydes, fusel alcohols, and fusel acids. Adapted from Hazelwood *et al.* (2008)

Branched-Chain	α-Keto acid	Fusel Aldehyde	Fusel Alcohol	Fusel Acid
Isoleucine	α-Ketomethylvalerate	Methylvaleradehyde	Active Amyl Alcohol	Methylvalerate
Leucine	α-Ketoisocaproate	Isoamyl Aldehyde	Isoamyl Alcohol	Isovalerate
Valine	α-Ketoisovalerate	Isobutanal	Isobutanol	Isobutyrate
Aromatic				
Phenylalanine	Phenylpyruvate	2-Phenylacetaldehyde	2-Phenylethanol	2- Phenylacetate
Methionine	α-Keto-γ-(methylthio)butyrate	Methional	Methionol	3-(Methylthio)propanoate
Tryptophan	3-Indole pyruvate	3-Indole acetaldehyde	Tryptophol	ρ-Hydroxy-phenylacetate
Tyrosine	ρ-Hydroxyphenylpyruvate	2-(4-Hydroxyphenyl)ethanal	Tyrosol	2-(Indol-3-yl)ethanoate

Following decarboxylation two alternative pathways exist; in the first proposed pathway the fusel aldehyde is reduced to a fusel alcohol (Ehrlich, 1907). The other is the oxidation of the fusel aldehyde into a fusel acid (Dickinson *et al.,* 1997; Dickinson *et al.,* 1998; Dickinson *et al.*, 2000; Dickinson *et al.,* 2003). The ratio of production of fusel alcohol to acid appears to be correlated with the redox state of the cell (Hazelwood *et al.*, 2008). Cells which requires the regeneration of NAD^+ favours the reduction pathway of the fusel aldehyde, producing a higher ratio of fusel acid to fusel alcohol. Conversely where $NADH^+$ regeneration is required the oxidation reaction will be favoured giving rise to higher concentrations of fusel alcohol. This concludes the Ehrlich degradation pathway. From here the resulting fusel alcohols and fusel acids are either excreted in to the extracellular environment or further utilized for the synthesis of esters.

2.4.3.3 Ester Formation from Higher Alcohols

Esters are the largest group of aroma compounds and are produced intracellularly as a result of yeast's metabolism (Nykanen & Nykanen, 1977). They represent the final degradation step before excretion from the cell of many flavour important compounds. The majority of esters are produced enzymatically as will be described in further detail later in this section, but some ester production is also the result of an enzyme-free equilibrium reaction between an alcohol and an acid (Saerens *et al.*, 2008). Ester production is influenced by: strain, temperature, pH, unsaturated fatty acid levels, oxygen and substrate concentration (Styger *et al.,* 2011). Two groups of esters which are important in fermented beverages are acetate and ethyl esters, both of which are largely as the result of enzymatic production (Table 2.3). Due to the link between to the metabolism of BCAAs and the production of some esters this section has been included to give a complete overview of the degradation of these amino acids and their resulting by-products.

Table 2.3 Acetate and ethyl esters and their corresponding alcohol or fatty acid precursors. Adapted from Lambrechts & Pretorius (2000).

Acetate Esters	Alcohol
Ethyl acetate	Ethanol
Isoamyl acetate	Isoamyl alcohol
Isobutyl acetate	Isobutanol
2-Phenylethyl acetate	2-Phenylethanol
Ethyl Esters	**Fatty Acid**
Ethyl butanoate	Butyric acid
Ethyl hexanoate	Hexanoic acid
Ethyl octanoate	Octanoic acid
Ethyl decanoate	Decanoic acid
Ethyl dodecanoate	Dodecanoic acid

2.4.3.4 Acetate Ester Production

The production of esters as described earlier is the result of the interaction between an alcohol and an acid. For the production of acetate esters the reaction requires the presence of an acid provided in the form of acetyl-CoA while the alcohol is the direct result of the yeast's metabolism of carbohydrates, nitrogen, or lipids (Table 2.3) (Saerens *et al.*, 2008). The enzymatic formation of these acetate esters from higher alcohols is carried out exclusively by the two alcohol acetlytransferases (AATases) Atf1p and Atf2p (Verstrepen *et al.,* 2003). Large

differences in ester production have been observed between yeast strains (Rojas *et al.*, 2003). Differences have also been observed in the activity and reactions favoured by these two enzymes, and it has been hypothesized that these strain differences may be as a result of the differences in ratio of AATase produced (Verstrepen *et al.,* 2003), but has yet to be confirmed and is likely to be more complex.

2.4.3.5 Ethyl Ester Production

The second group important to fermented beverages, the ethyl esters, are largely formed from the combination of ethanol with a medium-chain fatty acid (Table 2.3) (Saerens *et al.*, 2010). The enzymes known to be responsible for this reaction are the acyl-CoA:ethanol *O*-acyltransferases Eeb1p and Eht1p (Saerens *et al.*, 2006). The level of expression of these enzymes is does not appear to have an effect on ethyl ester production, which appears to be mostly affected by the fermentation media composition and temperature (Saerens *et al.,* 2008).

2.4.3.6 Branched-Chain and Aromatic Amino Acid Utilization Conclusion

The formation of esters is a highly complex reaction that is currently the subject of much research due to their importance to the flavour profile of fermented beverages. The formation of these flavour compounds is thought to be the by-product of important cellular functions (Saerens *et al.,* 2010). It has be hypothesized that these compounds may be the result of a detoxification growth inhibitory compounds as a result of metabolism are modified for easy transport across the plasma membrane (Nordström, 1964). Another hypothesis suggests that the generation of ester allows for the generation or regeneration of compounds required for cell metabolism (unsaturated fatty acid analogues and acetyl-CoA, respectively) (Saerens *et al.,* 2010). Much remains to be understood regarding the formation of esters by *S. cerevisiae* and it is beyond the scope of this review to further discuss the cellular importance of ester formation.

2.5 Connecting Grape Must Amino Acid Composition to Wine Aroma

To contextualize the importance of the catabolism of amino acids by *S. cerevisiae* in wine fermentations, amino acids represent one of the largest and most diverse groups of nitrogenous

compounds found in grape musts (Crépin *et al.*, 2012). As an essential macronutrient required for yeast growth nitrogen and amino acids have been well documented as playing an important role in the health of fermentations. It has been reported by Bell and Henschke (2005) that the minimum nitrogen concentration to support healthy wine fermentations is approximately 10 mmol N L^{-1}. Grape musts which contain insufficient nitrogen concentration often resulting in stuck and sluggish fermentations or the production of off flavours (Bisson, 1999). The composition of amino acids between different grape musts has been observed to be highly variable (Table 2.4). This variability has been observed to be affected by grape variety, vintage, viticultural practices, and geographical location (Spayd & Andersen-Bagge, 1996; Jiranek *et al.*, 1995; Hernández-Orte *et al.*, 1999). Additionally, the metabolism of amino acids by *S. erevisiae* is understood to affect the formation of many wine aroma impact compounds (Rapp and Versini, 1991; Lambrechts & Pretorius, 2000; Styger *et al.*, 2011).

Table 2.4 Approximate wine grape composition of the majority of amino acids of Chardonnay, Sauvignon Blanc, Gernache, Merlot, Cabernet Sauvignon, and Pinot Noir grapes. Values reported in terms of mmol N L^{-1}.

Amino Acid	Chard.*	Chard.†	Sauv. Blanc*	Sauv. Blanc†	Gernache*	Gernache†	Merlot*	Merlot†	Cab. Sauv.*	Cab. Sauv.†	Pinot Noir*	Pinot Noir†
ALA	1.032	n/a	1.671	n/a	0.875	n/a	0.807	n/a	0.662	n/a	2.355	n/a
ASP	2.498	1.193	2.843	3.353	0.390	1.500	1.493	0.218	1.163	0.600	3.750	2.498
ARG	1.311	0.897	1.403	1.150	2.737	0.897	0.805	1.196	0.368	0.506	2.415	1.196
GLN	n/a	1.152	n/a	2.002	n/a	1.426	n/a	0.069	n/a	0.987	n/a	0.974
GLU	0.794	0.774	0.869	1.140	0.475	0.421	0.461	0.353	0.428	0.319	1.221	0.943
GLY	0.067	0.040	0.134	0.040	0.053	0.013	0.147	0.067	0.067	0.053	0.240	0.080
HIS	1.045	0.755	1.239	0.794	1.374	2.110	0.658	0.465	1.026	0.465	1.220	0.755
ILE	0.237	n/a	0.283	n/a	0.222	n/a	0.229	n/a	0.161	n/a	0.283	n/a
LEU	0.290	n/a	0.367	n/a	0.252	n/a	0.290	n/a	0.199	n/a	0.390	n/a
LYS	0.274	0.137	0.302	0.165	0.343	0.041	0.398	0.206	0.411	0.000	0.329	0.206
MET	0.114	0.067	0.074	0.060	0.060	0.201	0.128	0.074	0.081	0.295	0.161	0.087
PHE	0.261	0.170	0.304	0.219	0.231	0.067	0.206	0.115	0.115	0.115	0.243	0.146
PRO	8.488	3.643	4.566	3.067	0.000	2.109	13.978	6.431	15.381	14.971	2.667	1.037
SER	1.330	0.817	0.931	0.684	0.475	0.200	0.713	0.447	0.361	0.333	1.045	0.570
THR	0.885	0.556	0.877	0.582	0.438	0.160	0.514	0.303	0.337	0.337	1.188	0.809
TYR	0.160	n/a	0.143	n/a	0.105	n/a	0.121	n/a	0.132	n/a	0.193	n/a
VAL	1.054	n/a	1.089	n/a	0.617	n/a	1.183	n/a	1.183	n/a	0.857	n/a
TOTAL	**19.840**	**10.200**	**17.092**	**13.255**	**8.647**	**9.145**	**22.131**	**9.942**	**22.073**	**18.982**	**18.557**	**9.300**

* Spayd & Andersen-Bagge, 1996
† Hernandez-Orte *et al.*, 2002

Considering the importance of amino acids for the health of fermentations and as the precursors of aroma compounds, it is surprising how little literature is available exploring the relationships between the initial grape must composition and the production of aroma compounds due to yeast metabolism. To date, few studies exist which attempt to identify

meaningful trends between aroma production and amino acid content. Current literature includes treatments with nitrogen compositions in both real (Hernández-Orte *et al.*, 2006; Miller *et al.*, 2007; González-Marco *et al.*, 2010) and synthetic grape must (Hernández-Orte *et al.,* 2002; Barbosa, *et al.*, 2009). These studies have clearly shown that the nitrogen composition of a grape or synthetic must significantly effects the final concentration of aroma compounds. Unfortunately, due to the complexity of the nitrogen treatments used in experiements under fermentative conditions it is difficult to explain the effect of specific amino acids. Another stumbling block is that much of our understanding of the metabolism of amino acids by *S. cerevisiae* has been obtained from laboratory strains under non-fermentative conditions (Smit, 2013). To properly evaluate the impact of amino acids composition of grape must on the aroma profiles of wine first requires a re-evaluation of the metabolic effect of amino acids under conditions resembling wine fermentations using simple and repeatable nitrogen conditions. Only then can a proper evaluation of the relationships between amino acid composition of grape musts and the formation of aroma compounds from a metabolic perspective be done.

2.6 Conclusion

Nitrogenous compounds, specifically amino acids, represent some of the most diverse and versatile macronutrients essential for the growth of *S. cerevisiae*. Amino acids are a complex group of compounds which can vary greatly in their effect on cell regulatory systems, biosynthesis pathways, production of permeases and enzymes, and formation of metabolic by-products. Under fermentative growth conditions with sufficient supplies of carbon and other nutrients, they are often the growth limiting factor for final biomass production (Jiranek *et al.,* 1995) making them the most important nitrogen compounds for wine production. They furthermore provide the precursors for many aroma impact compounds. Due to this, understanding the effect of amino acids on the metabolism of *S.cerevisiae* is important for the understanding of the process of converting of grape must into wine.

The accepted classification of amino acid efficiency is partly based on the empirical measurement of their generation time when supplied as the sole nitrogen source. This classification is complimented with the ability of each amino acid to cause the transcriptional

repression of uptake and catabolism of other amino acids via the NCR (Ljungdahl & Daignan-Fornier, 2012). Based on this criteria, amino acids are poorly labelled as ‘good’ or ‘bad’ sources of nitrogen and do not adequately reflect their effect on yeast metabolism. For example: the branched-chain and aromatic amino acids could be grouped together as flavour precursor amino acids (Table 2.2; Table 2.3). The degradation of these amino acids results in the production of important flavour compounds (higher alcohols, volatile acids, and acetate esters).

As stated earlier, the current classification of amino acids is heavily weighted upon the regulation of amino acid utilization and uptake by the NCR system. This fits excellent for the grouping of the preferred amino acids, but leaves much to be desired for the classification of the non-preferred amino acids. Though the NCR controls the general amino acid permease and several specific permeases, the NCR is not the sole amino acid regulatory system and does not control all permeases (Figure 2.1). Those permeases which are not under NCR control fall under SPS control with no overlapping control between the two regulatory systems (Crépin *et al.,* 2012). The SPS regulates the uptake of amino acids into the cell and is responsible for the cell’s ability to sense amino acids in the external environment. This scavenging of less preferred amino acids in the presence of good amino acids suggests that though preferred amino acids are the most efficient under single nitrogen conditions, a diverse pool of amino acids is even more efficient for cell growth.

The uptake and utilization of amino acids is an essential function for the survival of yeasts. Without this the cell would rely solely on the biosynthesis of these compounds from other nitrogen sources. The cell’s ability to scavenge for these compounds potentially decreases its energy requirements due to a reduction of biosynthesis and is therefore more efficiently able to meet its nitrogen demands. A better understanding of the NCR and SPS regulatory systems and its role to detect nitrogen content in the external environment will give greater insights into how the cell operates. Increasing our understanding of the effect of amino acids on the metabolism of the yeast cell may allow for future winemakers to better predict the final wines being produced. This would be desirable as winemakers could then manage their fermentations more effectively by optimizing nitrogen supplementation of musts and possibly

managing the final flavour profile of their wines by applying knowledge of the effects of amino acids on flavour formation.

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Chapter 3

Research Results

The Effect of Amino Acids on the Growth Kinetics and Major Volatile Formation by Industrial *S. cerevisiae* yeast.

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The Effect of Amino Acids on the Growth Kinetics and Major Volatile Formation by Industrial *S. cerevisiae* yeast

3.1 Introduction

The aroma of wine is one of its defining attributes and an important factor for the determination of its quality. The formation of wine aroma is the result of a multitude of factors and can be affected at every stage of the winemaking process. As the primary organism for the fermentation of grape must into wine, *S. cerevisiae* plays an important role in the determination of the final wine aroma. *S. cerevisiae* contributes to the formation of aroma impact compounds in a variety of ways, through the biocontrol of other yeasts and bacteria, the *de novo* synthesis of aroma compounds, and the transformation of mostly grape-derived aroma neutral compounds into aroma active ones (Fleet, 2003). The group of grape must compounds which have been shown to have the largest effect on the health *S. cerevisiae* yeast and the concentrations of aroma impact compounds are the nitrogenous compounds.

In grape musts the YAN composition is largely made up of α-amino acids and ammonium with the total concentration YAN usually being less than optimal for the formation of sufficient biomass (Bell & Henschke, 2005; Vilanova *et al.*, 2007). Insufficient YAN concentrations have been strongly correlated to stuck or sluggish fermentations and the production of undesirable aroma and flavour compounds due nitrogen limitation (Bisson, 1999). It has been reported by literature that 10 mmol N L^{-1} is the minimum required YAN concentration to support a healthy yeast population and prevent stuck or sluggish fermentations under winemaking conditions (Bell & Henschke, 2005; Carrau *et al.,* 2008). To overcome these nitrogen limiting conditions it has become common practice to supplement grape musts with di-ammonium phosphate. While ammonium supplementation is successful for supplying yeast with sufficient nitrogen to complete fermentation, the supplementation of ammonium has been reported to result in increased levels of acetic acid and glycerol (Vilanova *et al.,* 2007). These findings suggest that excessive ammonium supplementation could also have adverse effects on the final wine quality.

The metabolic fate and efficiency of many amino acids have been studied and reviewed extensively (Cooper, 1982; Monteiro & Bisson, 1992; Godard *et al.*, 2007; Ljungdahl & Dignan-Fornier, 2012). The widely accepted classification of amino acid efficiency based on the generation time of yeast identified ammonium, alanine, arginine, asparagine, aspartate, glutamate, glutamine, and serine as good nitrogen sources and all other nitrogen sources as poorer nitrogen sources to varying degrees dependent on the study`s classification system. Additionally, under laboratory conditions it has also been observed that the degradation of certain amino acids, most notably, the BCAAs are directly responsible for the formation of the aroma compounds fusel alcohols and fusel acids (Hazelwood *et al*., 2008). These studies provide important foundational metabolic datasets for the interpretation of the impact of amino acid composition in wine. However, due to the use of laboratory strains and non-winemaking conditions verification of the reported observations of these studies is required before these results can be applied to industrial wine strains under winemaking conditions.

The impact of amino acid composition in real and synthetic grape musts has also been the focus of extensive studies (González-Marco *et al.*, 2010; Barbosa *et al.*, 2009; Garde-Cerdán & Ancín-Azpilicueta, 2007; Hernández-Orte *et al.*, 2006; Hernández-Orte *et al.*, 2002). While these studies are often less fundamental in nature, they provide insights into the complexity and importance of the effects of amino acid composition on both the fermentation kinetics and aroma formation. In addition to demonstrating that the addition of more complex mixtures of nitrogen sources improved fermentation performance, the findings of these studies have shown that the composition of amino acids is highly correlated with the final concentration of aroma compounds derived from yeast metabolism. Unfortunately, due to the complexity of the nitrogen treatments and the lack of reproducibility of media used by studies utilizing real grape musts only non-causative observations can be made concerning the changes in growth kinetics and aroma compound formation.

To our knowledge this is the first study to systematically study the utilization of specific amino acids for growth and aroma production using industrial wine yeast under winemaking conditions. Furthermore, this study provides a novel analysis of the growth kinetics and the

relationships between amino acid concentration and major volatile compound formation of industrial wine yeast strains under conditions resembling wine fermentations.

3.2 Materials and Methods

3.2.1 Yeast Strains and Preculture Conditions

Two industrial S*accharomyces cerevisiae* strains VIN 13 (Anchor Yeast, Cape Town, South Africa) and BM45 (Lallemand Inc., Montreal, Canada) were used. The preculture procedure for fermentation experiments was as follows: A single colony of yeast was added into 100 ml YPD incubated overnight with agitation at 30°C. Cultures were spun down and washed with sterile distilled water and added again to 100 ml YPD at an OD_{600nm} of 0.1 so that the inoculation rate was 10^6 cfu mL^{-1} and again incubated overnight at 30°C. After washing, the fermentation media was then inoculated at an OD_{600nm} of 0.1 or 10^6 cfu mL^{-1}.

Two media were used for this study; Yeast Nitrogen Base (YNB) without amino acids and ammonium (Difco Laboratories) and synthetic grape must (SGM) based on the medium described by Henschke and Jiranek (1993). All media contained 10% m/v glucose as a carbon source, and the initial pH of all media used was adjusted to 3.8 with KOH or HCl as necessary. The YNB media contained one of 19 amino acids or NH_4^+ as the sole source of yeast assimilable nitrogen (YAN) in the media at a concentration of 10.71 mmol N L^{-1} (Table 3.1). The SGM contained acids (3.0 g L^{-1} potassium hydrogen tartaric acid, 2.5 g L^{-1} l-malic acid, and 0.2 g L^{-1} citric acid), salts (1.14 g L^{-1} K_2HPO_4, 1.23 g L^{-1} $MgSO_4.7H_2O$, and 0.44 g L^{-1} $CaCl_2.2H_2O$), trace elements (200 µg L^{-1} $MnCl_2.4H_2O$, 135 µg/l $ZnCl_2$, 30 µg L^{-1} $FeCl_2$, 15 µg L^{-1} $CuCl_2$, 5 µg L^{-1} H_3BO_3, 30 µg L^{-1} $Co(NO_3)_2.6H_2O$, 25 µg L^{-1} NaMoO4.$2H_2O$, and 10 µg L^{-1} KIO_3), vitamins (100 mg L^{-1} myo-inositol, 2 mg L^{-1} pridoxine.HCl, 2 mg L^{-1} nicotinic acid, 1 mg L^{-1} Ca pantothenate, 0.5 mg L^{-1} thiamin.HCl, 0.2 mg L^{-1} para-aminobenzoic acid, 0.2 mg L^{-1} Riboflavin, 0.125 mg L^{-1} Biotin, and 0.2 mg L^{-1} Folic acid), anaerobic factors (10 mg L^{-1} Ergosterol and 0.5 mL L^{-1} l Tween 80), and nitrogen sources of varied concentration and composition (Table 3.2; Table 3.3). All fermentation treatments were done in triplicate, contained 10% m/v glucose as a carbon source and the initial pH of all media used was adjusted to 3.8 with KOH or HCl as necessary. The utilization of 10% glucose as the carbon source was to allow for rapid completion

fermentation to mimic conditions similar to wine conditions. The source of ammonium (NH_4^+) utilized throughout this study was ammonium sulphate.

Table 3.1 19 amino acids found in grape must and ammonium, categorized in terms of efficiency adapted from Ljundahl & Daignan-Fornier (2012)

Good	Intermediate	Poor	Non-Utilized
Alanine	Proline	Isoleucine	Glycine
Arginine	Valine	Leucine	Histidine
Asparagine	Phenylalanine	Methionine	Lysine
Aspartate		Threonine	
Glutamate		Tryptophan	
Glutamine		Tyrosine	
$NH4^+$			
Serine			

Table 3.2 Amino acid treatments containing different concentrations of a branched-chain or aromatic amino acid and supplemented with either NH_4^+ or alanine for a total YAN of 21.43 mmol N L^{-1}.

Treatment Name	Amino Acid mmol N L^{-1}	NH_4^+ mmol N L^{-1}	ALA mmol N L^{-1}	Total YAN mmol N L^{-1}
LEU				
21.43	21.43	0	0	21.43
14.28	14.28	7.14	0	21.43
7.14	7.14	14.28	0	21.43
ILE				
21.43	21.43	0	0	21.43
14.28	14.28	7.14	0	21.43
7.14	7.14	14.28	0	21.43
VAL				
21.43	21.43	0	0	21.43
14.28	14.28	7.14	0	21.43
7.14	7.14	14.28	0	21.43
PHE				
21.43	21.43	0	0	21.43
14.28	14.28	7.14	0	21.43
7.14	7.14	14.28	0	21.43
ALA + VAL				
7.14 + 14.28	14.28	0	7.14	21.43
14.28 + 7.14	7.14	0	14.28	21.43
ALA				
21.43	21.43	0	0	21.43
NH_4^+				
21.43	21.43	0	0	21.43

Table 3.3 Complex nitrogen treatments containing 14.28 mmol N L^{-1} of YAN for treatments in SGM media from work by Smit (2013) with nitrogen concentration converted from mg N L^{-1} and expressed in mmol N L^{-1}.

Amino Acid Treatment	Nitrogen Source	mmol N/L
Preferred	**Total**	**14.28**
	NH_4^+	3.57
	ARG	2.41
	ASN	2.41
	ASP	2.41
	GLN	2.41
	GLU	2.41
B&A (Branched-Chain and Aromatic)	**Total**	**14.28**
	NH_4^+	3.57
	ILE	2.41
	LEU	2.41
	PHE	2.41
	TYR	2.41
	VAL	2.41
Non-Preferred	**Total**	**14.28**
	NH_4^+	3.57
	ALA	2.41
	GLY	2.41
	SER	2.41
	THR	2.41
	TRP	2.41
Non-Utilized	**Total**	**14.28**
	NH_4^+	3.57
	HIS	3.57
	LYS	3.57
	PRO	3.57

3.2.2 Sampling

All fermentations were sampled in duplicate at endpoint for the analysis of major volatile compounds. For the creation of growth curves sampling under sterile conditions was carried out every 2 to 4 hours for the first 20 hours followed by every 6 to 24 hours until weight loss ceased. This point was assumed as the end of sugar consumption. During this period optical density (OD) of the fermentations were measured at 600nm in triplicate for each flask for each time point to determine biomass formation.

3.2.3 Measurement of Metabolites and Fermentation Parameters

Growth curves were constructed for each treatment from the averaged values of each sampling point. From these growth curves the average values with <10% error were obtained for biomass, exponential growth rate, lag phase, and fermentation time for each treatment.

3.2.3.1 Biomass Determination

Fermentations were sampled in triplicate every 8 hours. 2 mL of fermentation medium was aseptically removed from the fermentation vessel, spun down at 5000 rpm for 5 min, and supernatant was removed. Pellets were resuspended in distilled water, centrifuged and washed again. Samples were dried for 48 hours at 100^{o}C and weighed. To obtain a calibration curve for the biomass, the weight of the cell sample was plotted against the OD reading at each of these sampling points.

3.2.3.2 Exponential Growth Rate and Lag Phase Determination

The average exponential growth rate was determined by performing a semi-log transformation the growth curve of each single amino acid treatment. From this transformed growth curve the linear section was isolated and a trendline was created. The slope of this trendline was used as the value for the rate of exponential growth. Since the lag phase end once the exponential growth phase begins the average lag phase was assumed to stop at the first point of the linear section of the transformed growth curve.

3.2.3.3 Determination of Time to Complete Fermentation

Fermentation vessels were weighed every 24 hours until no further weight loss was experienced. To confirm that fermentation had gone to dryness sugar levels were determined through enzymatic analysis.

3.2.3.4 Gas Chromatographic Analysis of Major Volatile Compounds

Major volatiles were extracted from fermentation samples via a modified liquid-liquid extraction as described by Louw *et al.* (2009) for the analysis by gas chromatography. For each 5 mL sample of media 100 µl of internal standard (4-methyl-2-pentanol) and 1 mL of solvent (diethyl ether) were added and then mixture was then placed in an ultrasonic bath for 5 minutes to facilitate extraction. The mixture was then centrifuged for 3 min at 4000 rpm after which Na_2SO_4 was added to remove any water from the non-polar layer and the sample was again centrifuged for another 3 min at 4000 rpm.

The quantification of major volatiles was carried out by gas chromatography with a flame ionization detector (GC-FID) using a Hewlett Packard 6890 Plus gas chromatograph (Agilent, Little Falls, Wilmington, USA), with a split/splitless injector. The split flow rate was set at 49.4 ml/min and the split ratio was set to 15:1 at a temperature of 200^{o}C. The separation of compounds was done using a J&B DBFFAP capillary GC column (Agilent, Little Falls, Wilmington, USA) with the dimensions of 60 m x 0.32 mm and a 0.5 µl coating film thickness with the flow rate of the hydrogen carrier gas set at 3.3 ml/min. Once the FID oven temperature reached the temperature of 240^{o}C 3 µl of extracted sample was injected into the gas chromatograph at an initial temperature of 33^{o}C and held for 8 min; the temperature was then increased by 21^{o}C/min to 130^{o}C and then held for 17 min; increased by 12^{o}C/min to 170^{o}C and held for 5 min; increased by 21^{o}C/min to 240^{o}C and held for 2.5 min. A post run step at the end of each sample was carried out at 240^{o}C for 5 min. Each sample was injected in duplicate. Approximately every 20 samples the column was cleaned by the injection of hexane into the GC-FID with a run time of 10 min. Manual data collection and peak integration was done using the HP ChemStations software (Rev. B01.03 [204]).

3.2.3.5 Measurement of pH

The pH of the fermentation media was measured using a Crison Basic 20 pH meter with a 50 11 T pH electrode (Crison Instruments, S.A., Barcelona, Spain). The values were then averaged for the treatment.

3.2.3.6 Measurement of glucose concentration

For the determination of d-glucose concentration 2 mL of filtered sample was analyzed using the Arena 20TX photometric analyzer (Thermo Electron Oy, Finland) to measure the concentration of NADH at the ultraviolet wavelength of 340 nm. This method is based on the reaction between hexokinase and D-glucose as depicted by the following reaction:

D-Glucose + ATP <---Hexokinase---> Glucose-6-phosphate + ADP

Glucose-6-phosphate + NAD^{+} <---G6P-Dehyrogenase---> Gluconate-6-Phosphate + NADH + H^{+}

3.2.3.7 Prediction of theoretical major volatile production due to initial amino acid concentration

The prediction of volatile compounds was calculated by plotting the linear slope of the final molar concentration of major volatile compounds vs. initial molar concentration of specific amino acid concentrations of media (Figure 3.1). It was assumed that the linear correlation between the initial amino acid concentration and final major volatile compound concentration remained constant when the initial concentration of amino acids was lower than the parameters of the those found in Table 3.2. By extrapolating the trend from this linear slope, the final major volatile compound concentrations were predicted (Table 3.3)

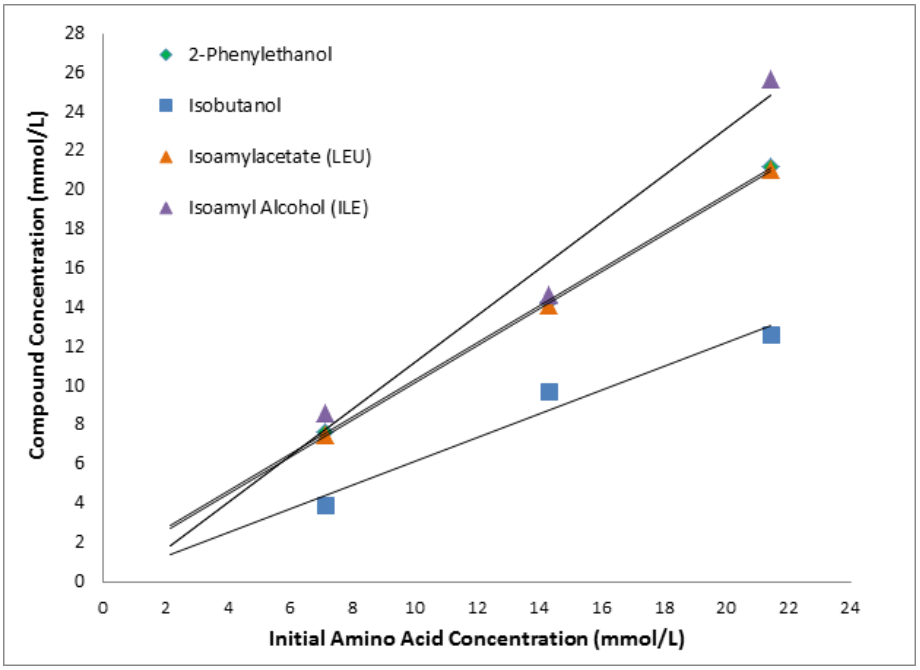

Figure 3.1 Extrapolation graph for the prediction of final higher alcohol concentration from the initial BCAA concentration based on linear correlations observed between major volatile formation and initial amino acid concentration.

3.3 Results

3.3.1 Classification of Amino Acids as Sole Nitrogen Source in wine yeast strains under fermentative conditions

Amino acids as the sole source of nitrogen were evaluated for their ability to support growth in *S. cerevisiae*. Two yeast strains VIN 13 and BM45 previously shown by Rossouw (2009) to have divergent growth characteristics were used. These two strains were grown in YNB media to characterize growth kinetics when grown with one of 19

amino acids as a sole source of nitrogen at 10.71 mmol N L^{-1}. Fermentations were monitored by taking OD_{600nm} measurements and weight loss measurements to obtain lag phase, exponential grow rate, length of fermentation and biomass formation for the evaluation of growth kinetics. All treatments with the exception of those containing tryptophan were observed to go to dryness (less than 4 g/L residual sugar). Significant differences in growth kinetics were observed due to both amino acid treatment and yeast strain with the amino acid treatments having the most dramatic effect.

3.3.1.1 Biomass Formation

High biomass formation was obtained for the aspartate, alanine, glutamine, glutamate, phenylalanine, serine, and valine treatments for both yeast strains, while tryptophan, threonine, and asparagine treatments gave the poorest biomass formation for both yeast strains (Figure 3.1). The treatments which gave rise to lower final biomasses include arginine, isoleucine, tyrosine, methionine, ammonium, proline, and leucine. Strain differences in final biomass production was noted for the arginine, isoleucine, tyrosine, methionine, and NH_4^+ treatments with VIN 13 giving rise to lower biomasses. Differences in biomass formation between strains were found to be much smaller at the end of fermentation as compared to 24 hours after inoculation. Glutamate was observed to give rise to one of the largest differences in after 24 hours with VIN 13 having a much higher biomass than BM45, however no differences were found between the yeast strains at the end of fermentation. In general those treatments which were found to give high biomass after 24 hours were also found to give rise to the highest final biomasses. Exceptions to this were the proline, phenylalanine, valine treatments. Proline was found to give some of the highest biomass after 24 hours, however, at the end of fermentation it was found have one of the lowest biomasses. This is likely due to the presence of dissolved oxygen in the media which is consumed during the first 24 hours. Conversely, phenylalanine and valine were found to be give intermediate biomass formation after 24 hours and at the end of fermentation were found to be the treatments which had some of the highest biomasses. Differences in biomass formation were found to be smaller between treatments at the end of fermentation as compared to after 24 hours after inoculation. The glycine, histidine, and lysine

treatments gave no biomass formation for VIN 13 or BM45 after 24 hours and were excluded from further experiments.

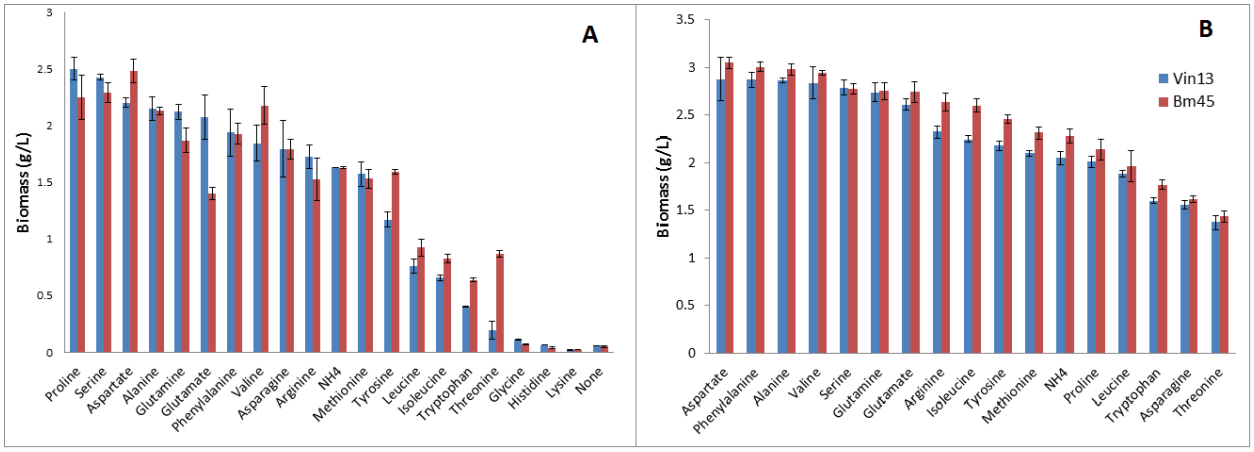

Figure 3.1 Biomass formation of VIN 13 and BM45 yeast strains in YNB media containing 10% glucose and 10.71 mmol N L^{-1} of a single nitrogen source 24 hours after inoculation (A) and at the end of fermentaion (B).

3.3.1.2 Exponential Growth Rate

High exponential growth rates for the glutamine, arginine, glutamate, serine and aspartate treatments were found for both strains (Figure 3.2). The highest growth rate was observed with the BM45 strain in the glutamate treatment. High rates of growth were also observed in the asparagine and alanine treatments for VIN 13 and BM45, respectively. Low growth rates were observed in both strains for NH_4^+, threonine, proline, methionine, leucine, tyrosine, isoleucine, and tryptophan treatments with slightly higher values observed for the NH_4^+ and threonine treatments with VIN 13. Treatments which were observed to have intermediate growth rates included: phenylalanine and valine for both strains with BM45 giving higher growth rates for both treatments. Also included in the intermediate growth group are asparagine and alanine for BM45 and VIN 13, respectively.

3.3.2.1.3 Time to Complete Fermentation

The glutamine and arginine treatments gave the shortest fermentation duration, completing after 72 hours (Figure 3.2). Glutamate, alanine, aspartate, serine, phenylalanine, and valine treatments also lead to short fermentation times reaching completion after 81 hours. Fermentations with long duration were those lasting longer than 100 hours. Included in this

group were tyrosine, isoleucine, leucine, methionine, tryptophan, NH_4^+, proline, and threonine treatments which took between 105 to 120 hours to complete. Also included in the long fermentation group was the asparagine treatment which had the longest duration of 169 hours.

3.3.1.4 Duration of Lag Phase

The amino acid treatments with the shortest lag phase included glutamate, glutamine, alanine, asparagine, aspartate, arginine, phenylalanine, serine, valine, threonine, and NH_4^+ with glutamate having the shortest lag phase in both strains (Figure 3.2). While proline, tyrosine, methionine, leucine isoleucine, and tryptophan treatments gave rise to long lag phases. Strain differences were noted with threonine, isoleucine, and tryptophan having longer lag phases for VIN 13 with isoleucine and tryptophan having the longest lag phase for this strain. In the case of BM45 leucine was observed to give the longest lag phase.

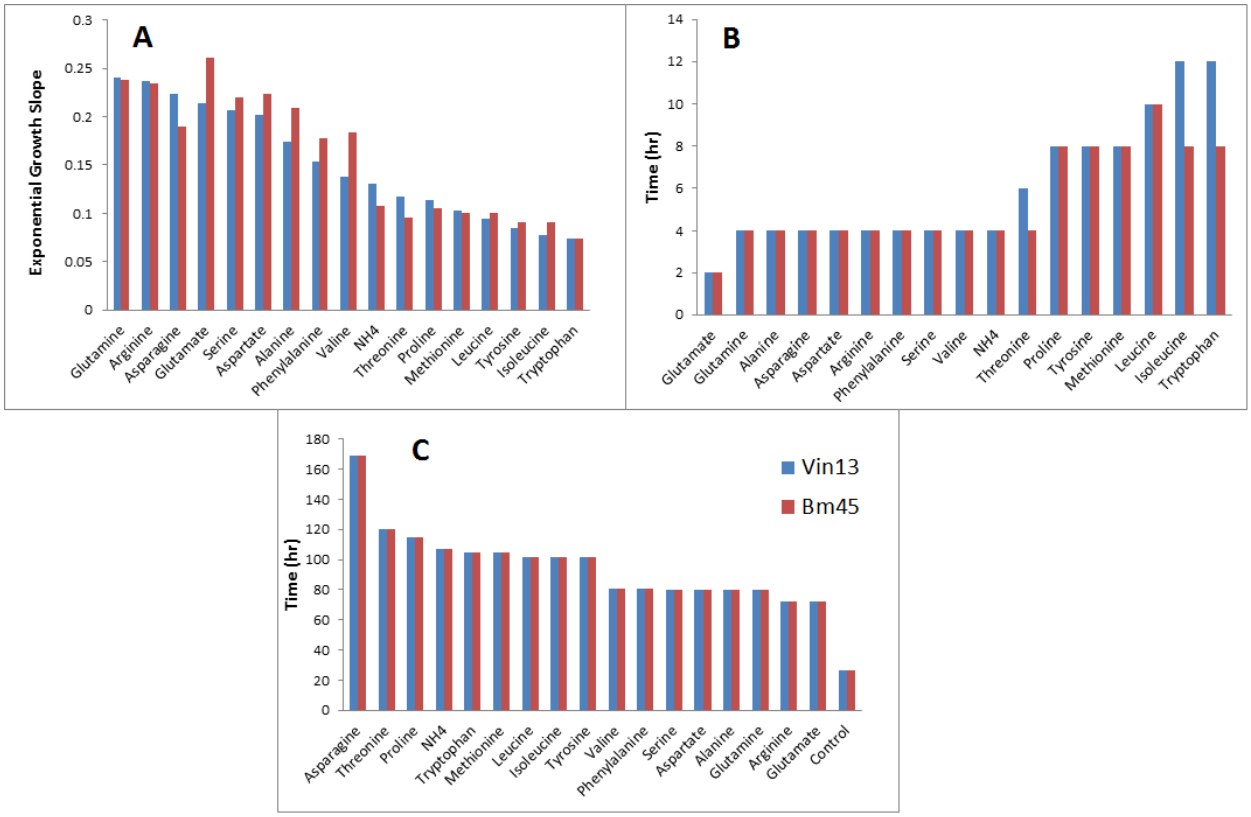

Figure 3.2 Average (< 10% error) growth characteristic values for exponential growth rate (A), time to complete fermentation (B) and assumed duration of lag phase (C) of VIN 13 and BM45 grown in YNB containing 10% glucose and 10.71 mmol N L^{-1} of a single amino acid as the sole source of nitrogen.

Table 3.4 Tabulated characteristics of the growth parameters from Figure 3.2 including for the yeast VIN 13 grown in YNB media containing one of 17 nitrogen source as the sole source of nitrogen at a concentration of 10.71 mmol N L^{-1}.

Nitrogen Source	Biomass Formation (g L^{-1})	Exponential Growth Rate	Lag Phase (hr)	Fermentation Duration (hr)
Efficient				
Alanine	2.87	0.175	4	80
Aspartate	2.88	0.202	4	80
Glutamate	2.61	0.214	2	72
Glutamine	2.74	0.240	4	80
Serine	2.79	0.219	4	80
Intermediate				
Arginine	2.32	0.236	4	72
Phenylalanine	2.87	0.153	4	80
Valine	2.84	0.138	4	80
Inefficient				
Asparagine	1.55	0.224	4	169
NH_4^+	2.05	0.131	4	107
Threonine	1.37	0.118	6	120
Proline	2.01	0.114	8	115
Methionine	2.10	0.103	8	105
Leucine	1.88	0.094	10	102
Tyrosine	2.18	0.085	8	102
Isoleucine	2.45	0.078	12	102
Tryptophan	1.60	0.074	12	105

Table 3.5 Tabulated characteristics of the growth parameters from Figure 3.2 including for the yeast BM45 grown in YNB media containing one of 17 nitrogen source as the sole source of nitrogen at a concentration of 10.71 mmol N L^{-1}.

Nitrogen Source	Biomass Formation (g L^{-1})	Exponential Growth Rate	Lag Phase (hr)	Fermentation Duration (hr)
Efficient				
Alanine	2.98	0.209	4	80
Aspartate	3.05	0.224	4	80
Glutamate	2.74	0.261	2	72
Glutamine	2.75	0.238	4	80
Serine	2.78	0.219	4	80
Intermediate				
Arginine	2.64	0.234	4	72
Phenylalanine	3.01	0.178	4	80
Valine	2.95	0.183	4	80
Inefficient				
Asparagine	1.62	0.190	4	169
NH_4^+	2.28	0.108	4	107
Threonine	1.43	0.095	4	120
Proline	2.14	0.106	8	115
Methionine	2.31	0.100	8	105
Leucine	1.97	0.100	10	102
Tyrosine	2.45	0.091	8	102
Isoleucine	2.60	0.091	8	102
Tryptophan	1.77	0.074	8	105

3.3.2 Major Volatile Compound Analysis at the end of fermentation

The concentration of major volatile compounds at the end of fermentation was analyzed for the treatments containing a single amino acid as the sole nitrogen source. In general, few strain differences were observed for the production of major volatiles with the general trends of volatile production being conserved. The production of acetic acid and ethyl acetate appeared to be positively correlated as they followed the same relative trends in production regardless of treatment effect. Furthermore, many aroma compounds were observed to be produced in high concentration by specific amino acid treatments.

Those treatments containing BCAAs as the sole source of nitrogen yielded the highest observed concentration of major volatiles. For these treatments, high levels of fusel alcohols and fusel acids, specifically; isobutanol, and isobutyric acid for the valine treatment (Figure 3.3), isoamyl alcohol, isovaleric acid, and isoamyl acetate for the isoleucine and leucine treatments (Figure 3.4), and 2-phenylethanol and ethyl phenylacetate for the phenylalanine treatment (Figure 3.5) with the corresponding fusel alcohol always present in higher concentrations than the fusel acid. The other amino acid treatments were not observed to give rise to significant amounts of these higher alcohols or higher acids.

Other treatments with strong effects on the formation of major volatiles are threonine, proline, and NH_4^+ (Figure 3.6). The threonine treatment gave rise to the highest levels of butanol and propionic acid, moderate levels of isoamyl alcohol, isovaleric acid, acetic acid, and ethyl acetate. The proline treatment resulted in the production of high concentrations of propanol and ethyl acetate by the yeast, and the NH_4^+ treatment led to high acetic acid and ethyl acetate concentrations. Along with producing high levels of isobutanol and isobutyric acid the valine treatment was observed to give relatively high levels of propanol, acetic acid, and ethyl acetate with VIN 13 producing higher levels of each of these compounds (Figure 3.6). Other strain differences observed include higher propanol levels produced by all VIN 13 treatments. Also, the production of acetic acid was highest for BM45 treatments with the exception of the treatment containing valine as the only nitrogen source.

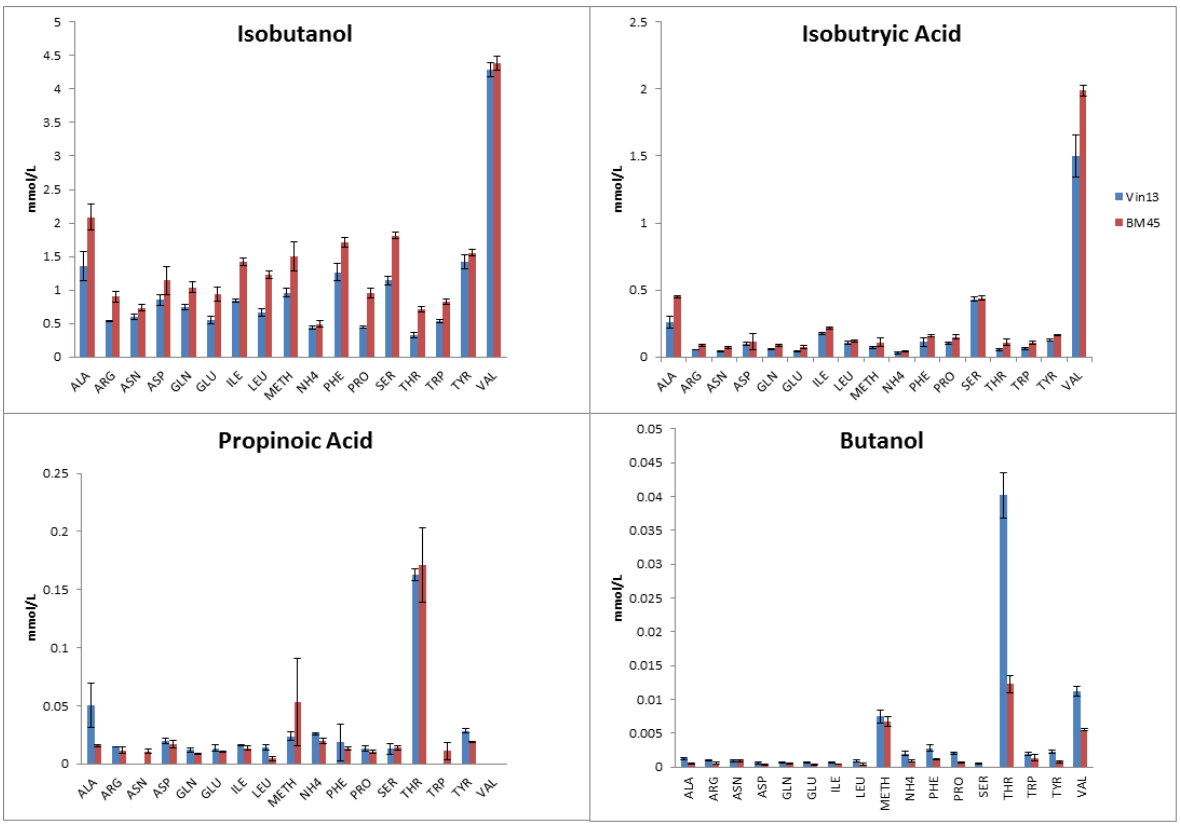

Figure 3.3 GC-FID analysis of volatile compounds related to valine and threonine metabolism, produced by BM45 and VIN 13, measured at the end of fermentation in YNB with 10% glucose and 10.71 mmol N L^{-1} of a single nitrogen source.

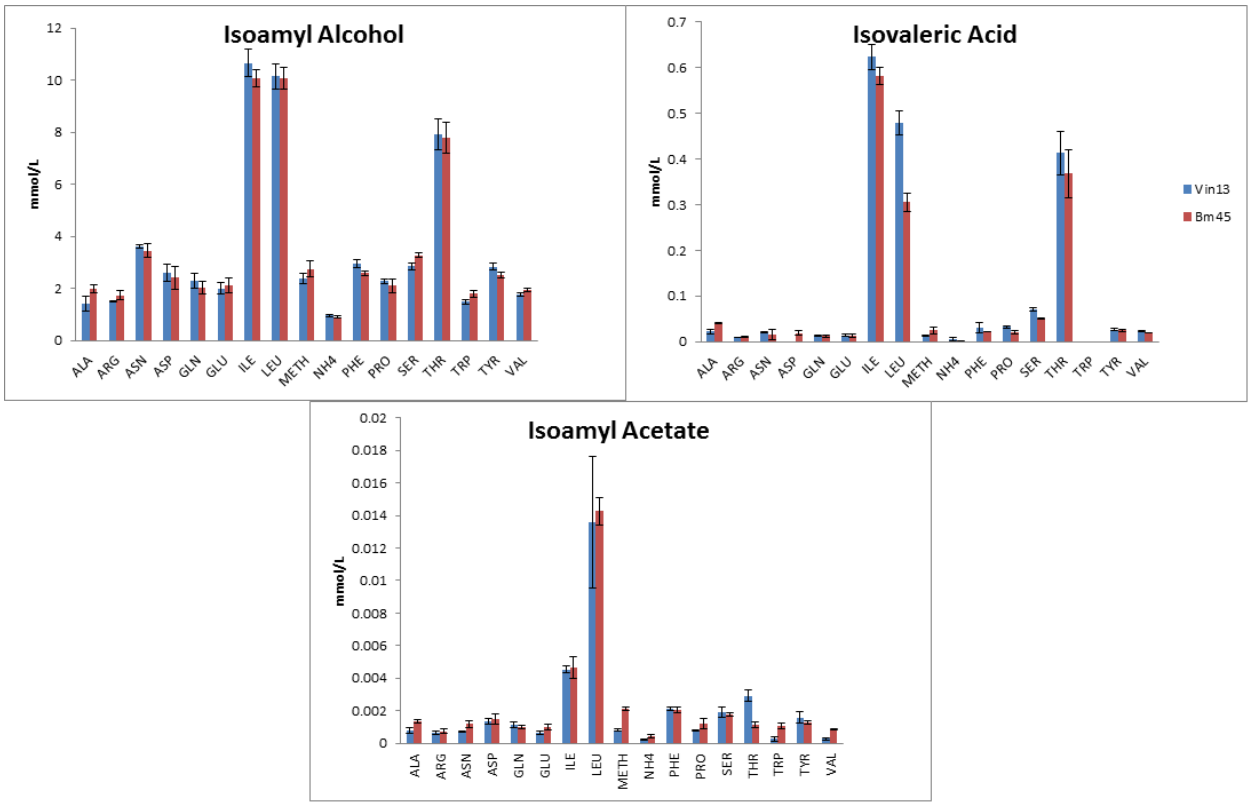

Figure 3.4 GC-FID analysis of volatile compounds related to isoleucine and leucine metabolism, produced by BM45 and VIN 13, measured at the end of fermentation in YNB with 10% glucose and 10.71 mmol N L^{-1} of a single nitrogen source.

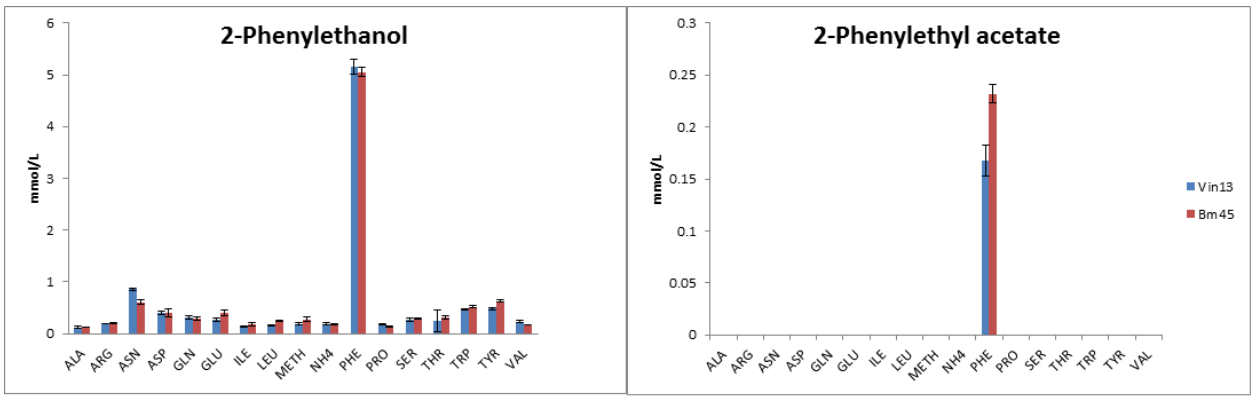

Figure 3.5 GC-FID analysis of volatile compounds related to phenylalanine metabolism, produced by BM45 and VIN 13, measured at the end of fermentation in YNB with 10% glucose and 10.71 mmol N L^{-1} of a single nitrogen source.

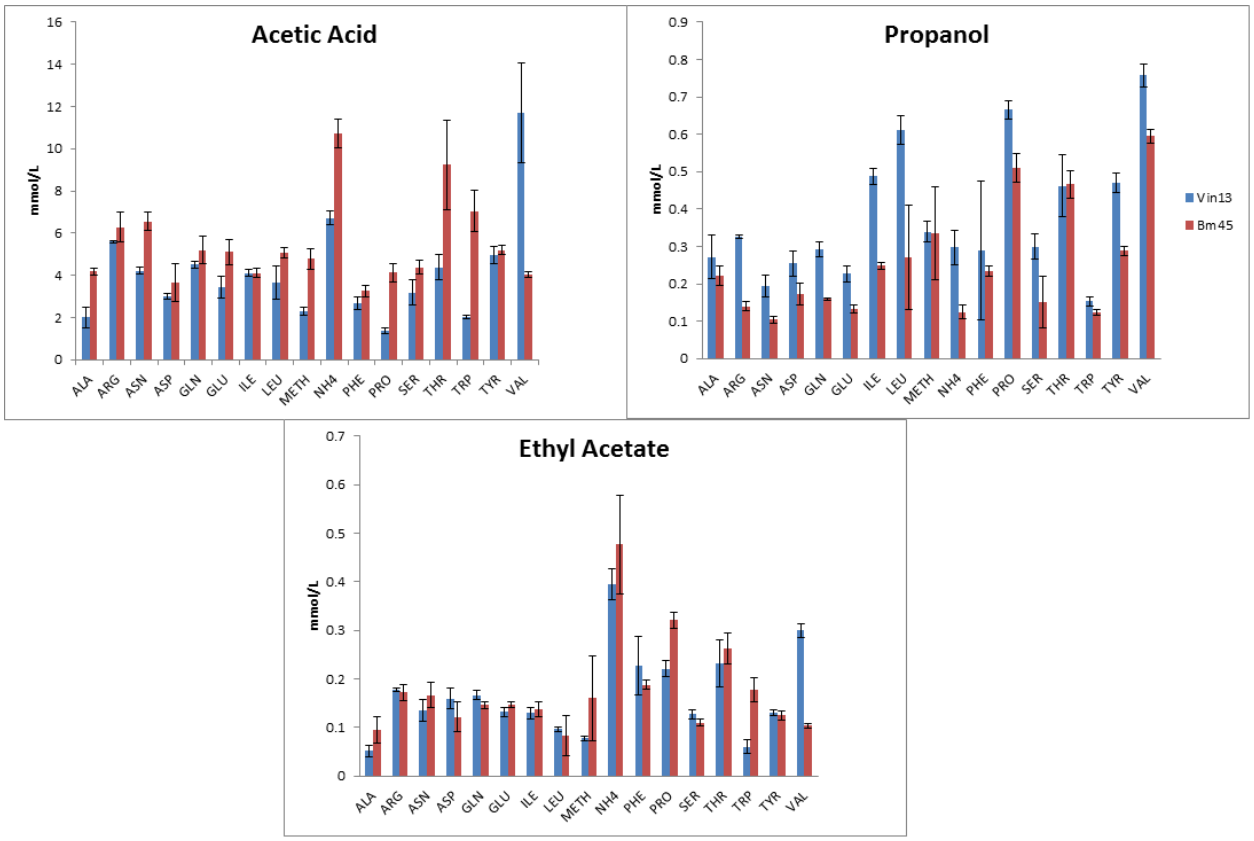

Figure 3.6 GC-FID analysis of volatile compounds related to general yeast metabolism, produced by BM45 and VIN 13, measured at the end of fermentation in YNB with 10% glucose and 10.71 mmol N L^{-1} of a single nitrogen source.

3.3.3 Investigation of NH_4^+ as a Sole Nitrogen Source in Different Synthetic Media

The NH_4^+ treatment in YNB media resulted in unexpected poor biomass formation for both *S. cerevisiae* strains as it is typically characterized as a good sole source of nitrogen (Cooper, 1982; Magasanik and Kaiser, 2002; Godard *et al.*, 2007). When the same treatment was used

in the SGM media the yeast growth was as expected resulting in good biomass formation (Figure 3.7).

To increase the number of parameters captured to discover the cause of the difference in biomass formation between these two media, measurements of the final pH were taken for each treatment. It was found that drops in pH from the initial 3.8 pH of greater than 1.0 pH were observed for YNB treatments and less than 0.5 for SGM treatments.

As a result of the observed differences in final pH for the two media the organic acids found in SGM were added to or excluded from YNB and SGM. In both YNB and SGM media containing SGM organic acids the yeast gave growth characteristics with NH_4^+ similar to other good nitrogen sources (Figure 3.7). Conversely, when these organic acids were excluded from YNB and SGM media the NH_4^+ treatment gave rise to a long fermentation time, low biomass formation, and low exponential growth rate.

Measuring of the pH of these treatments revealed that those treatments containing organic acids had a much greater buffering capacity resulting in a pH change of less than 0.5. Those treatments which did not contain the SGM organic acids were observed to have pH decreases greater than 1.0 pH. Also, a slight difference was observed between the two media, with SGM consistently giving higher biomass formation than YNB under the same conditions.

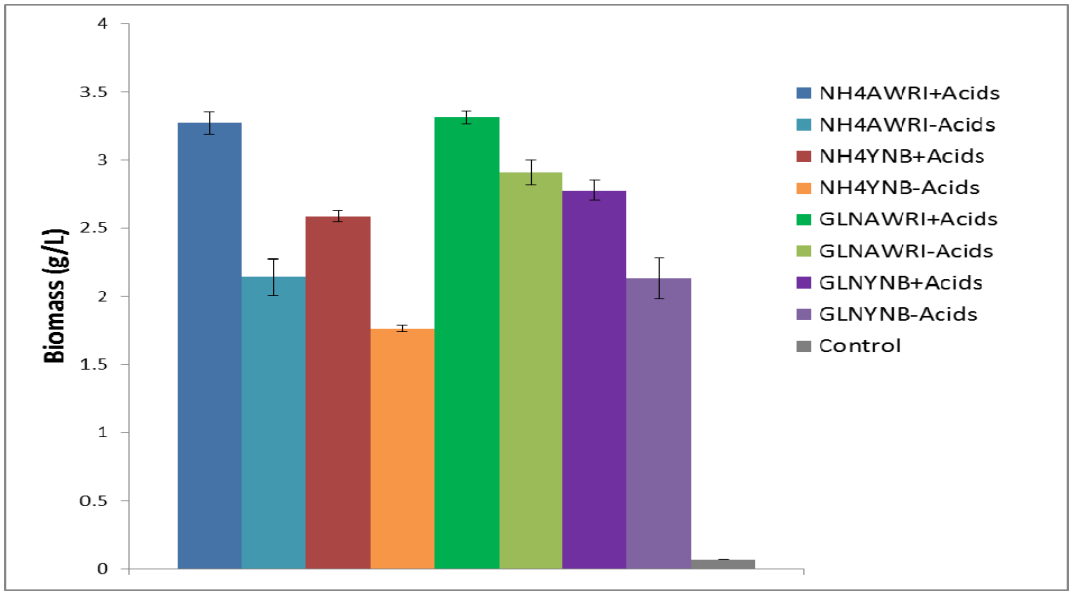

Figure 3.7 Effect of presence of SGM organic acids on VIN 13 final biomass formation in YNB and SGM media containing 10% glucose and 10.71 mmol N L^{-1} of either glutamine or NH_4^+.

3.3.4 Effect of Branched-Chain and Aromatic Acids Concentration on Growth and Aroma Production

To investigate the relationship between the concentration of the BCAAs and the final concentration of aroma products, VIN 13 was grown in SGM at a total YAN concentration of 21.43 mmol N/L containing a single BCAA; phenylalanine, isoleucine, leucine, or valine at concentrations varied between 7.14, 14.28, and 21.43 mmol N L^{-1}. Treatments were supplemented with NH_4^+ to reach a total nitrogen content of 21.43 mmol N L^{-1}. An additional set of valine treatments supplemented with alanine in place of NH_4^+ to control for any confounding results as a result of the NH_4^+ addition. For controls, a treatment containing only NH_4^+ and another containing only alanine as the sole nitrogen source at a concentration of 21.43 mmol N L^{-1} were also used. The supplementation with alanine and the alanine only control were used to control for possible confounding results due to the presence of NH_4^+ on flavour compound production. Dependent upon the concentration of the BCAA used some variation in growth kinetics was observed, however, all fermentations completed to dryness.

3.3.4.1 Growth Characteristics

The treatments which were supplemented with another source of nitrogen (NH_4^+ or alanine) gave rise to higher biomass formation and higher exponential growth rates than those treatments which contain only a single amino acid (Figure 3.8). The valine and leucine treatments display a linear correlation between the quantity of $NH4^+$ and the final biomass and exponential growth rate; the greater the quantity of NH_4^+ in the initial media the higher the biomass and growth rate observed. The valine treatment supplemented with alanine gave a low exponential growth rate regardless of the quantity of alanine present as seen in comparison to the only alanine control. The treatment containing only isoleucine was observed to have the poorest growth kinetics giving the lowest biomass, exponential growth rate, longest time to complete, and longest lag phase as compared with the other treatments.

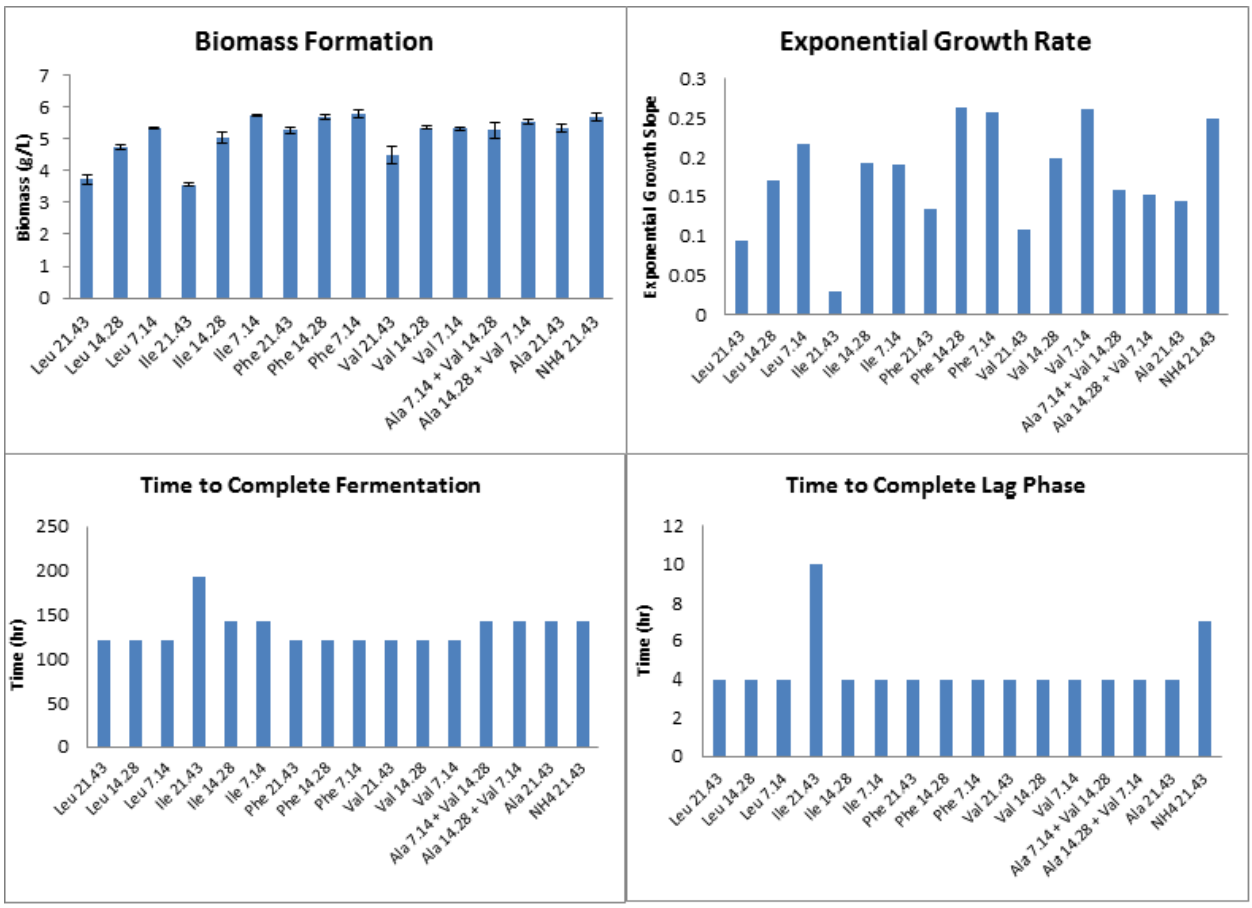

Figure 3.8 Gro-wth characteristics: biomass formation, fermentation duration, lag phase duration, and exponential growth rate of VIN 13 in SGM containing 10% glucose and 21.43 mmol N L^{-1} YAN.

3.3.4.2 Major Volatile Analysis

A linear correlation was observed in the production of fusel alcohols and fusel acids when the initial concentrations of several BCAAs were varied. For all treatments the concentration of fusel alcohols produced was much higher than that for the fusel acids. The amino acids valine, isoleucine and leucine, and phenylalanine were found to be positively correlated with production of isobutanol and isobutyric acid, isoamyl alcohol and isovaleric acid, and 2-phenylethanol and 2-ethylphenyl acetate, respectively (Figure 3.9; 3.10; and 3.11).

Two treatment sets containing varying concentrations of valine were used to compare and contrast the effects of supplementation with different nitrogen sources. One set was supplemented with NH_4^+ and the other with alanine (Figure 3.9). The valine treatments which contained alanine were found to give rise to higher concentrations of isobutanol and isobutyric acid than those containing NH_4^+. The exception to this was the treatment which contained only

valine which was found to produce the highest concentrations of these two compounds. Additional compounds positively correlated to the presence of valine in the initial media are propionic acid and butanol, however, only when the media also contained only valine or valine supplemented with NH_4^+ as well. Treatments where valine was supplemented with alanine show no propionic acid or butanol formation (Figure 3.9).

Isoamyl acetate formation was positively correlated with leucine and to a lesser extent with isoleucine which gave a linear correlation with concentration of leucine, but, no such trend was observed for isoleucine (Figure 3.10). Valeric acid and decanoic acid were positively correlated to the presence of phenylalanine as a nitrogen source with valeric acid displaying a linear correlation to phenylalanine concentration, while decanoic concentration remained consistent for these treatments (Figure 3.11).

Finally, the fermentation products acetic acid, propanol, and ethyl acetate all appear correlated to the concentration of NH_4^+, with acetic acid and ethyl acetate being proportional and propanol being inversely proportional to the NH_4^+ concentration (Figure 3.12). The exceptions to these trends are phenylalanine treatments for acetic acid and propanol, treatments containing valine for ethyl acetate, and alanine treatments for all three. These treatments gave equal concentrations of their respective aroma compounds regardless of the NH_4^+ concentration. Regardless, the production of acetic acid and ethyl acetate was found to be positively correlated and they followed the same relative trends in production for each treatment.

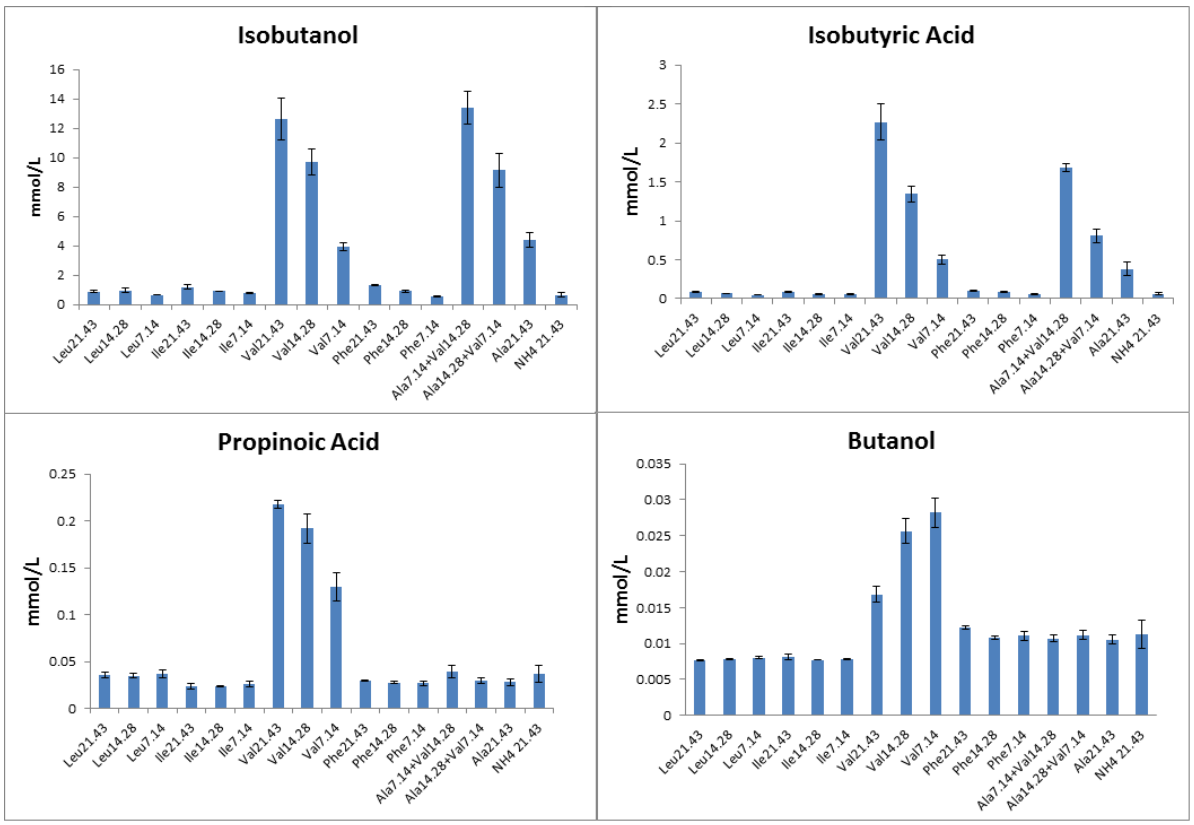

Figure 3.9 GC-FID analysis of volatile compounds related to valine metabolism, produced by VIN 13, measured at the end of fermentation in SGM media with a total YAN concentration of 21.43 mmol N L^{-1}

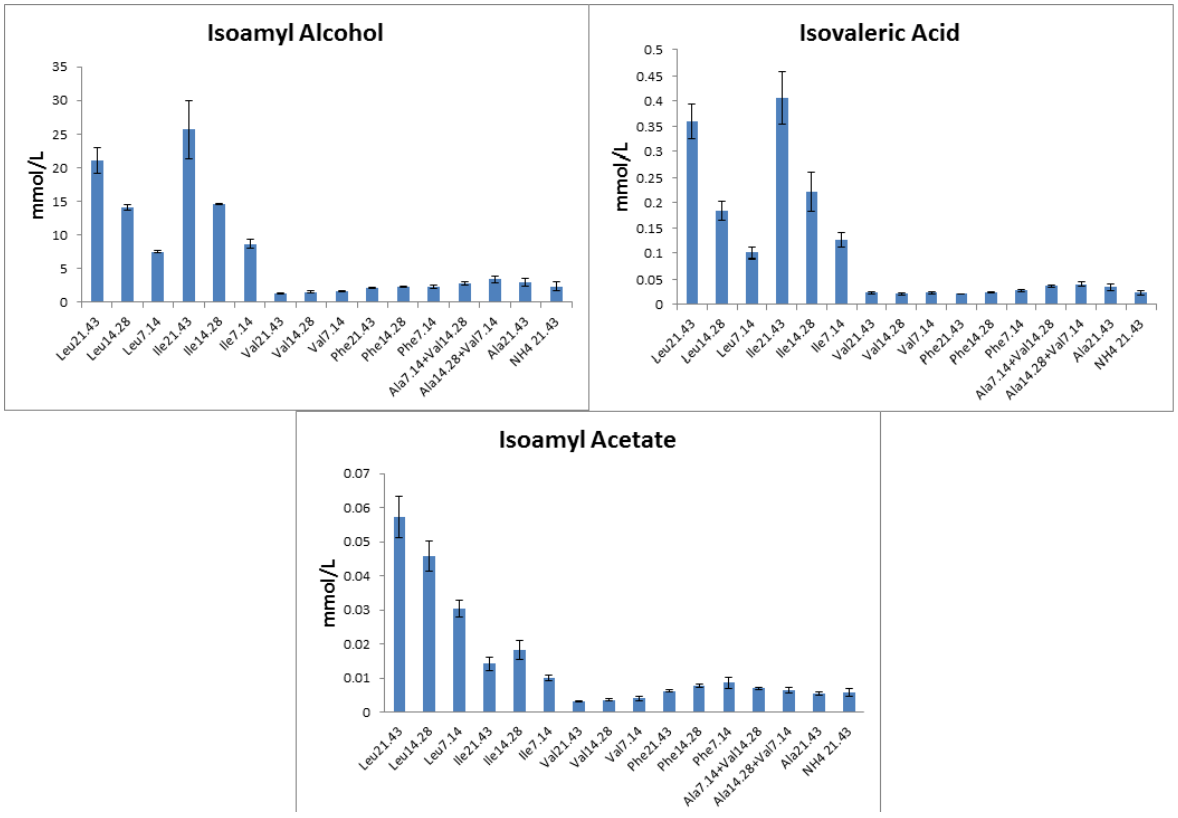

Figure 3.10 GC-FID analysis of volatile compounds related to isoleucine and leucine metabolism, produced by VIN 13, measured at the end of fermentation in SGM media with a total YAN concentration of 21.43 mmol N L^{-1}

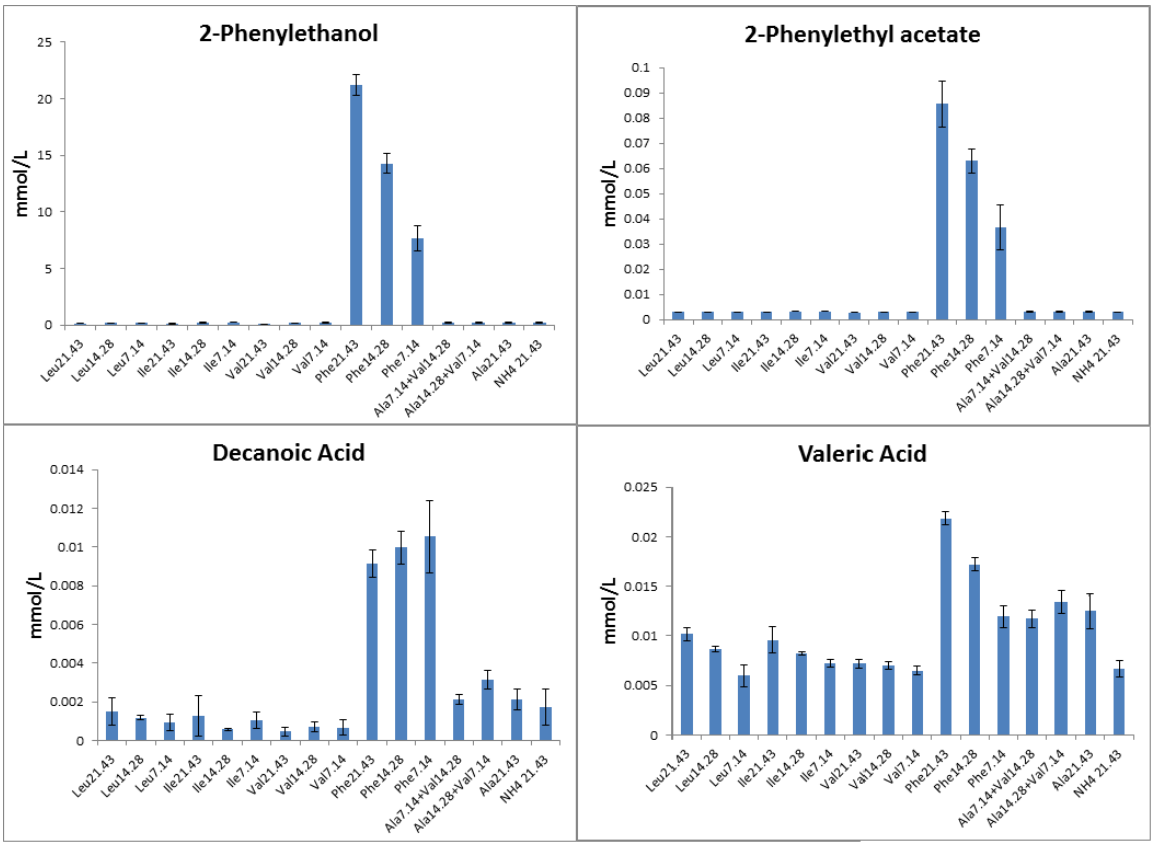

Figure 3.11 GC-FID analysis of volatile compounds related to phenylalanine metabolism, produced by VIN 13, measured at the end of fermentation in SGM media with a total YAN concentration of 21.43 mmol N L^{-1}

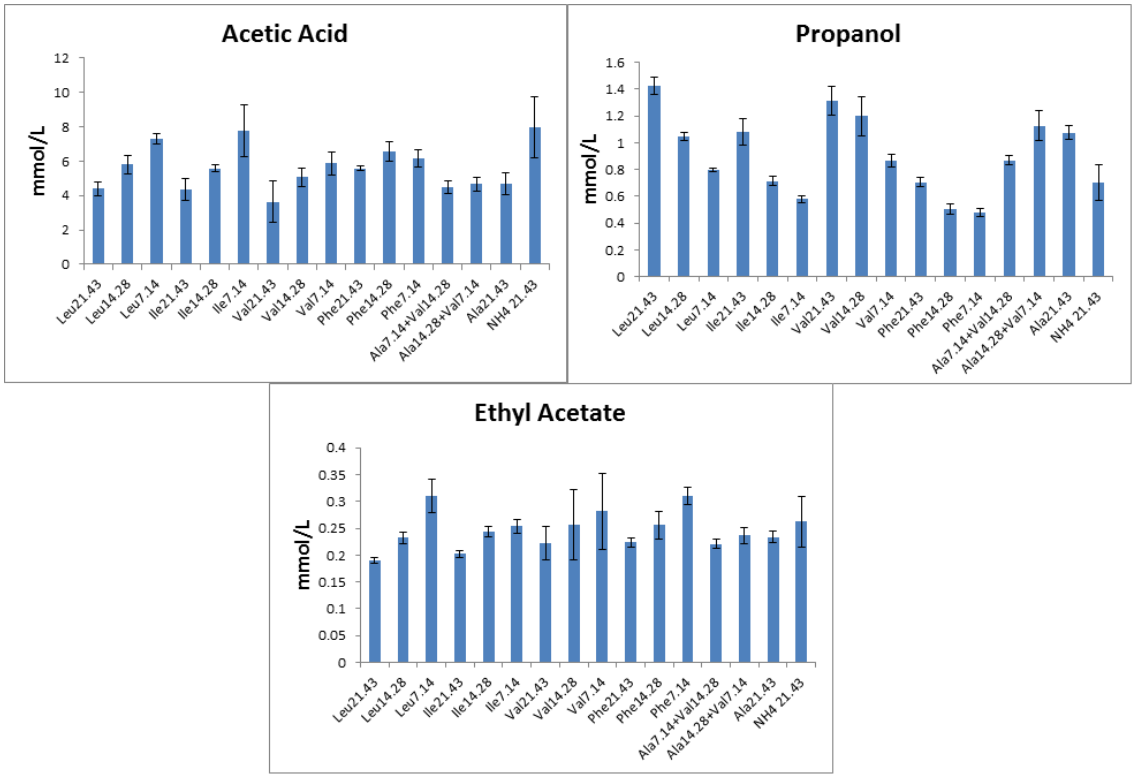

Figure 3.12 GC-FID analysis of volatile compounds related to general yeast metabolism, produced by VIN 13, measured at the end of fermentation in SGM media with a total YAN concentration of 21.43 mmol N L^{-1}

3.3.5 Effect of Complex Nitrogen Treatments on the Predictability of Major Volatile Production

To further investigate the relationship between the final concentration of aroma compounds and the initial BCAA concentration, SGM media containing a complex mixture of nitrogen sources was fermented by VIN 13. This experiment used similar nitrogen treatments from previous work done by Smit (2013) containing a total YAN concentration of 14.28 mmol N L^{-1}. Four treatments were used, containing either only preferred amino acids, only non-preferred amino acids, only non-utilized amino acids or only BCAAs (Table 3.3). All treatments were supplemented with an equal concentration of NH_4^+. A control treatment was also included containing no nitrogen. Many of the aroma compounds previously found to be linearly correlated to BCAAs were predicted within a reasonable error. Few differences in growth kinetics were observed for these treatments which contained more complex nitrogen treatments and all fermentations rapidly completed to dryness.

3.3.5.1 Growth Characteristics

The growth of the preferred, non-preferred, and BCAAs treatments appear to be almost identical (Figure 3.13). All three treatments reached a similar biomass and experienced an equal duration of the lag phase with a slightly faster generation time was experienced by the preferred treatment. The non-utilized treatment gave rise to approximately half the level of biomass than the other treatments. All treatments completed fermentation to dryness over the same duration.

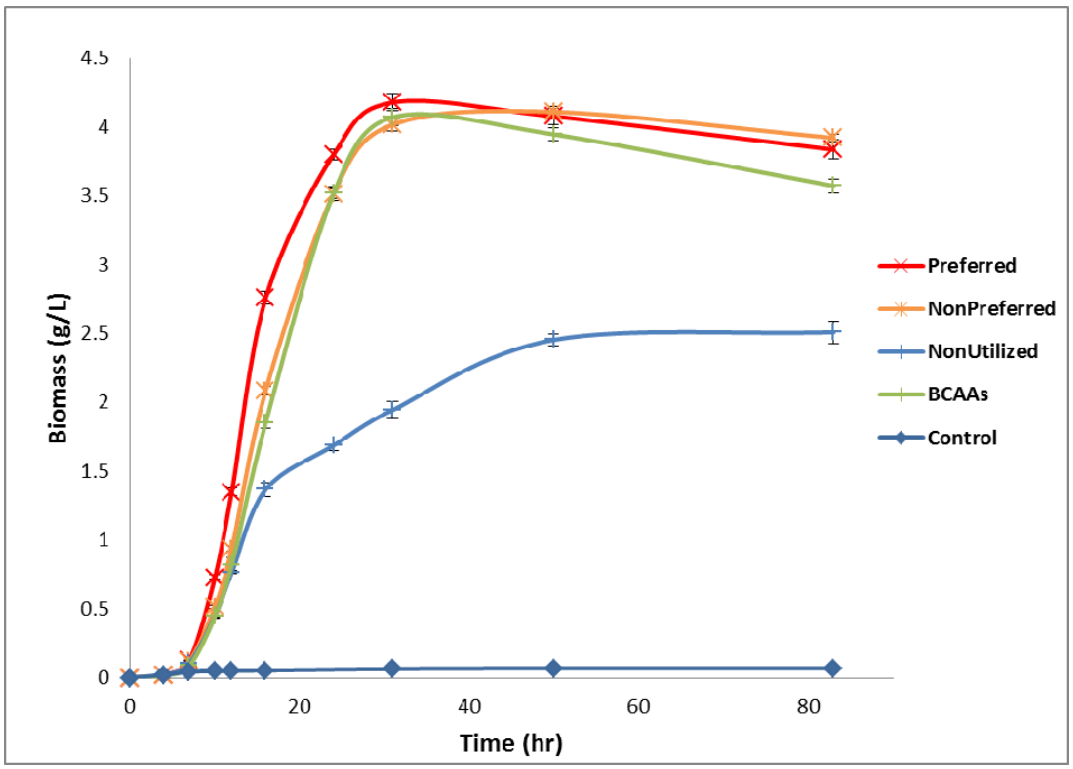

Figure 3.13 Biomass formation by VIN 13 grown in SGM with nitrogen treatments used by Smit (2013) containing 14.28 mmol N L^{-1} YAN.

3.3.5.2 Major Volatile Compound Production from Complex Nitrogen Treatments

The treatment containing only BCAAs gave rise to high concentrations of fusel alcohols and acids as well as lower relative levels of propionic acid and butanol and relatively higher levels of valeric acid as compared with other treatments (Figure 3.14; 3.15; and 3.16).

The preferred amino acid treatment gave rise to high levels of acetic acid and slightly elevated levels of ethyl acetate with the lowest levels of propanol as compared with the other treatments (Figure 3.17). The preferred treatment also resulted in lower production of propionic acid as compared with the non-preferred and non-utilized treatments as well as the lowest level of valeric acid production. Finally, the lowest ethyl acetate production was observed for the non-utilized amino acid treatment.

3.3.5.3 Predicting Volatile Compound Production Due to Branched-Chain and Aromatic Amino Acid Metabolism

From the linear correlations observed between initial amino acid concentration and final volatile compound composition the final concentrations of these compounds were predicted for

the BCAAs treatment containing equal quantities of phenylalanine, isoleucine, leucine, and valine.

Of the compounds which were observed to have a linear relationship only the fusel alcohols and fusel acids (Figure 3.14; 3.15; and 3.16) isobutanol, isobutyric acid, isoamyl alcohol, isovaleric acid, and 2-phenylethanol, as well as the ester isoamyl acetate (Figure 3.15) maintained their linear predictability at lower concentrations in a more complex nitrogen treatment. The volatile acids (Figure 3.14 and 3.16) decanoic acid, valeric acid, and propionic acid and the alcohol butanol (Figure 3.14) did not appear to follow the same linear trend for these more complex nitrogen treatments.

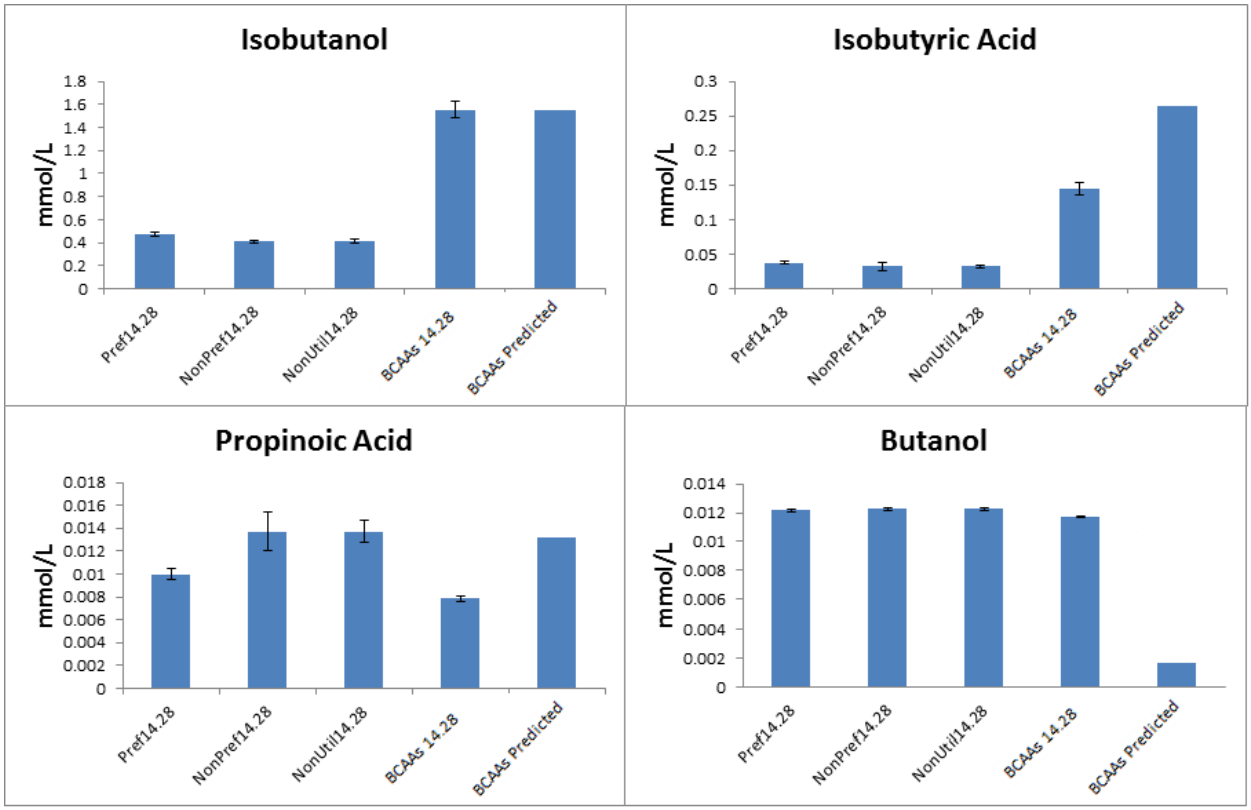

Figure 3.14 Products of valine metabolism and predicted yields by VIN 13 in SGM media containing a complex mixture of 14.28 mmol N L^{-1} YAN

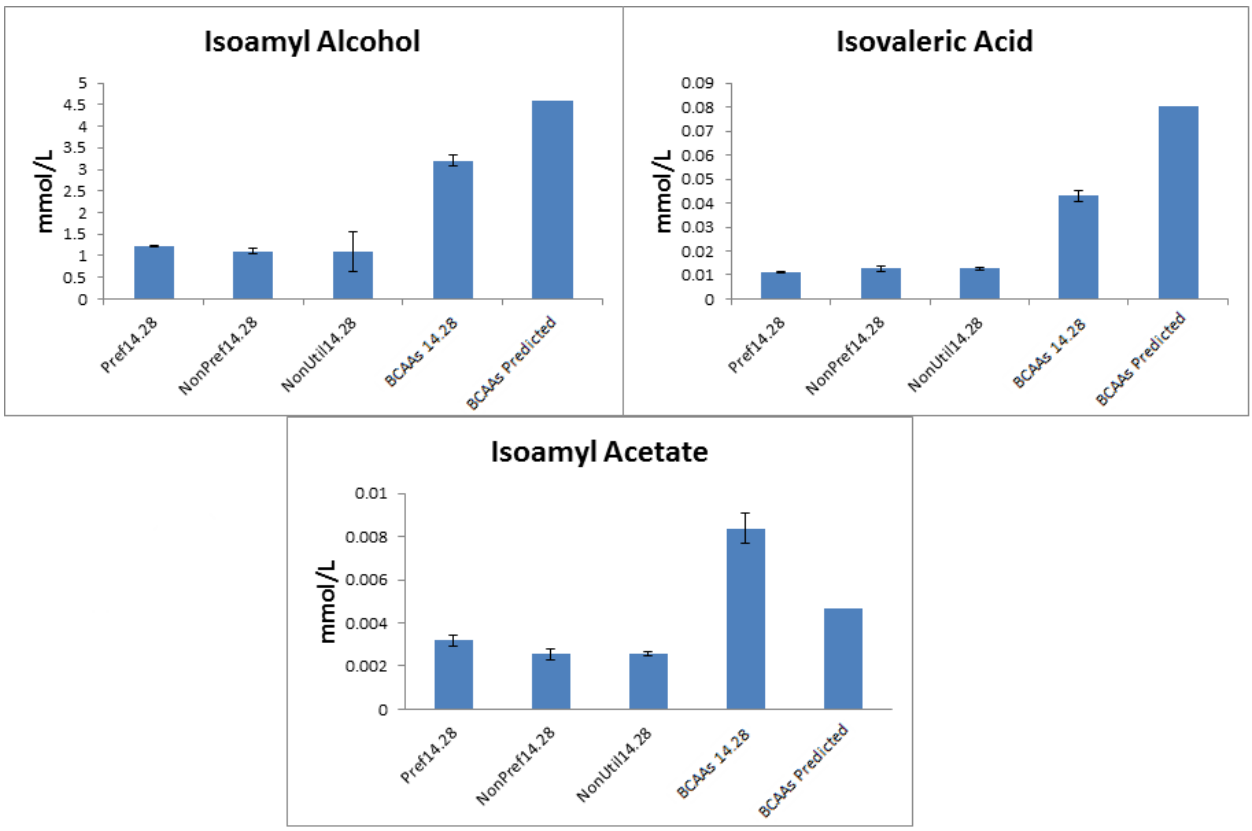

Figure 3.15 Products of isoleucine and leucine metabolism and predicted yields by VIN 13 in SGM media containing a complex mixture of 14.28 mmol N L^{-1} YAN

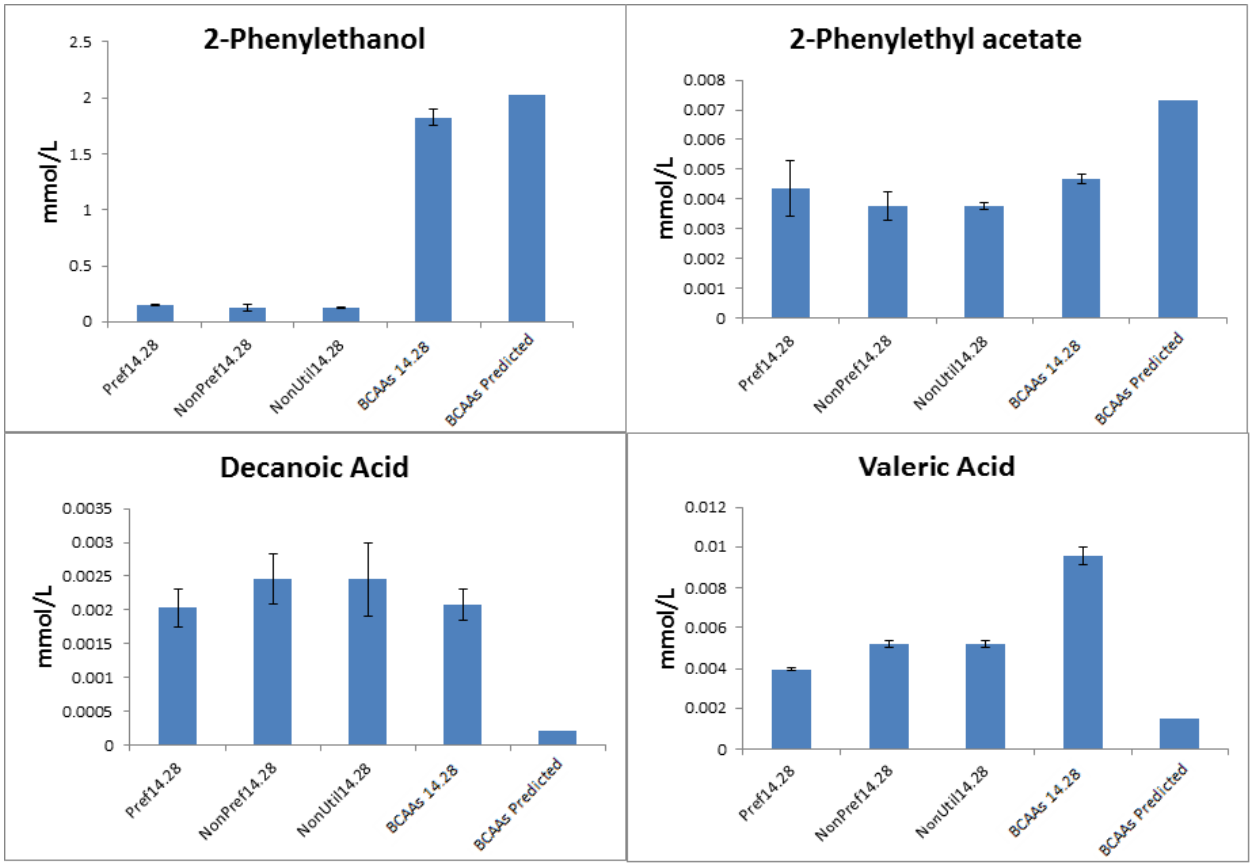

Figure 3.16 Products of phenylalanine metabolism and predicted yields by VIN 13 in SGM media containing a complex mixture of 14.28 mmol N L^{-1} YAN

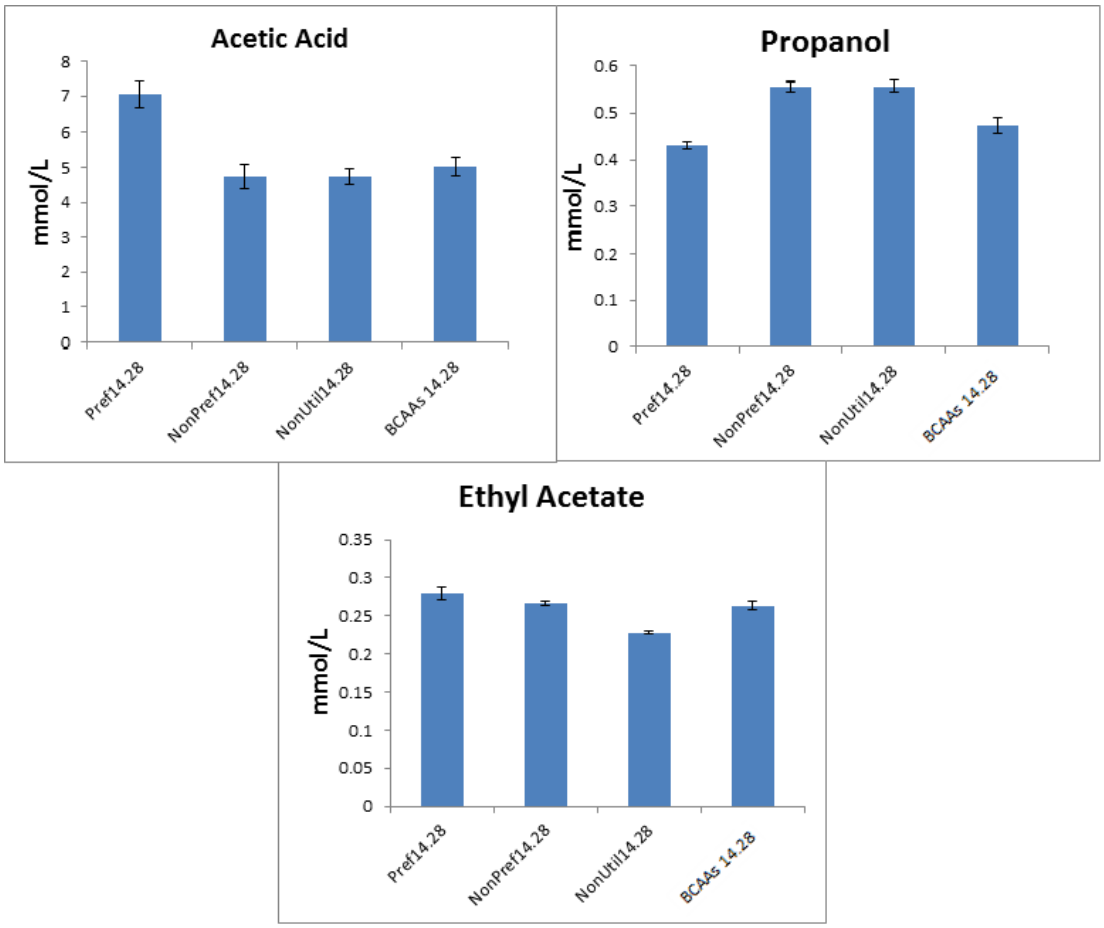

Figure 3.17 General aroma products by VIN 13 under complex nitrogen conditions in SGM containing 14.28 mmol N L^{-1} YAN.

3.4 Discussion

This study focused on identifying the impact of two fermentation parameters: the genetic background of industrial *S. cerevisiae* yeast and the effect of nitrogen content on the growth performance and major volatile compound production. Although generalizing the findings of this comparison of two wine yeasts to all industrial *S. cerevisiae* wine yeast may be problematic, this study provides novel comparison of industrial yeast strain growth and major volatile production. Furthermore, the logistics of monitoring a large number of yeast strains with highly variable growth curves with sufficient replicates for statistical evaluation would be beyond the scope of this single study. Therefore, two yeast strains which were previously observed by Smit (2013) and Rossouw (2009) to be the most divergent in their growth characteristics were utilized to capture the behaviour of the majority of industrial wine yeasts.

3.4.1 Effect of Amino Acids on *S. cerevisiae* Growth Kinetics

Traditionally the sole growth parameter for the evaluation of the efficiency of nitrogen sources to support growth under single nitrogen source conditions is the exponential growth rate and is expressed in terms of generation time (Cooper, 1982; Godard 2007). This study increased the number of growth parameters evaluated, which allowed for a more rigorous and complete study of the efficiency of single amino acids as the sole nitrogen source to support growth. Since amino acids are provided as the sole source of nitrogen, no regulatory interference in their utilization occurs from the presence other nitrogen sources (Ljungdahl & Diagnan-Fornier, 2012; Godard *et al.*, 2007). Therefore, the efficiency of these nitrogen sources was dependent solely on the cell's ability to take up and utilize that amino acid for its nitrogen requirements.

In general, the efficiency of single amino acids to support growth for wine yeast strains under fermentative conditions was in agreement with that of previous studies by Cooper (1982) and Godard *et al.* (2007). Those nitrogen sources which gave the best fermentation kinetics (alanine, aspartate, glutamine, glutamate, and serine) were also the amino acids which are easily incorporated into the cell's metabolic pathways. After these amino acids are transaminated to form glutamate, the remaining carbon skeleton can then be easily incorporated into the TCA cycle (Cooper, 1982; Magasanik & Kaiser, 2002; Godard *et al.*, 2007 Ljungdahl & Diagnan-Fornier, 2012).

For the treatments containing single BCAAs, the phenylalanine and valine treatments were observed to be the most efficient of this group and overall good sources of nitrogen. The reduced utilization of BCAAs in general was likely limited due to a lack of sufficient BCAA permeases initially present in the cell's plasma membrane. This does not, however, explain the better performance of phenylalanine and valine over the other BCAA treatments. Looking at the physical structures of these two amino acids, phenylalanine and valine contain the least complex side chains for the aromatic and branched-chain groups, respectively. These smaller side chains may allow for these amino acids to be more easily incorporated into the cell's metabolic pathways allowing for better growth than other BCAAs.

Along with examining the trends found for each parameter, correlations were observed between the four growth parameters. The formation of biomass was found to be correlated with the length of the lag phase and the exponential growth rate was found to be correlated to the fermentation time. In general, the shorter the lag phase the higher the biomass formation observed, and the higher the exponential growth rate observed the shorter the fermentation time. Exceptions to these correlations due to strain differences were found for both the biomass and lag phase (arginine, isoleucine, and threonine treatments) and exponential growth rate and fermentation time (valine and threonine treatments) correlations. Furthermore, the asparagine and NH_4^+ treatments were found to be exceptions for both correlations due to their irregular growth performance in the four growth parameters.

The correlation of fermentation time based on exponential growth rate is supported by previous literature which suggests that sufficient biomass is required to prevent stuck or sluggish fermentations (Bisson, 1999). However, for the lag phase and biomass formation correlation, the lag phase is affected by the presence of amino acid permeases as well as the ability of the yeast to meet its nitrogen requirements with the nitrogen source supplied. Thus, this correlation is less likely to be maintained as the complexity of the nitrogen content increases. This underlines the importance of capturing several growth parameters to allow for a more complete interpretation of the effects of single amino acids as the sole source of nitrogen in the context of wine fermentations.

3.4.2 Effect of Yeast Genetic Background on Growth Kinetics

Though most amino acids were found to have similar trends in efficiency for both yeast strains, some strain differences were observed in particular for the treatments containing less efficient amino acids. These differences in nitrogen utilization efficiency could be attributed to certain strains being better adapted to growth in certain nitrogen conditions due to their different genetic backgrounds. Similar observations have been made in previous studies as reviewed by Lambrechts and Pretorius (2000), where it has been observed that differences in nitrogen demands have been found with some strains requiring higher concentrations of total YAN to complete fermentation (Torrea *et al.*, 2003). This underlines the importance of defining the

measure of efficiency of nitrogen sources. While the exponential growth rate has been used in the past to define efficiency this may be an incomplete understanding of the contribution of amino acids to wine fermentations. From a winemaking perspective, including the parameters of fermentation time and biomass formation for the evaluation of amino acids provides a greater understanding of the effect of these compounds on fermentation performance. Furthermore, the impact of the amino acid metabolites on the final wine would also be a consideration as in the case of the BCAAs as they may improve the health of fermentations while also affecting the final wine aroma.

3.4.3 Effect of YAN Concentration and Complexity on Growth Kinetics

The concentration and composition of YAN in the fermentation media were both found to be important factors in determining growth kinetics. Under the conditions of this study, when treatments contained sufficient nitrogen to support good growth, the concentration was the rate-limiting parameter for biomass formation. This was found to be the case regardless of the complexity of the nitrogen treatments provided. Interestingly, as the complexity of the nitrogen treatments increased the similarity in growth kinetics between treatments also increased. In the treatments containing single nitrogen sources the growth kinetics were highly variable while complex nitrogen treatments containing a combination of only non-preferred amino acids or only BCAAs preformed equally to the treatment containing only efficient amino acids.

The treatment containing amino acids which are unable to support growth or support poor growth as the sole source of nitrogen completed fermentation to dryness within the same time as the other complex nitrogen treatments. The combinatory effect of these non-utilized and less efficient nitrogen sources appears to provide sufficient nitrogen diversity to meet the cell's nitrogen requirements which these amino acids cannot meet when they are provided as the sole source of nitrogen. While the combination of the poorest single nitrogen sources allowed for completion of fermentation, this treatment gave rise to only half as much biomass as the other treatments and therefore is at a greater risk of becoming a stuck or sluggish fermentation. While the fact that each nitrogen treatment did contain a small and equal concentration of NH_4^+ would

have contributed to the improvement in growth kinetics, it is unlikely that this was solely responsible due to the small concentration.

The reason why providing yeast with combinations of poor or inefficient amino acids allows for improved fermentation performance remains unclear. It is possible that the side chains of these amino acids are not easily utilized by the cell for the biosynthesis of other nitrogen compounds, increasing the diversity of substrate may allow the cell to successfully meet its nutritional demands. Additionally, the resulting compounds due to the degradation of these amino acids may be inhibitory to cell growth when found at high concentrations. Utilizing a diverse number of nitrogen sources may reduce the total concentration of inhibitory compounds.

In wine, amino acids make the largest group of YAN compounds found in grape musts (Jiranek *et al.,* 1995; Spayd & Andersen-Bagge, 1996; Bell & Henschke, 2005). Preferred amino acids make up approximately 40% of the total amino acids found in grape musts (Table 2.4). While poor amino acids usually account for approximately only 1-2% of the total amino acid make up of grape musts. It is therefore highly unlikely for yeast to be subjected to amino acid conditions as extreme as found in this study. Regardless, this ability to complete fermentation when yeast are supplied with a combination of amino acids that as the sole sources of nitrogen lead to incomplete fermentations was also reported by Smit (2013). To our knowledge, this effect has never before been investigated or reported by previous literature and represents a novel and highly interesting finding.

3.4.4 Effect of Buffered Media on Amino Acid Efficiency

Surprisingly, NH_4^+ and asparagine which are often reported as efficient nitrogen sources by literature (Cooper, 1982; Godard, 2007) were found in this study to give rise to poor growth kinetics in YNB-based media. This was investigated for the NH_4^+ treatment and was found to be linked to the lack of buffering capacity by the YNB media. The presence of organic acids in the SGM media allowed for a much greater buffering capacity than that of the YNB media.

Therefore the poor growth experienced by NH_4^+ and asparagine in YNB media is attributed to the acidification of the extracellular environment as a direct result of the utilization of these nitrogen sources. The conversion of NH_4^+ into glutamate for the synthesis of amino

acids is done by the glutamate dehydrogenase (GDH1) (Cooper, 1982) which requires NADPH and results in the release of H^+. This excess H^+ appears to be exported extracellularly resulting in an acidification of the external environment. Since NH_4^+ is a direct by-product of asparagine degradation (Dunlop & Roon, 1975), a similar effect can be seen in this treatment but to a lesser extent. Thus, the decreased pH of the external environment results in an increased osmotic stress on the cell by increasing the susceptibility of the plasma membrane's structural proteins to denaturation (Gibson *et al.*, 2007).

The importance of a buffered media was not highlighted until after the first fermentation set had been completed. In retrospect, SGM was a superior media to YNB for the classification of single amino acids as the sole source of nitrogen. Despite YNB and SGM both being minimal media, SGM contains buffering organic acids which resulted in significantly different levels of biomass formation for yeast supplied with NH_4^+ as the sole source of nitrogen.

3.4.5 Effect of Amino Acids on Major Volatile Production by Industrial Wine Yeasts

The production of aroma compounds by wine yeast was influenced by yeast strain and to a larger extent the nitrogen composition of the media. Similar tends in the final concentrations of major volatile compounds due to the amino acid treatments were observed for the majority of compounds for both yeast strains, but strain differences in final concentration of acetic acid, ethyl acetate, and propanol concentrations were noted. With the exception of the valine treatment, BM45 was found to produce higher concentrations of both acetic acid and ethyl acetate. Acetic acid is a primary metabolite of alcoholic fermentation and not directly linked to amino acid metabolism. However, when under nitrogen limiting conditions (insufficient 'good' nitrogen sources) acetic acid is produced to maintain the yeast cell's redox balance through the regeneration of NADH (Bely, *et al.,* 2003). This would explain the large variation in acetic acid between single amino acid treatments. Since many amino acids will have a unique metabolic impact on the redox balance of the cell, those treatments which put more redox stress on the cell theoretically produce higher levels of acetic acid. Considering that ethyl acetate is the result of the enzymatic interaction between an acid and ethanol (Saerens *et al.,* 2010), and observing the consistency of the correlation between ethyl acetate and acetic acid production suggests

that the majority of ethyl acetate formation is due to the interaction between acetic acid and ethanol. Propanol and butanol though usually associated with the metabolism of threonine (Giudici *et al.*, 1993) were found to be produced in high levels from the threonine only treatment. However, the production of propanol was also found to be produced in relatively high levels by the majority of treatments, suggesting that it also plays an important role in general metabolism of yeast as it was not found to be correlated with any specific treatment.

These trends in compound production due to redox stress were also observed in the more complex nitrogen treatments with the differences between treatments decreasing as the complexity of the treatments increased. In general, those treatments which were supplemented with NH_4^+ were observed to produce higher levels of acetic acid and lower levels of propanol. These higher levels of acetic acid due to ammonium supplementation are in agreement with current literature (Vilanova *et al.*, 2007). This should be an important consideration when supplementing grape musts with ammonium in the form of DAP as a preventative measure against stuck or sluggish fermentations. As the addition of DAP may prevent poor fermentation performance while inadvertently altering the final wine flavour and aroma due to increased redox stress. Further research should investigate alternative nitrogen compounds for the supplementation of grape musts which are less of a risk to negatively impact the final wine.

3.4.5.1 Effect of BCAA Concentration on Major Volatile Production

In agreement with literature, the degradation of BCAAs into their corresponding fusel alcohols and fusel acids was observed to be positively linked (Ehrlich, 1907; Hazelwood *et al*., 2008). Examining the relationship between the molar concentration of BCAAs and the production of aroma compounds, the combined final molar concentration of fusel alcohols and fusel acids was close to a 1:1 ratio with their related BCAA initial concentration, indicating a 100% conversion rate. If this same rate of conversion is found in real grape must the possible contribution of BCAAs on the final flavour and aroma of wines would be highly significant. Consider that the majority of YAN in grape musts is from amino acids and that BCAAs comprise approximately 10% of these amino acids (Table 2.4). If the degradation of BCAAs results in

100% conversion into aroma impact compounds their degradation could account for a large proportion of secondary metabolite aroma compounds.

Additionally, it was demonstrated that the initial BCAA concentration could be used to predict the formation of their related fusel alcohols and fusel acids. This is in agreement with the understanding that the production of these aroma compounds is the direct result of metabolic degradation of the resulting carbon backbone of these amino acids after transamination (Dickinson *et al.*, 1997; Dickinson *et al.*, 1998; Dickinson *et al.*, 2000; Dickinson *et al.*, 2003). Furthermore, it appears that the conversion of BCAAs is not impacted by the presence of other BCAAs. These findings suggest that under fermentative conditions the final concentrations of fusel alcohols and fusel acids due to BCAA degradation by industrial *S.* cerevisiae yeast strains could be predicted. This would be possible provided that the initial concentration of their related BCAAs was known and if all of these amino acids are completely degraded during fermentation.

Aside from limiting nutritional factors preventing the complete consumption of BCAAs from the fermentation, a possible factor which could theoretically affect the predictability of BCAA correlated aroma compound formation is the availability of associated degradation enzymes. The degradation of BCAAs requires that these amino acids are first transaminated by transaminases which are unique to either the branched-chain (Bat1p & Bat2p) or aromatic (Aro8p & Aro9p) group. After which the resulting carbon backbone is decarboxylated by one of four decarboxylases which are shared between all BCAAs (Aro10p, Pdc1p, Pdc5p, Pdc6p) and a fifth decarboxylase (Thi3p) which is specific for only the branched-chain amino acids (Hazelwood *et al.*, 2008). Since nitrogen was not supplied in excess and the BCAAs in the treatments were supplied at equal concentrations, all BCAAs appear to be degraded equally allowing easy predictability of their associated aroma compounds. However, it could be expected that these amino acids have different affinities for these degradation enzymes and therefore different rates of degradation. Therefore, a fermentation containing a mixture of BCAAs, where nitrogen is not the growth limiting factor could result in incomplete BCAA degradation due to differences in enzymatic affinities between BCAAs. Thus, an incomplete degradation of all or some of these BCAAs could be experienced and a loss of predictability of their associated aroma compounds.

Another possible limiting factor for the availability of BCAA degradation enzymes could be due to the transcriptional repression of the production of these degradation enzymes. The NCR is known to facilitate the selective utilization of preferred nitrogen sources by repressing the utilization of less preferred nitrogen sources (Magasanik & Kaiser, 2002). In the presence of preferred amino acids the NCR limits the utilization of less preferred nitrogen sources by repressing the transcription of genes required for the production of permeases and degradation enzymes required for the catabolism of these amino acids. However, when preferred nitrogen sources are limiting the transcription of amino acid premeases and catabolic enzymes become de-repressed (Magasanik & Kaiser, 2002). Though the NCR is one of the most extensively studied nitrogen regulation systems, the mechanisms of regulation remain to be fully understood and the findings have yet to be verified in industrial yeast strains under winemaking conditions. Provided the NCR has the same effect on industrial yeast strains under winemaking conditions, the production of BCAA degradation enzymes may also be limited due transcriptional repression. Fermentations containing mixture a of all 19 amino acids commonly found in grape musts (Table 2.1) with an excess of YAN may also result in the incomplete degradation of BCAAs and therefore, a possible loss in predictability of BCAA associated major volatile compounds.

Along with fusel alcohols and fusel aldehydes each BCAA was found to be correlated with the production secondary metabolites not directly related to BCAA degradation. However, this was not the case when BCAA treatments were supplemented with alanine in place of NH_4^+. The supplementation with a more complex nitrogen compound precursor such as an amino acid negated correlation between BCAA concentration and the production of these secondary metabolites. This loss of predictability of secondary metabolites was also found to be the case when yeast were provided with more complex nitrogen treatments. As a generalization, the production of these secondary metabolites became more homogeneous and decreased with the increased complexity of the nitrogen treatments. The exception to this rule was valeric acid which was produced in higher levels than predicted for the treatment containing a mixture of BCAAs. This loss of predictability when the complexity of the nitrogen treatment increases suggests that these compounds (decanoic acid, valeric acid, butanol, and propionic acid) are

products of more complex secondary metabolic functions not necessarily directly linked but correlated to the degradation of BCAAs. This underlines the complex nature of aroma production as small increases in media complexity can have dramatic effects on aroma predictability. Thus, highlighting another consideration for the understanding of aroma compound production; the diversity and make-up of the YAN content of grape must.

3.4.5.2 Effect of Temperature on Major Volatile Production

Though analysis of the specific compounds which make up the YAN content in musts is often neglected in wine making, careful attention is paid to the effect of fermentation temperature on aroma formation. The formation of high levels of fusel alcohols is known to be affected by temperature and the use of higher than typical wine fermentation temperatures as used in this study favours their production (Garcia *et al.,* 1993; Molina *et al.*, 2007). The higher fermentation temperatures utilized for this study were to ensure a rapid completion of the screening study and to limit the risk of stuck fermentations allowing for proper comparison of treatment effects.

As the goal of this exploratory study was to determine any relationships between initial amino acid content and aroma production it was beyond the scope of this experiment to examine the effect of temperature on the aroma trends observed. However, this higher fermentation temperature may have resulted in a reduction of fusel alcohol degradation. It is understood that temperatures (between 10-25^{o}C) are the optimal temperature for the formation of esters (Verstrepen *et al.*, 2003). Furthermore it is well documented that fusel alcohols and fusel acids are substrates for the enzymatic formation of some esters (Lambrechts & Pretorius, 2000; Styger *et al.*, 2011). This higher fermentation temperature may have inhibited the enzymatic degradation of these fusel alcohols and formation of acetate esters, allowing for the observation of the relationship between the production fusel alcohols and acids due to BCAA degradation.

While the trends found by this study are significant, the results of this study require further verification. The formation of final wine aroma is the result of numerous non-linear interactions and the result of several microorganisms including non-*Saccharomyces* yeast and bacteria.

While many of the parameters and the simplicity of synthetic media do not reflect real grape must, synthetic grape must has been shown to be suitable as a model for both red and white grape musts by Smit (2013) and Rossouw (2009). Nevertheless, it is recommended further studies on this topic be done to obtain more validity to the findings of this study as well as to better assess their application in real wine fermentations.

3.5 Conclusions

This novel study allowed for the verification of previous metabolic data on the impact of single amino acids on the growth kinetics and aroma production by industrial yeast strains under wine making conditions to be similar to that of laboratory yeast strains under respiratory and glucose limiting conditions. Interesting insights in the relationship between initial must composition on the growth characteristics and major volatile production of industrial wine yeast strains were observed.

Factors which were found to be of importance for the formation of biomass and overall health of fermentation were the concentration and the composition of nitrogen provided for the yeast. Biomass formation and fermentation kinetics were improved when inefficient or non-utilized amino acids were combined to increase the diversity of nitrogen sources available for the yeast. Some mild differences of yeast performance were observed for the single amino acid treatments classified as intermediate and inefficient amino acids. These observations emphasize the importance of capturing as many parameters as possible to allow for a better evaluation of yeast growth kinetics.

This study provides an important foundation for the study of the relationships between initial must constituents and the production of aroma compounds in wine fermentations. The production of specific fusel alcohols and fusel acids as a direct result of BCAA degradation was found to be a predictable and linear relationship based on the initial concentration of the corresponding BCAA. The potential to predict the total or a fraction of the total concentration of aroma compounds produced due to BCAA degradation is an exciting prospect.

The results of this study provide important insights into the metabolic impact of amino acids on aroma production by industrial wine yeasts. In real grape must the diversity of amino

acids is much greater and the relative concentration of BCAAs is much lower than in the treatments in this study. Due to the simplicity of the media used the findings of this study must be validated in real grape must to assess their practical application. Nevertheless, this study presents novel findings and exciting prospects for the understanding and prediction of the effect of initial constituents on final wine aroma.

3.6 References

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Chapter 4

General Discussion and Conclusions

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4.1 Concluding Remarks and Future Prospects

The composition and relative concentrations of amino acids in grape must are important factors for the determination of fermentation performance and aroma compound production. *S. cerevisiae* requires sufficient YAN compounds for the synthesis of proteins that allow for structural cell maintenance, enzyme production, and biomass formation. The metabolism of many amino acids results directly and indirectly in the production of aroma impact compounds. As the largest and most diverse group of YAN compounds found in grape must (Spayd & Andersen-Bagge, 1996; Bell & Henschke, 2005) and considering their variability between grape musts (Hernández-Otre *et al.*, 2002; Huang & Ough, 1989), amino acids are arguably the most important nitrogen compounds found in the initial grape must.

This study provided a novel evaluation of the efficiency of single amino acids as the sole nitrogen source by industrial wine yeast strains under conditions resembling wine fermentations through the observation of four growth characteristics; exponential growth rate, biomass formation, lag phase, and fermentation duration. Previous literature on amino acid efficiency by Cooper (1982) and Godard *et al.* (2007) utilized exponential growth rate as the sole growth characteristic to evaluate efficiency. From a practical winemaking perspective, the evaluation of amino acids utilizing a greater number of growth parameters is more valuable than exponential growth rates alone. It could be argued that amino acids that support good biomass formation and shorter fermentation times would be of greater value.

In general, the trends observed in industrial wine yeast strains under fermentative conditions were in agreement with the observations of literature of laboratory yeast strains under respiratory growth and glucose limiting conditions. In general, those amino acids whose carbon backbones are readily incorporated into the TCA cycle after transamination (alanine, asparagine, aspartate, glutamine, glutamate, and serine) were found to be the most efficient sources of nitrogen as the single source of nitrogen. Furthermore it appears that the effect of amino acid composition on aroma production appears to be conserved between industrial wine yeast strains.

It was found that the total YAN concentration was the rate limiting factor for biomass formation for yeast that were supplied with an efficient source of nitrogen. However, as the complexity of amino acid treatments increased it was found that differences in growth kinetics between treatments also decreased. To our knowledge this is the first study to observe that combinations of inefficient amino acids could provide yeast with sufficient nitrogen diversity to give similar growth characteristics as a combination of efficient amino acids. While the efficient amino acids usually make up a little under half of the total amino acid concentration, this effect suggests that the diversity of nitrogen sources may be just as important for the health of fermentations as the total YAN concentration. Furthermore, the supplementation of grape musts with a single nitrogen compound such as ammonium is known to affect the production of aroma and flavour impact compounds such as acetic acid due to an increase in redox stress (Bely *et al.,* 2003; Vilanova *et al.*, 2007). Supplementation with a combination of amino acids may be an alternative to ammonium supplementation to reduce the changes to the final wine flavour and aroma composition while improving fermentation performance.

The ability to predict and modulate the final aroma of wine based on the initial composition of the grape must is an exciting tool of great potential value. Many studies have focused on the effects of amino acid supplementation on the final production of major volatiles in real grape must (Garcia *et al.*, 1993; Hernández-Orte *et al.*, 2006; Miller *et al.*, 2007) as well as in synthetic media (Albers *et al.*, 1996; Vilanova *et al.*, 2007; Carrau *et al.*, 2008; Barbosa *et al.*, 2009). The findings of these studies suggest that amino acids play an important role in the formation of aroma impact compounds, however, due to the complex nature of the nitrogen treatments it is unclear what the effect of specific amino acids on the formation of these compounds are. In this work it was found that the production of higher alcohols, volatile acids, and a few associated esters formed from the degradation of the BCAAs isoleucine, leucine, phenylalanine, and valine were able to be predicted with a reasonable accuracy when the initial concentration of the amino acid was known. Further evaluation of the robustness of these predictions is required for fermentations in real grape must. However, there is evidence that this relationship between major volatile production and amino acid concentration found in synthetic media will be reflected in real grape must fermentations (Smit, 2013).

From a practical perspective, understanding the relationships between the initial amino acid composition of grape musts and the evolution of aroma impact compounds would be a powerful tool. The future applications of this information could drastically improve current nitrogen supplementation practices to improve fermentation health with little effect on the final wine. Other applications could be the supplementation of wine musts with amino acids for the modulation of the wine aroma.

While these future applications are speculative, the findings of this study underline the importance of nitrogenous compounds in grape must as they are crucial for the health of fermentations. Furthermore, changes to the YAN composition appear to have a dramatic effect on final major volatile composition. Understanding how YAN composition affects the final wine aroma and flavour could be a valuable tool to help winemakers to optimize their wines.

4.2 References

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