Universidade Trás-os-Montes e Alto Douro

Evaluation of starter cultures for application in Oenology

Versão Definitiva

Mestrado em Biotecnologia e Qualidade Alimentar

Fabiana Oliveira Quintas

Prof. ªDoutora Ana Alexandra Ferreira

Prof.ª Doutora Elsa Cristina Dantas Ramalhosa

VILA REAL, 2017

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Fabiana Oliveira Quintas

Orientador: Prof. ªDoutora Ana Alexandra Ferreira

Co-orientador: Prof.ª Doutora Elsa Cristina Dantas Ramalhosa

Composição do Júri:

Prof.ª Doutora Elsa Cristina Dantas Ramalhosa

Prof. Doutor Luís Pereira do Amaral

Doutora Catarina da Costa Rodrigues Barbosa

VILA REAL, 2017

Este trabalho foi realizado na unidade de Microbiologia e Biotecnologia do Vinho da Universidade de Trás-os-Montes e Alto Douro, integrada no BioISI - Instituto de Biossistemas e Ciências Integrativas, no âmbito do projeto SMARTWINE – *Smarter wine fermentations: integrating OMICS tools for the development of novel mixed-starter cultures for tailor-made wine production*, com a referência. PTDC/AGR-TEC/3315/2014 - POCI-01-0145-FEDER-016834, financiado pela Fundação para a Ciência e Tecnologia e co-financiado pelo Fundo Europeu de Desenvolvimento Regional (FEDER) através do COMPETE 2020 – Programa Operacional Competitividade e Internacionalização (POCI) e do Programa Operacional Regional de Lisboa".

“Seja feliz do jeito que você é, não mude sua rotina pelo o que os outros exigem de você, simplesmente viva de acordo com o seu modo de viver “

Bob Marley

Aos meus pais

ACKNOWLEDGEMENTS

Finalizada mais uma etapa académica, não podia deixar de expressar os meus agradecimentos a todas as pessoas que contribuíram e me motivaram no decorrer deste ano de trabalho.

Agradeço aos meus orientadores, à Professora Doutora Alexandra Mendes Ferreira e Professora Doutora Elsa Ramalhosa, por terem aceite orientar a minha dissertação de mestrado, pelos conselhos e ensinamentos transmitidos, por se demonstrarem sempre disponíveis para ouvirem atentamente as minhas questões e pela dedicação prestada.

Agradeço à Professora Doutora Arlete Faia pela ajuda e disponibilidade sempre demonstradas e pelos conhecimentos transmitidos.

Agradeço a todos os funcionários, técnicos e grupo de investigação do Departamento de Enologia da Universidade de Trás-os-Montes e Alto Douro, pela força, carinho e amizade.

Agradeço de um jeito especial aos meus pais, Mário Camarinha e Fernanda Fonseca, por todo apoio incondicional que me deram, todo o carinho,compreensão, porque nunca deixaram de acreditar em mim, sem vocês nada disto teria sido possivel. Um muito obrigada.

Agradeço ao meu irmão, Ludovic Quintas, pela compreensão, boa disposição, e apoio durante o meu percurso academico.

Agradeço ao resto da minha familia, que são das pessoas mais importantes na minha vida, e sempre me apoiaram e ajudaram a ultrapassar todas as divergências da vida.

Agradeço á Andreia Alves, por todo carinho, simpatia e acima de tudo por toda amizade demonstrada em todo o meu percurso académico. Por todas as conversas, desabafos, videos partilhados, risota sem qualquer motivo válido obrigada por tornares os meus dias mais felizes. E um especial agradecimento a todos os moradores do Andreia Alves, Bruno Lopes, Cátia Rodrigues, Rita Machado e Pedro Rodrigues pela estadia 2ºesquerdo, por me receberem tão bem, com um grande avontade, mimo e aconchego , não há nada que pague isso, confesso que vou sentir saudades desse ambiente familiar.

Agradeço á Carina Guedes, por todo o apoio, por estar sempre presente em todos os momentos importantes da minha vida, e acima de tudo nunca me deixar desistir nem baixar os braços. Agradeço por todos os cafés, todas as conversas de Domingo, todas as palestras de psicologia, e apesar de todos os obstáculos, tem sempre uma palavra amiga e uma solução inteligente. Obrigada mesmo, já não me vais aturar mais com a frase:" Tenho que escrever a tese".

Agradeço á Raquel Invulgar, pelo apoio e carinho, pelos conselhos fantásticos que consegue transmitir, pela alegria contagiante e pelo sentido bom de viver a vida, todos os problemas tornam-se uma animação.

Por fim mas não menos importante, agradeço em especial aos meus amigos, que sempre me ajudaram, demonstrando que o sorriso é a melhor arma para ultrapassar problemas e alcançar objetivos.

Abstract

Over the last years, the role of *non-Saccharomyces* yeasts in winemaking, previously diabolized and neglected has been re-evaluated, and their use has been proposed in controlled mixed fermentation towards the improvement of wine complexity, aroma profile and control of spoilage microorganisms. Based on previous studies, where the yeast strain *Hanseniaspora guilliermondii* UTAD222 was found to significantly alter the profile of aroma compounds found at the end of the fermentation when inoculated in consortium with *S. cerevisiae* UCD522, in this study we aim to increase knowledge on this yeast consortium. For this purpose, Response Surface Methodology (RSM) based on central composite (CCD) was employed to statistically evaluate the combined effect of different conditions, on yeast growth and fermentative activity and on the production of sensory relevant wine compounds. Four independent variables i.e., fermentation temperature (10ºC-30ºC), initial nitrogen (100-500 mg/L) and sugars concentration (150–300 g/L) and inoculum levels of *H. guillliermondii* (0 – $1x10^6$CFU/ml) were studied. The statistical analysis of the experimental data showed that overall, nitrogen was the main factor impacting fermentation kinetics and metabolites produced, followed by sugars levels and fermentation temperature. More important, the effects seen due to *H. guilliermondii* presence prompt the future application of this *non-Saccharomyces* strain in mixed-starter culture wine fermentations.

Keywords: wine, non-*Saccharomyces*, mixed-cultures, Central Composite Design, Response Surface Methodology

Resumo

Nos últimos anos, o papel das leveduras não-*Saccharomyces* na vinificação, previamente diabolizado e negligenciado, tem sido reavaliado tendo sido proposta a sua utilização em fermentações com vista à melhoria e/ou distinção do perfil aromático dos vinhos. Tendo por base estudos prévios, em que se verificou que a estirpe *Hanseniaspora guilliermondii* UTAD222 altera significativamente o perfil de compostos aromáticos encontrados no final da fermentação quando inoculado em consórcio com *S. cerevisiae* UCD522, neste estudo foi objetivo aumentar o conhecimento sobre este consórcio. Para isso, foi utilizada a metodologia de superfície de resposta (RSM) associado a um desenho experimental de compósito central (CCD) para avaliar o efeito combinado de diferentes condições de fermentação no crescimento e atividade fermentativa das leveduras bem como na produção de compostos do vinho sensorialmente relevantes. Quatro variáveis independentes, ou seja, temperatura de fermentação (10ºC - 30ºC), concentração de azoto (100-500 mg/L) e de açúcares (150-300 g/L) iniciais e níveis de inóculo de *H. guillliermondii* (0-1x10^6 CFU/ml) foram estudados. A análise estatística dos dados experimentais mostrou que, em geral, o azoto foi o principal fator que afetou a cinética de fermentação bem como os metabolitos produzidos, seguido pelos níveis de açúcares e temperatura de fermentação. Mais importante, os efeitos relevantes associados à presença de *H. guilliermondii* reforçam o interesse na sua aplicação futura em fermentações vínicas conduzidas por culturas mistas.

Palavras-chave: vinho, não-*Saccharomyces*, cultura mista, modelo composito central, metodologia de superfície de resposta

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Abbreviations

DAP - Diammonium phosphate

GJM - Grape juice medium

Hg - *H.guilliermondii* UTAD222

Sc - *S.cerevisae* UCD522

S- Sugar

N – Nitrogen

T- Temperature

I- Inoculum

CFUs – Colony forming units

WL - Wallerstein Laboratory agar

YAN – Yeast assimilable nitrogen

YPD - Yeast Peptone Dextrose agar

RSM – Response surface metodology

CHAPTER I

Literature Review

I.1. Microbial communities in grapes and wine: Particular focus on yeasts.

The wine is a complex matrix consisting of ethyl alcohol, several other alcohols, sugars, other carbohydrates, polyphenols, aldehydes, ketones, enzymes, pigments, at least half a dozen vitamins, 15 to 20 minerals, more than 22 organic acids, volatile-, and non-volatile compounds, defined as a chemical symphony for the first time by Amerin *et al.*,(1972). These compounds come from grapes, from the metabolic activity of yeasts during alcoholic fermentation and from phenomena that may occur during conservation and aging. The wine quality is strongly dependent on the concentration of each one of the compounds and on equilibrium of the various chemical compounds.

Grape-berries harbor diverse microbial communities, consisting of yeasts, filamentous fungi and bacteria. Yeasts have a pivotal role on the transformation of grape must into wine, since yeasts catalyze the rapid, complete and efficient conversion of sugars into ethanol, carbon dioxide and other metabolites such as higher alcohols, esters, among others which give sensorial attributes to the wine, the so called ‘‘fermentation bouquet’’ (Rapp *et al.*,1991; Mendes-Ferreira *et al.*,2011). Thus, routine inoculations with selected pure strain of active dry yeasts of *Saccharomyces cerevisae* is still a common practice in most wine-producing regions since the middle of the 20th century, since this fermentative specie occurs at extremely low populations on healthy and undamaged grape-berries (Pretorius *et al.*,2000). The inoculation practice also insures reliable fermentations and allows to obtaining wines with an adequate final quality, that means with higher concentration of desirable fermentation products and less amounts of undesirable metabolites that may reduce the quality of the wine such as hydrogen sulfide, acetic acid and aldehydes. On the other and the inoculation of grape-juice with *S. cerevisiae,* will also reduce the persistence and contribution of the native grape-berry-yeasts like the so-called apiculate yeasts (*Kloeckera apiculata/ Hanseniaspora uvarum*) and other less prevalent yeasts of the genera *Candida, Brettanomyces Cryptococcus, Kluyveromyces, Metschnikowia,* and *Pichia* (Fleet *et al.*,1993; Fleet *et al.*,2003; Pretorius *et al.*,2000),but does not necessarily suppress growth of these indigenous species, nor does it ensure that the inoculated strain will become dominant over indigenous strains of *S. cerevisiae* (Fleet *et al.*,1990; Fleet *et al.*,2003).

In fact, the dominant yeasts in grapes initiate grape-must fermentation but their growth, rather than being limited to the first two or three days of fermentation due to their weaker ethanol tolerance and inability to survive to the increasing concentrations of ethanol produced by *S. cerevisiae* as thought previously, can persist or even dominate longer periods either in spontaneous or inoculated fermentations, (Fleet *et al.*,1993; Fleet *et al.*,2003; Fleet *et al.*,2008). The persistence of *non- Saccharomyces* yeasts throughout alcoholic fermentation or being eliminated early depends on winemaking conditions, grape juice composition, winery practices, and type of inoculation used (Bisson *et al.*,1999; Bisson *et al*.,2010). Growth of these yeasts could strip the grape juice of thiamine and other micronutrients needed by *S. cerevisiae,* leading eventually to stuck or sluggish fermentation (Bisson *et al.*,1999). Thus, *non- Saccharomyces* yeasts are capable of altering the fermentation dynamics, composition and flavor of wine.

In recent years, and considering consumer's demands for "terroir" wines, with personality and character of a given region, winemakers have been looking for the potential use of natural yeasts to start must fermentation to promote the regional characteristics of flavor and character of such wines. In fact, under certain conditions, some of the indigenous yeast may produce significantly higher amounts of aromatic esters than *Saccharomyces*, conferring increased perceived complexity in wine either positively or negatively (Romano *et al.*,2003; Moreira *et al.*,2011). Thus in the last two decades, several research groups have examined various *non- Saccharomyces* yeasts as potential adjuncts to *S. cerevisiae* to exploit their flavor complexing properties (Jolly *et al.*,2014; Jolly *et al.*,2006; Bely *et al.*,2008), considering that the more diverse the microbial communities, the more diversity of final metabolites the wine will have.

Despite the increasing interest in the industrial application of *non- Saccharomyces*, more research is still needed for a better understanding their succession and dominance within the total population during alcoholic fermentation (Fleet *et al.*,2008; Mills *et al.*,2008).

I.2. The yeasts and the wine fermentation

The term "wine fermentation" is used for the result of the set of biochemical reactions due to the metabolic activity of the yeasts during wine vinification, leading to the transformation of grape-must into wine. We intend to distinguish this process from the biochemical process

known as alcoholic fermentation, consisting on the anaerobic transformation of sugars, mainly glucose and fructose, into ethanol and carbon dioxide, given by the overall chemical equation of Gay-Lussac (Zamora *et al*.,2009) presented below.

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$$

Yeasts are chemoorganotrophic microorganisms deriving their chemical energy, in the form of ATP, from the breakdown of carbon substrates. Sugars are their preferred carbon and energy sources. In grape-juice, the sugars are almost entirely glucose and fructose, ranging from 150 to 300 g/L, other sugars exist in very small amounts, such as sucrose, melibiose, raffinose and traces of pentoses (Boulton *et al.*,1996). Most wine-related yeasts ferment glucose more rapidly than fructose, while others, such as *Zigosaccharomyces bailii,* preferentially ferment fructose (Sand *et al.*,1983) and others, such as *S. uvarum*, indifferently utilize glucose and fructose (Bell *et al.*,2005).

The biochemical pathway used by yeasts to produce energy from hexoses, namely glucose, is the glycolysis, also called Embden-Meyerhof-Parnas pathway, until the formation of 2 moles of pyruvate. Under aerobic conditions, and /or low sugar concentration, oxidation will be the predominant process and, under these conditions, pyruvate can be completely oxidized to CO_2 via TCA cycle. Conversely, under anaerobic conditions or at high glucose concentrations (such as in grape-juice) even in aerobic conditions in those yeasts that exhibit the Crabtree effect such as *S. cerevisiae* (Bisson *et al.*,1993) pyruvate is instead decarboxylated through the pyruvate decarboxylase, resulting in acetaldehyde which is then reduced to ethanol with the concomitant oxidation of the coenzyme NADH $+H^+$. The coenzyme NADH +H was previously generated in the oxidation of glyceraldehyde-3-phosphate to 1,3-diphosphoglyceric acid. Regeneration of NAD+ is necessary to maintain the redox balance and to prevent the halt of glycolysis, Figure 1 (Bisson *et al*.,1993).

Besides ethanol, other different strategies are used by yeast to regenerate NAD^+, leading to several other end products such as glycerol, succinic acid, acetic and lactic acids, acetaldehyde, α-keto acids, aldehydes and the correspondent alcohols, diacetyl, acetoin and 2,3-butanediol, as well as several other metabolites with impact on the organoleptic characteristics and thus on the final complexity of the wine.

Glycerol is quantitatively the most important secondary metabolite in wine fermentation (Ciani *et al.*,1996). The production of glycerol is modulated by fermentation conditions, especially those that affect growth or physiological stress (Ugliano *et al.*,2009).It is formed by yeasts in the initial phase of the alcoholic fermentation, just after the addition of the inoculum and its formation appears to result from the competition between the enzymes alcohol dehydrogenase and glycerol-3-phosphate dehydrogenase to re-oxidize the reduced coenzyme NADH $+H^+$ (Fleet *et al.*,2003; Bisson *et al.*,1999). Acetaldehyde is the preferred electron acceptor; however, when it exists in media is insufficient in the media possibly because it is combined with SO_2 being the oxidation of the coenzyme NADH + H^+ carried out through the dihydroxyacetone-phosphate followed by dephosphorylation to glycerol. Unlike aerobic growth which use O_2 as the terminal electron acceptor, anaerobic growth depends on glycerol production to restore NAD+:NADH balance (Bisson *et al.*,1999).

The amount of glycerol produced is dependent on the yeast strain, the temperature of vinification and on the amount of sulfur dioxide used as antiseptic in musts (Ciani *et al.*,1996; Viana *et al.*,2008). SO_2 addition to grape-must can stimulate glycerol production by forming a hydroxysulfonate adduct with acetaldehyde, although that increase is relatively small at the rates of SO_2 added in winemaking (Ugliano *et al.*,2009). At high sugar concentration, there is an increase on the glycerol production due to osmotic stress (Pigeau *et al.*,2005).Nitrogen availability stimulates yeast biomass formation and, consequently, more NADH production, leading to more glycerol production (Vilanova *et al.*,2007). Growth on inorganic nitrogen (ammonium salts) compared to mixtures of amino acid also generates NADH due to amino acid biosynthetic reactions, which stimulates glycerol production (Taillandier *et al.*,2007).

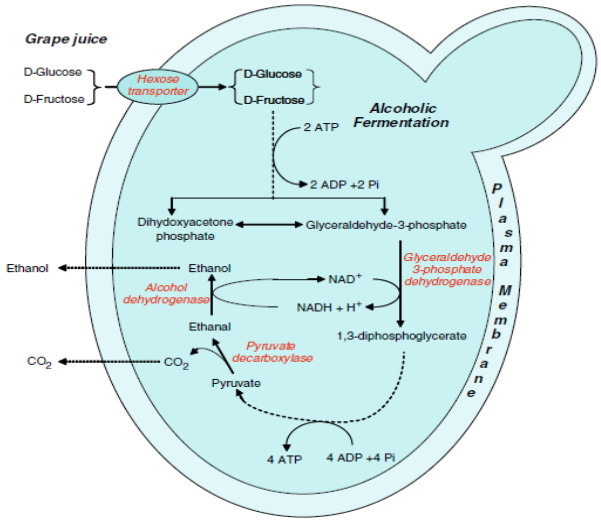

Fig.1: The alcoholic fermentation (Zamora *et al.*,2009).

The biochemical mechanism proposed for the production of diacetyl, acetoin and 2,3-butanediol involved the α-acetolactate, formed by the condensation of pyruvate with acetaldehyde-TPP, which is decarboxylated to diacetyl. Acetoin can also be formed by directly reduction of diacetyl and 2,3-butanediol is formed in yeast by reducing acetoin through acetoin reductase (Zoecklein *et al*.,2004; Bisson *et al.*,1999). At low levels (5 mg/L), diacetyl is considered to add complexity to wine aroma since it can impart positive nutty or caramel characteristics, the most significant ketone produced by yeast is diacetyl (2,3-butanedione), although malolactic bacteria are a more important source, when malolactic fermentation occurs in the wine. At low levels and according to the type of wine, is considered to add complexity to wine aroma since giving a 'nutty', 'toasty' or 'buttery' aroma depending on its concentration (Ugliano *et al.*,2009). At levels above 5 mg/L it can result in spoilage, creating an intense buttery or butterscotch flavor, and is perceived as a defect (Ugliano *et al*.,2009).

Higher alcohols are aliphatic and aromatic alcohols containing at least six carbons atoms, which are the largest group of the flavor compounds detected in alcoholic beverages (Mills *et al*.,2008). In concentrations exceeding 400 mg/L higher alcohols can impart a strong, pungent smell and taste, whereas optimal levels below 300 mg/L confer fruity characters (Lambrechts *et al.*,2000; Pretorius *et al.*,2000; Swiegers *et al.*,2005). It is generally accepted that higher alcohols come from the 1) pathways involved in the synthesis of branched-chain amino acids and 2) catabolism of valine, leucine, isoleucine, and 2-phenylalanine, known as the Ehrlich pathway. Through this pathway, the α-keto acids derived from the sugars via pyruvate or from transamination/deamination of amino acids are decarboxylated to the corresponding aldehyde, which is then reduced to the corresponding alcohol (Wang *et al.*,2016; Moreira *et al.*,2011; Moreira *et al.*,2008; Mendes-Ferreira *et al.*,2011).

The majority of esters found in wine results from the activity of yeasts during the alcoholic fermentation (reviewed by Mendes-Ferreira *et al.*,2011). Esters are synthesized throught via of the lipid and acetyl-CoA metabolism during fatty acid biosynthesis or are produced by chemical esterification of alcohols with acids during ageing (Lambrechts *et al.*,2000; Saerens *et al.*,2008). Ethyl esters arise from the condensation of acyl-CoA compounds with ethanol by acyl-CoA:ethanol O-acyltransferases, whereas acetate esters result from the condensation of acetyl-CoA with higher alcohols, by alcohol acetyltransferases (Boulton *et al.*,1996; Miller *et al*.,2007)

During fermentation, the metabolic activity of yeast is not circumscribed to the degradation of sugars. In fact, some of the acids of the grape-musts undergo more or less relevant modifications and other acids appear during the process. The tartaric and citric acids are not used by yeasts under wine fermentation conditions while malic acid is partially used (3 to 45%) by *S. cerevisiae*. The biochemical mechanism involves the malic enzyme and oxidative decarboxylation of malate to pyruvate and then through pyruvate decarboxylase and alcohol dehydrogenase, it is converted to ethanol and CO_2 (Cordente *et al.*,2012; Fleet *et al.*,2003).

Succinic acid is the main acid produced by yeast during wine fermentation. Most of the succinic acid is formed by yeasts during the early stages of alcoholic fermentation (Klerk *et al.*,2010), but its formation has been also detected during yeast stationary phase (Styger *et al.*,2011). The biochemical mechanism of succinate formation is not completely elucidated: some authors consider succinate formation appears not to be associated with the intracellular concentration of reduced coenzyme, indicating its formation by an oxidative pathway in agreement with the conclusions of (Styger *et al.*,2011; Schnierda *et al.*,2014). Other authors consider that the functioning of TCA cycle is seriously limited and not operative in wine fermentation conditions. The amount of succinic acid produced during alcoholic fermentation was found to depend on the yeast strain, fermentation temperature and chemical composition of the synthetic grape juice (Klerk *et al.*,2010). Acetic acid is quantitatively and sensory the most important volatile organic acid produced during alcoholic fermentation (Ugliano *et al*.,2009). It is formed by yeast during fermentation in the range of 0.1 to 0.3 g/L and later by malolactic bacteria at concentrations rarely exceeding 0.7 g/L. Acetic acid gives a negative sensory attribute (vinegar-character) to wine still remains when present at high concentrations, with an acceptable concentration of 0.2 to 0.7 g/L. The biochemical mechanism of acetic acid formation by wine yeasts not completely elucidated (Boulton *et al.*,1996; Ribéreau-Gayon *et al.*,2006). The possible biochemical reactions in yeast that can lead to acetic acid formation are as follows: 1) reversible formation from acetyl Co-A and acetyl adenylate through acetyl Co-A synthetase; 2) cleavage of citrate by citrate lyase; 3) production from pyruvate by pyruvate dehydrogenase; 4) reversible formation from acetyl-phosphate by acetyl kinase and 5) oxidation of acetaldehyde by aldehyde dehydrogenase (Boulton *et al.*,1996).

I.3. Factors affecting yeasts growth and fermentative performance

There are several variables which can affect the fermentation process and final quality of wine. Grape-must is far from being an optimal growth medium for yeast hence numerous factors can interfere with yeast growth and fermentative performance namely: grape-must composition (high sugar concentration, variable nitrogen concentration, low pH, deficiency of vitamins and or minerals), presence of antimicrobials, variation of temperature during vinification, among others (Bisson *et al.*,1999), In this review we will focus on the following factors: sugar and nitrogen concentration, temperature variation and presence of *non-Saccharomyces*.

I.3.1. Sugar concentration

The growth and survival of yeasts during the winemaking process are affected early by the composition of the must, in particular, by the initial concentration of sugars, which varies between 125-250 g/L (Fleet *et al.*,1993). Some yeasts species can grow well in high sugars solutions, containing something like 40% of sugars, although it must be considered a stressful condition. By increasing the medium osmolarity through addition of salt or glucose, yeast initiates a complex adaptive responses are mostly governed by the high osmolarity glycerol (HOG) signaling pathway, (Saito *et al.*,2012). Therefore, it is expected that the initial concentrations of sugars in grape-juice, glucose and fructose, will selectively influence the species and/or strains of yeasts that are better adapted to grow under such conditions (Arroyo-López *et al*.,2009).

Grape juice composition and fermentations conditions significantly influence the growth and metabolism of yeasts that consequently impact the final composition of wines. Wine strains of *Saccharomyces* are typically resistant to 16-17% ethanol while many wild strains are not as tolerant. If the grapes are harvested at a high Brix level, these strains might not be able to complete the fermentation (Bisson *et al.*,1999). Besides the initial sugar concentration will significantly affect the several metabolites with relevance for the wine complexity and quality. The formation of fusel alcohols, and esters, is parallel to ethanol formation and is mainly produced by the fermentation of sugars by yeasts, thus the more sugars in grape-must, the higher

the potential of alcohols formation. The formation of acetic acid by yeasts during alcoholic fermentation was found to depend on sugar and vitamins concentration (Nordström *et al.*,1964). Also, at high sugar concentration, there is an increase on the glycerol production due to osmotic stress (Pigeau *et al.*,2005; Remize *et al.*,2000).

I.3.2. Nitrogen concentration

Following carbon, nitrogen is the second nutrient that most affects yeast growth and fermentative performance. In wine production, the amount of sugars present in grape-musts largely exceeds the nutritional requirements of yeasts, while nitrogen levels are highly variable, ranging from 60 to 2400 mg/L, and are often limiting to their growth. An insufficient concentration of nitrogen in grape-juice results in poor degradation of the sugars leading to slow and stuck fermentations and formation of hydrogen sulfide which can irreversibly depreciate the quality of the wines. A mean value of 140 mg/L (Tesnière *et al.*,2015) of assimilable nitrogen has been mentioned as being sufficient for complete fermentation of reasonably ripened grapes, whereas (Mendes-Ferreira *et al*.,2004) established a minimum of 267 mg/L of nitrogen for complete fermentation of 200 g/L of glucose in an industrially reasonable time. Thus the concentration of nitrogen needed to achieve total sugar degradation is not only dependent on the initial sugar concentration and fermentation conditions but also most significantly on the yeast strain used. Low levels of assimilable nitrogen lead to slow growth and low biomass accumulation, resulting in sluggish fermentation (Garde *et al.*,2008; Gutiérrez *et al.*,2012; Mendes-Ferreira *et al.*,2004). Yeast growth, like the fermentation rate, is an exponential function of the initial nitrogen content of must (Kemsawasd *et al.*,2015; Medina *et al.*,2012; Taillandier *et al.*,2007), being highly sensitive to low (less than 300 mg/L) and less responsive to higher nitrogen concentrations.

Nitrogen availability also significantly affects the metabolites released by yeast during alcoholic fermentation. The amount of glycerol is dependent on nitrogen availability, which may stimulate yeast biomass formation and, consequently, more NADH production, leading to more glycerol production (Taillandier *et al.*,2007; Vilanova *et al.*,2007). Comparison of yeast growth on ammonium salts or on a mixture of amino acids shows that the former N source

generates NADH due to amino acid biosynthetic reactions, which stimulates glycerol production.

Higher alcohols formed during fermentation depends on several factors namely the initial nitrogen content of the media (Vilanova *et al.*,2012; Moreira *et al.*,2008; Vilanova *et al.*,2007; Mendes-Ferreira *et al.*,2009). The less the nitrogen concentration in the medium, the more higher alcohols are formed (Mendes-Ferreira *et al.*,2009; Mendes-Ferreira *et al.*,2011). The amount of acetic acid produced by yeast appears to be inversely correlated with the initial nitrogen levels (Mendes-Ferreira *et al.*,2011; Kemsawasd *et al.*,2015; Koker *et al.*,2015). The lowest acetic acid concentrations occur around 200–250 mg/L of yeast assimilable nitrogen with increases of up to twofold at nitrogen concentrations outside this range (Vilanova *et al.*,2007). These levels are apparently unaffected by the initial sugar concentration and osmotic stress (Ugliano *et al.*,2009).

The formation of medium-chain fatty acids (MCFA) is affected by assimilable nitrogen content in grape-: hexanoic, octanoic and decanoic, and their respective ethyl esters ethyl hexanoate, ethyl octanoate, and ethyl decanoate, is stimulated by high assimilable nitrogen levels (Mendes-Ferreira *et al.*,2009). Also, the timing of nitrogen addition affects MCFA released by yeasts (Barbosa *et al.*,2009), a later addition of DAP, during stationary growth-phase, leads to a significant decrease in the production of these compounds. Factors that stimulate fatty acid biosynthesis also stimulate fatty acid ester formation (Saerens *et al.*,2008).

Succinic acid production is also dependent on the yeast assimilable nitrogen (YAN) content and genetic factors (yeast strain). In general, several commercial yeast strains produced more succinic acid under the low-nitrogen regime, being the quantity of succinic acid produced by the yeasts directly correlated with the final biomass (Barbosa *et al.*,2014). In contrast, Klerk *et al.*,(2010) verified that succinic acid production is favored by moderate amounts of metabolically available nitrogen (300 ± 50 mg/L).

The amount of esters formed by yeast during fermentation also depends on nitrogen availability. Addition of ammonium to grape-musts, increases the concentration of acetate esters and ethyl butyrate without any significant change on ethyl esters, ethyl hexanoate, ethyl octanoate and ethyl decanoate (Beltran *et al.*,2005; Barbosa *et al.*,2009; Saerens *et al.*,2008) using completely different fermentation conditions and a brewer's yeast strain verified that high

content of nitrogen in the fermentation medium resulted in only moderate changes in ethyl ester production.

Despite the innumerous studies carried out on this topic, important gap in knowledge still remains, hence it is not yet possible to completely predict and overcome fermentation problems, still common in modern oenology.

I.3.3. Temperature variation

Wines are generally not fermented at optimal temperatures for yeast growth. White wines are fermented at temperatures below 18°C, sometimes ranging from 12-14°C, while red wines are fermented at temperatures as high as the yeast can tolerate 35°C, to facilitate colour extraction. These temperatures can be tolerate by yeasts however, it causes a stress condition then affects several growth parameters like yeast specific growth rate (Fleet *et al.*,1993; Arroyo-López *et al.*,2009; Beltran *et al.*,2008; Canbafi *et al.*,2007) and consequently fermentation length. Low temperature restrict yeast growth and increases fermentation length (Torija *et al.*,2003), slower fermentations, with lower fermentation rates and Vmax (Beltran *et al.*,2008), resulting in changes on chemical and sensory properties of wines.

The use of low temperature in alcoholic fermentations is becoming more frequent due to the wish to produce wines with more noticeable aromatic profiles, but with high risk of having fermentation problems like too slow or stuck fermentations.

Temperature of vinification also affects the dynamics of yeast populations during alcoholic fermentation. At low temperatures, some non-Saccharomyces species have a higher chance of surviving, as they have low tolerance to ethanol subjected to these temperatures have a greater persistence in the fermentation, since ethanol does not become toxic. (Fleet *et al.*,1993; Llaurado *et al.*,2002). In fact, strains of *Kloeckera apiculata* dominate fermentations conducted at temperatures of 10-15°C, whereas *S. cerevisiae* dominates fermentations conducted at temperatures of 30°C (Llaurado *et al.*,2002; Merritt *et al.*,1966).

In sum, the temperature of fermentation affects growth and fermentative performance of wine yeasts and also the end products metabolites released by yeast namely glycerol, acetic

acid, acetaldehyde and ethanol (Torija *et al.*,2001). The amounts of ethanol, glycerol and higher alcohols showed a pick at 30°C (Merritt *et al.*,1966). Higher alcohols (Molina *et al.*,2007) and esters formation by yeast during fermentation (Romero-Gil *et al.*,2013; Salvadó *et al.*,2011) is dependent on the fermentation temperature. High temperatures encourage the production of fusel alcohols, while a lower fermentation temperature tends to increase the production of esters not (Molina *et al.*,2007) due to retention of volatile esters or inhibition of esterase activity , but due to changes in the expression profiles of several genes involved in esters synthesis under low temperature conditions (Saerens *et al.*,2008) using completely different fermentation conditions. Ethyl octanoate and decanoate production incresed with temperature when this varied between 14 and 26ºC, whereas ethyl hexanoate production decreased but only up to a temperature of about 20ºC (Saerens *et al.*, 2008). According to Suomalainen *et al.*,(1981), gives an explanation for his own results assuming that the increase in the fermentation temperature releases higher levels of esters through more efficient excretion and/or enhanced autolysis of the yeast.

Volatile compounds are, in general, significantly higher in wines produced at lower temperatures (Torija *et al*.,2003). Also the formation of fatty acids (Torija *et al*.,2003) and acetic acid produced by yeasts are affected by the temperature of vinification (Beltran *et al*.,2008). The amount of glycerol released by yeasts and succinic acid production are also dependent on the temperature (Klerk *et al.*,2010; Llaurado *et al.*,2002). Under wine fermentation conditions, succinic acid is favored by moderate to high fermentation temperatures (20°C to 28°C) in grape juice with a nicotinic acid and/ or nicotinamide deficiency, high sugar content (200 g/L to 240 g/L), moderate amounts of metabolically available nitrogen (300 ± 50 mg/L), the presence of flavonoids and large supplies of unsaturated long-chain fatty acids (Klerk *et al.*,2010). The author reports that fermentation temperatures below 18°C, too much metabolizable nitrogen (> 450 mg/L MAN), very high concentrations of fermentable sugar (> 240 g/L), lipid deficiencies and a lack of pantothenic acid, thiamine, biotin or pyridoxine will decrease the amount of succinic acid produced by fermenting yeasts.

I.3.4. Interaction with *non- Saccharomyces*

Yeasts occurring in grape-musts at the early stages of fermentation have two main origin, the vineyard, the grapes, and from surfaces and equipment in the winery (Bisson *et al.*,1999). *S. cerevisiae* occurs at extremely low populations on healthy and undamaged grape-berries, but it is a natural resident in the winery (Pretorius *et al.*,2000; Andorrà *et al.*,2012; Ciani *et al.*,2016). Very recently, Guilloux *et al*.,(2016) and co-workers were able to isolate two genera of yeast, *Hanseniaspora* and *Saccharomyces*, from grape-must and from the winery environment before harvesting but not on grape berries. The genus *Hanseniaspora* represented 27% of isolates in the grape-must and 35% of isolates in the winery environment (Grangeteau *et al.*,2015). Thus, this work opens new avenues about the implantation and persistence of some *non- Saccharomyces* species from one year to another in the winery environment, as has been described before for *S. cerevisiae* (Ciani *et al.*,2009; Cordente *et al.*,2012; Hierro *et al.*,2007). However it should be pointed out that both climate and vineyard management may affect microbial communities in grape berries and musts and its composition as recently confirmed by Brilli *et al*.,(2014). In fact the authors have studied the long-term relationship (5 years) between yeast quantity and composition and the meteorological variables (air temperature, relative humidity, and rainfall) in a vineyard located at Tuscany. Their results indicated total yeast were correlate with the weather conditions (rainfall and relative humidity) 25 to 30 days before harvesting whereas the population of *K. apiculate* and *C. zemplinina* were correlated with temperature 10 days before harvest (Brilli *et al*.,2014).

As an example of yeast-yeast interaction is the fact already mentioned before that the *non-Saccharomyces* species died early because they are less tolerant to ethanol and high temperature and more oxygen dependent, than *S. cerevisiae* (Jolly *et al.*,2006; Lleixà *et al.*,2016). Others authors claim that *non- Saccharomyces* yeasts have negative impact on the growth of *S. cerevisiae* (Domizio *et al*.,2011), by competing for nutrients or by producing toxic end products, such as medium chain fatty acids and killer factors (Bisson *et al.*,1999), that may limit growth and fermentative ability of *S. cerevisiae* strains (Fleet *et al.*,1999; Medina *et al.*,2012). Others consider that the dominant behavior of *S. cerevisiae* against yeasts of some species is only expressed when they sense other yeasts in the same environment.

Thus, the interactions among wine microorganisms may act in a dual way: in some cases, the presence of some microorganisms may promote or inhibit the growth of others species or strains. But the mechanism(s) underlying yeast responses to the presence of other microorganisms, of the same or other species, are not completely elucidated. Additional studies

are needed to understand better the complex metabolic interactions and to describe the major impacts on wine composition and flavor (Ugliano *et al*.,2009).The persistence of yeasts during alcoholic fermentation is affected by the other microorganisms present. In general, *S. cerevisiae* strains have however, evolved to withstand and grow in most of the stress conditions that grape must and wine fermentations offer (Kemsawasd *et al*.,2015).

At the onset of natural alcoholic fermentation, the dominant yeasts will advance during the winemaking process, and various species can cohabitate, some better than others, and even interact with one another for short or longer time periods. Depending on the vigour of *S.cerevisae* active dry yeast (ADY) used for inoculation grape-musts, the natural yeasts will grow at different extent, and will contribute to final wine character, hence ADY does not necessarily suppress growth of these indigenous species, nor does it ensure that the inoculated strain will become dominant over indigenous strains of *S. cerevisiae* (Lambrechts *et al.*,2000*;* Albergaria *et al.*,2016).

It is generally recognized that, the more microbial diversity and longer coexistence, the more distinct and diverse characteristics the final wine will have. Thus, winemakers have been combining strains of *S.cerevisiae* for several decades on the basis of their empirical observations that mixed-cultures produce more flavour diversity and balanced wines, by introducing a greater range of flavor notes and moderating the intensity of distinctive estery/fruity notes, such as pineapple and banana in Chardonnay wines. Ugliano *et al.*,(2009); and Lage *et al.*,(2014) when using mixed cultures with more than one strains of *S. cerevisae,* verified that this cohabitation affected yeast biomass, fermentation kinetics and by-product formation; the couple *S. cerevisiae, S. uvarum* hybrid produced more acetaldehyde, suggesting that acetaldehyde exchange between the two strains could play an important role in inhibiting some yeast strains and allowing the growth of others. Also, Arroyo-López *et al*.,(2009) who investigated the cofermentation of *S. cerevisae* T73, *S. kudriavzevii* and the hybrid strain *S. cerevisiae* × *S. kudriavzevii* described the competition between two species at different temperatures (17 °C, 24 °C and 31 °C). Both yeasts showed a reduction in their maximum specific growth rates in mixed fermentations and the antagonistic effect between them was influenced by the temperature. In respect to aroma profiles of wines fermented with more than one species of *Saccharomyces*, Cordente *et al*.,(2012) found that cofermentation not only produced a metabolite profile different from wines made by single culture fermentation, but also in blends of single cultures, whereas Nordström *et al*.,(1964) obtained similar

concentrations of ethyl esters and higher alcohols tended to be produced by the highest yeast producer in monoculture, whereas ethanol, glycerol and fatty acids tended to be produced in intermediate concentrations (Suomalainen *et al.*,1981).

Indeed, there are only a few studies on the interaction between the yeasts (competition/complementarity) during natural or inoculated fermentation that can influence the quality of the final wine. An interaction of *Saccharomyces* with other species is reported in the literature (Lage *et al.*,2014). In this study, conducted in our laboratory, we clearly provide evidence that *H. guillermondii* negatively affected *S. cerevisiae* growth and fermentative kinetics, and differences, despite of being minor, were detected on the aroma profiles of mixed-culture fermented wines compared to those obtained by *S. cerevisiae* in single-culture. Some of them are perceived and the wines preferred by sensory tests. Tofalo *et al.*,(2016) reported a single class of aroma compounds produced for a specific yeast, when the aroma profile of wines fermented with natural *Saccharomyces* were compared with those co-inoculated with and *non-Saccharomyces*.

The role of *non- Saccharomyces* yeasts on aroma profiles is due to a diversity of enzymes that can modify some molecules in wine. Fernández *et al.*,(2000), have surveyed enzymatic activities of 182 *non- Saccharomyces* yeasts isolates from the La Mancha Appellation of Origin in Spain. Nearly 80% of the yeasts possessed one or more enzymes of biotechnological interest. The enzyme β-glucosidase was more linked to the species *Metschnikowia pulcherrima*, whereas polygalacturonase activity was common in most of the species tested. Proteolytic activity was observed in *Pichia membranifaciens* and in *Metschnikowia pulcherrima*. *Non- saccharomyces* due to their high β-glycosidase activity can hydrolyze part of the bound monoterpenes, thus enhancing the wine aroma and flavor. New isolates of *non- Saccharomyces* yeasts have been screened for enzymatic activities and some yeast showed high activity of β-glucosidase and β-xylosidase, confirming previous data that wines fermented with selected mixed-cultures could have a better and distinct quality than others (López *et al.*,2015).

On the other hand, as consequence of the re-evaluation of the role of *non- Saccharomyces* yeasts, there is an increasing interest on the use of different species in mixed inoculated fermentations where the yeast interactions play a fundamental role (Sun *et al*.,2014).

Wine-related *non- Saccharomyces* yeasts, such as species of *Torulaspora delbrueckii*, can contribute significantly to the fermentation by-product profile of wine because this yeast

produces low concentration of acetic acid, ethyl acetate, acetaldehyde and produce high amounts of glycerol (Ugliano *et al*.,2009). *Kluyveromces thermotolerans* is also moderate to high tolerant to ethanol and is characterized by moderate acetic acid, low acetaldehyde and off-flavors production. *Issatchenkia orientalis* produces relatively high concentrations of esters, higher alcohols and succinic acid together with moderate concentrations of acetic acid and acetaldehyde (Ugliano *et al.*,2009). This thermotolerant, acidophilic yeast was found to degrade L-malic acid, and in co-fermentation with *S. cerevisae* could produce wine with acceptable quality (Henschke *et al.*,2002). *Metschnikowia pulcherrima*, despite of being low tolerant to ethanol, this yeast when used as adjunct of *S. cerevisiae* gives interesting sensorial characteristics to the wines, thus in sensory tests these wines were preferred (Jemec *et al.*,2005; Jolly *et al.*,2003). *Hanseniaspora uvarum* is commonly the major yeast present on the grape berry and in musts and juices, but due to low tolerance to ethanol, populations decline quickly in the presence of *S. cerevisiae.* In single culture produce very high concentrations of acetic acid, ethyl acetate and acetaldehyde, which is a negative predicate to this yeast. However, when used with *S. cerevisiae* can produce wines with an acceptable balance of volatile and non-volatile compounds and sensory scores (Ciani *et al.*,2006; Jemec *et al.*,2005; Jolly *et al.*,2003). *H.guilliermondii* can produce more ethanol than *H. uvarum* and has a better balance of volatile and non-volatile compounds, including production of 2-phenylethyl acetate, which elicits a fruity, honey, rose-like aroma (Rojas *et al.*,2003; Swiegers *et al.*,2005; Lage *et al.*,2014).Taking this into account, this yeast strain when used as adjunct of *S.cerevisiae* has shown potential for aroma enhancement of wine.

Traditionally, the effects of different fermentation conditions on yeast performance and wine quality has been studied altering one-variable-at-a-time. This approach is extremely time consuming and expensive and unable of detecting optimum conditions particularly due to the interactions among the factors.

Nowadays, design of experiments and surface response methodology (RSM) have been used to optimize culture conditions and medium composition of fermentation processes, conditions of enzyme reaction and processing parameters in the production of food, drugs and enzymes by fungi, bacteria and yeasts (Hamouda *et al.*,2015; Shankar *et al.*,2015; Singh *et al.*,2014; Rollero *et al.*,2015; Ratnam *et al.*,2005; Gomes *et al.*,2013). Indeed, RSM allows the elucidation of how variables are related, obtaining statistically significant results independently of the class of variables. Taking advantage of the use of this kind of approaches, in the present

study a central composite model and RSM were used in order to understand the impact of sugar, nitrogen, temperature and inoculum on the fermentation kinetics, as well as on the aromatic composition of the wines.

I.4. Aims of the work

Evalution of the combined impact of fermentation conditions, namely initial sugar and nitrogen concentrations, fermentation temperature and co-inoculation with *H. guilliermondii* UTAD222 on fermentation activity and on the modulation of the quality of the final wines.

I.5. References

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CHAPTER II

Materials and Methods

Chaper II - Materials and Methods

II.1. Yeasts strains and maintenance conditions.

The strain *H. guilliermondii* UTAD222, previously isolated in our laboratory from a fermenting grape-juice from Douro Region, was selected for this study based on interesting oenological traits such as high ethanol tolerance and low potential for hydrogen sulfide production. *S. cerevisiae* UCD522 was supplied by the Enology Culture Collection, Department of Viticulture and Enology, University of California, Davis, USA. Pure cultures were routinely maintained at 4°C on YPD (Yeast Peptone Dextrose) slants, containing glucose 20 g/L, peptone 10 g/L, yeast extract 5 g/L and agar 20 g/L and the stocks were stored at -80°C with glycerol (40% v/v).

II.2. Synthetic grape juice media

In this study, the chemically-defined Grape-Juice Medium (GJM) used was similar in composition to typical grape juice, as previously described (Heard et al., 1988), with some modifications. Three different levels of initial yeast assimilable nitrogen (YAN) and sugar content were used (Table 2.1). YAN was supplied as a mixture of amino acids and ammonium in a proportion of 60:40, at final concentrations of 100, 300 or 500 mg/L. Ammonium was supplied as Di-ammonium phosphate (DAP) with a stock solution of 90 g/L, and amino acids (AA) with a stock solution 10x. These nitrogen levels were selected based on previous results obtained by our group (Mendes-Ferreira *et al.*, 2009) in which the effect of YAN on *S. cerevisiae* UCD522 growth, fermentative activity and metabolite production is demonstrated. The carbon and energy source was composed by an equimolar mixture of glucose and fructose (1:1) at final concentrations of 150, 225 or 300 g/L. This wide range of sugars concentrations were chosen in order to mimic a broader spectrum of "type of wines". For instance, Vinho Verde wines are known to have low alcohol levels, around 8.5 to 11%, while in another wine regions grapes naturally reaching the potential alcohol of 16%-18% are frequently found.

II.3. Inoculum and fermentation conditions

For all experiments, starter cultures were prepared by pre-growing the yeast overnight in 100 ml shake flasks, containing 70 ml of YPD: glucose 50 g/L, peptone 10 g/L and yeast extract 5 g/L, with pH adjusted to 3.5 with NaOH 10 M. The flasks were incubated overnight, at 25°C in an orbital shaker set at 150 rpm. These starter cultures of *S. cerevisiae* and *H.guilliermondii* were used for the inoculation of GJM, at the cell count considered for each experimental run. Inoculum levels consisted of an unchanging cell count of 7 $x10^5$ CFU/mL for *S. cerevisiae* UCD522, in single and in mixed culture with *H. guilliermondii* UTAD222. The fermentations were conducted in 250 ml-flasks filled to 2/3 of their volume fitted with a side-arm port sealed with a rubber septum to allow anaerobic sampling. Aseptic sampling was accomplished using a syringe type system. To avoid medium accumulation in the system, a stylet was inserted in the needle holder. The flasks were incubated at the appropriate temperatures (10-30°C) in an orbital shaker set at 120rpm. The choice of these temperature levels took into account that *non-Saccharomyces* yeast persistence during fermentation is highly influenced by this parameter, with low temperatures decreasing the sensitivity of these species to ethanol (Heard *et al.*,1988) and, consequently, wine fermentations conducted at temperatures less than 15–20 ºC may show a greater contribution from *H. guilliermondii*. Fermentation was monitored by weighing the fermentation flasks on a laboratory scale, assuming that once CO_2 saturation of the juice is reached, weight loss corresponds to CO_2 evolution, which in return is proportional to sugar consumption. In this way, R25 was estimated, corresponding to 25% of sugars consumed and fermentations were allowed to proceed until no further weight loss was observed (R100). Maximum fermentation rate (MFR) was determined from the slope of the linear dependence of the steepest decline in weight (g) at the corresponding time points (h).

II.4. Experimental design

A one block with an α-value equal to 1 and a central composite design (CCD) was constructed to investigate the influence of the following four independent factors: initial sugar content, initial nitrogen concentration, temperature of fermentation, and inoculation level. The dependent variables were: R25, R100, MFR, acetic acid, glycerol, ethanol,

succinic acid, as well as several aroma compounds produced. In this experimental design, there were three-coded factor levels: -1, 0, +1, in which -1 corresponded to the low level of each factor, 1 to the high level, and 0 to the mid-level. The correspondence between coded and uncoded variables is indicated in Table 2.1. Thirty-one experiments with seven replications in the central point (Experiments 1, 5, 14, 19, 20, 28 and 30) were performed (Table 2.2). In order to minimize the influence of systematic errors, the experiments were randomly performed.

Table 2.1- Levels of each one of the factors tested: Sugars, Nitrogen, Temperature and Inoculum.

Level	Sugar (g/L)	YAN (mg/L)	Temperature (ºC)	Inoculum level*
-1	150	100	10	0
0	225	300	20	$5x10^5$
1	300	500	30	$1x10^6$

*CFU/ml of *H. guilliermondii* UTAD222. the level -1 of inoculum corresponded to *S. cerevisiae* UCD522 in single culture at a cell count of $7x10^5$.

II.5. Statistical analysis

All statistical analysis was carried out using JMP 11 software (SAS Institute Inc, Cary, NC, USA). The relationship found between the dependent variables and the independent variables was established by the following second order polynomial model:

$$\mathbf{Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{23} x_2 x_3 + \beta_{24} x_2 x_4 + \beta_{34} x_3 x_4 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{44} x_4^2 + \varepsilon}$$

where x_1, x_2, x_3 and x_4 represent the independent variables: initial sugar content, initial nitrogen concentration, temperature of fermentation, and *H. guilliermondii* inoculation level, respectively, Y is the predicted dependent response, β_0 is the intercept term (corresponding to the estimated response at the center point), β_i is the linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the interaction coefficient term of variables i and j and ε is the independent N (0, σ^2) error term. Based on the value and sign of the β coefficients of the polynomial models we were able to determine the relative

contribution of the different independent variables and whether their effect on the different responses evaluated was positive or negative respectively.

The adequacy of the models was predicted through the analysis of variance (ANOVA) and the determination regression coefficient (R^2 and adjR^2). Furthermore, the lack-of-fit *p*-value of the models was used to check the quality of the second-order polynomial models. For some dependent variables (ethanol, glycerol, acetic and succinic acids), and in order to minimize the lack of fit of the obtained models ($p<0.05$), stepwise regression was undertaken with minimum AICc (Akaike information criterion) as stopping rule with progressive selection of the effects removing non-significant model terms. Whenever lack-of-fit was non-significant, response surface plots were generated for different interactions of any two independent variables, while holding the others constant to analyse the behaviour of the system within the experimental design.

Table 2.2 - Experimental conditions for the 31 fermentations.

RunOrder	PtType	Blocks	Sugars	Nitrogen	Temperature	Strain
1	0	1	0	0	0	0
2	1	1	-1	-1	1	-1
3	1	1	-1	1	-1	-1
4	1	1	-1	-1	-1	-1
5	0	1	0	0	0	0
6	-1	1	0	0	1	0
7	1	1	1	1	-1	1
8	1	1	1	-1	-1	1
9	1	1	-1	-1	1	1
10	1	1	1	1	1	-1
11	-1	1	1	0	0	0
12	-1	1	0	0	0	-1
13	-1	1	0	0	-1	0
14	0	1	0	0	0	0
15	-1	1	0	0	0	1
16	1	1	-1	1	1	-1
17	1	1	-1	1	1	1
18	1	1	-1	1	-1	1
19	0	1	0	0	0	0
20	0	1	0	0	0	0
21	1	1	1	1	1	1
22	-1	1	-1	0	0	0
23	1	1	1	-1	-1	-1
24	1	1	1	-1	1	1
25	-1	1	0	-1	0	0
26	1	1	-1	-1	-1	1
27	1	1	1	1	-1	-1
28	0	1	0	0	0	0
29	1	1	1	-1	1	-1
30	0	1	0	0	0	0
31	-1	1	0	1	0	0

II.6. Determination of viable cell number

The yeast growth was followed by counting the colony forming units (CFU's) in YPD agar, WL nutrient agar medium (Wallerstein's Laboratory), and/or Lysine agar (Oxoid) plates. WL was used for viable cell count of both *H. guilliermondii* and *S. cerevisiae* during fermentations, since this medium is a differential mean that allows identification of wine yeasts, based on the color and morphology of the colonies. *S. cerevisiae* colonies are white and bigger than those of *H. guilliermondii*, which are green and small. Moreover, in order to monitor the loss of viability of the *H. guilliermondii* during fermentation, Lysine medium was used, since it is a selective medium as *Saccharomyces* yeast cannot utilize lysine as a sole source of nitrogen and thus only the *non-Saccharomyces* yeasts are able to grow

II.7. Analysis of fermentation metabolites by liquid chromatography

The glucose and fructose consumed, ethanol converted, glycerol, succinic and acetic acids produced were determined in a high-performance liquid chromatography (HPLC Flexar, PerkinElmer, Shelton, Connecticut, USA) system equipped with the ion exclusion, cation exchange column Aminex HPX-87H (Bio-Rad Laboratories, Hercules, CA, USA) and refractive index and UV/VIS detectors. The column was eluted using sulfuric acid (0.005 N) at 60°C and a 0.6 ml min^{-1} flow rate. The samples were filtered through a membrane (Millipore, 0.22 μm pore size) before injecting 6 μl. The components were identified through their relative retention times compared with the respective standards, using the Perkin Elmer Chromera Software.

II.8. Analysis of volatile compounds by gas chromatography/mass spectrometry

At the end of alcoholic fermentations, samples were taken from different fermented media to be screened for aroma compounds production. Aliphatic higher alcohols (1-propanol, isobutanol, 2-methyl-1-butanol and 3-methyl-1-butanol) and ethyl acetate were analysed as previously described (Moreira et al. 2011) using a Hewlett- Packard 5890 gas

chromatograph equipped with a flame ionisation detector and connected to a HP 3396 Integrator. 50 μL of 4-methyl-2-pentanol at 10 g/L was added to 5 mL of wine as internal standard. The sample (1 μL) was injected (split, 1:60) into a CP-WAX 57 CB column (Chrompack) of 50 m × 0.25 mm and 0.2 μm phase thickness. The temperature programme was 40 °C (5 min) to 80 °C (0 min) at 3 °C. Injector and detector temperatures were set at 250 °C. Carrier gas was H_2 at 1–2 mL/min.

The determination of 2-phenylethanol, acetates of higher alcohols (isoamyl acetate, 2-phenylethyl acetate and hexyl acetate) and ethyl esters of fatty acids (ethyl butyrate, ethyl hexanoate, ethyl octanoate, ethyl decanoate and ethyl dodecanoate), ethyl lactate, volatile fatty acids (butyric, isobutyric, isovaleric acids) and free fatty acids (hexanoic, decanoic and dodecanoic acids) was performed in a Hewlett Packard 5890 gas chromatograph, equipped with a flame ionisation detector. For that purpose, 50 mL of wine, with 4-decanol at 1.5 mg/L as internal standard, was extracted successively with 4, 2 and 2 mL of etherhexane (1:1 v v−1) for 5 min. The organic phase (1 μl) was injected (splitless, 0.3 min) into a a CP-WAX 58 (FFAP)-CB column (Chrompack) of 50 m x 0.32 mm and 0.3 μm phase thickness. Temperature programme was 40 °C (1 min) to 220 °C (15 min) at 2 °C min^{-1}. Injector and detector temperatures were set at 220 °C. The carrier gas used was H_2 at 1–2 mL/min.

II.9. References

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CHAPTER III

Results and Discussion

III. Results and discussion.

III.1. Effect of fermentation conditions on fermentation kinetics

Thirty-one experiments were conducted to evaluate the effect of temperature, nitrogen and sugar initial concentrations and inoculation level with *H. guilliermondii* on wine fermentations, resulting in a variety of fermentation profiles (Table 1). Given the amount of experiments conducted in Figure 3.1, is depicted the fermentation profiles for selected experimental conditions. In order to evaluate the effects of the four independent variables on the fermentation kinetics three parameters were considered in this study, R25, R100 and MFR determined based on weight loss of the flasks (see Material and methods).

Table 3.1- Experimental conditions and R25, R100 and MFR for the 31 experiments.

Run	Sugars (g/L)	Nitrogen (mg/L)	Temperature (ºC)	Inoculum level	R25 (h)	R100 (h)	MFR (gCO_2/h)
1	225	300	20	1	48	216	0.267
2	150	100	30	0	24	144	0.192
3	150	500	10	0	120	360	0.117
4	150	100	10	0	120	480	0.083
5	225	300	20	1	48	216	0.263
6	225	300	30	1	24	168	0.354
7	300	500	10	2	144	1600*	0.125
8	300	100	10	2	360	1896*	0.037
9	150	100	30	2	48	192	0.171
10	300	500	30	0	24	216*	0.354
11	300	300	20	1	48	720*	0.250
12	225	300	20	0	48	216	0.283
13	225	300	10	1	120	648	0.121
14	225	300	20	1	48	216	0.242
15	225	300	20	2	48	216	0.262
16	150	500	30	0	nd	96	0.442
17	150	500	30	2	18	96	0.457
18	150	500	10	2	96	576	0.088
19	225	300	20	1	48	216	0.254
20	225	300	20	1	48	216	0.225
21	300	500	30	2	24	264*	0.367
22	150	300	20	1	48	312	0.162
23	300	100	10	0	240	1636*	0.092
24	300	100	30	2	72	984*	0.142
25	225	100	20	1	120	936	0.083
26	150	100	10	2	168	816	0.042
27	300	500	10	0	144	1296*	0.108
28	225	300	20	1	48	216	0.246
29	300	100	30	0	48	456*	0.192
30	225	300	20	1	48	216	0.250
31	225	500	20	1	48	168	0.350

Shaded lines represent the seven replicates included at the center of the experimental domain.
MFR - Maximum Fermentation Rate nd – not determined * Stuck fermentations
Level 0: Sc $7x10^5$ CFU/ml; level 1: Sc $7x10^5$ Hg $5x10^5$ CFU/ml; level 2: Sc $7x10^5$ Hg $1x10^6$ CFU/ml

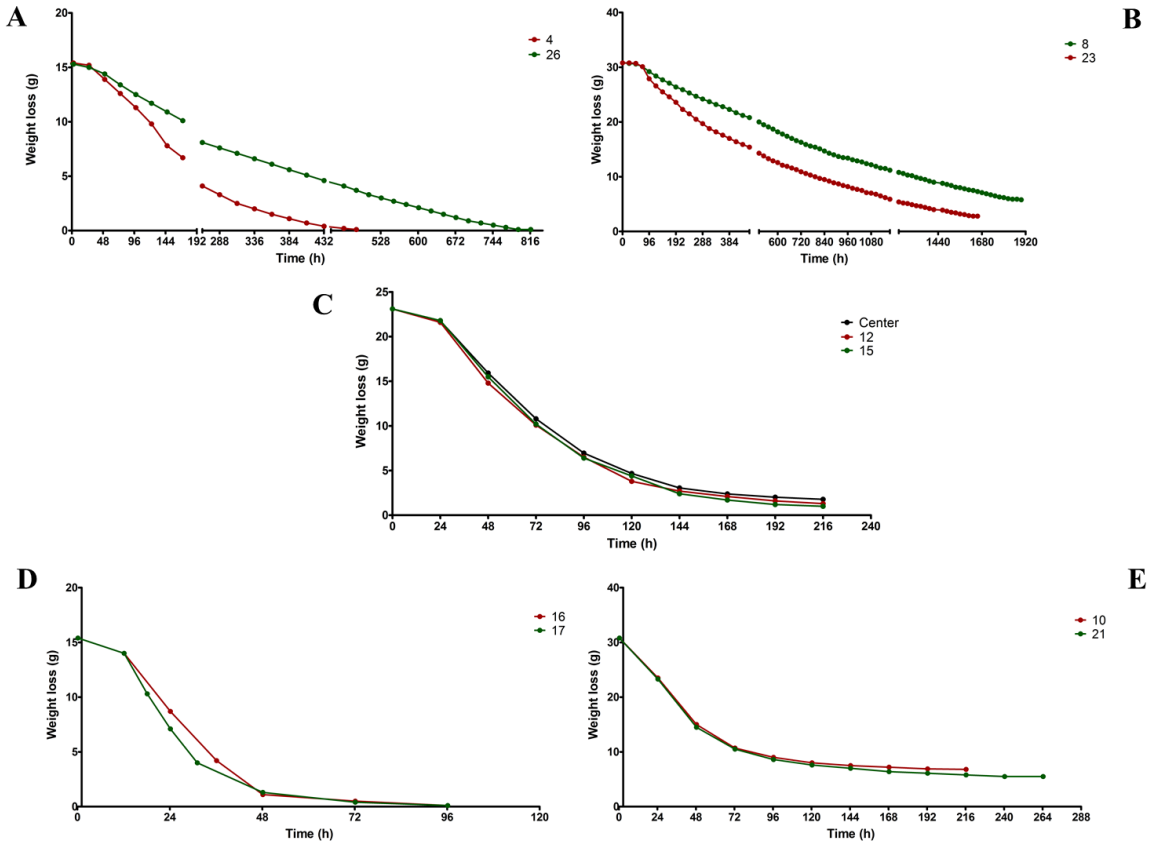


Figure 3.1 - Fermentation profiles in selected experimental conditions **A** (150g/L of sugar, 100mg/L YAN, 10 ºC); **B** (300g/L of sugar, 100mg/L YAN, 10 ºC); **C** (225g/L of sugar, 300mg/L YAN, 20 ºC) – Center conditions; **D** (150g/L of sugar, 500mg/L YAN, 30 ºC); **E** (300g/L of sugar, 500mg/L YAN, 30 ºC) inoculated with *S. cerevisiae* UCD522 in single (red) or in mixed-culture (green)

The R25 ranged from 18 to 360 h, R100 ranged from 96 to 1896 h and MFR from 0.037 to 0.457 g CO_2 /h (Table 1). At central point (runs 1, 5, 14, 19, 20, 28 and 30), R25 and R100 were 48 and 216h, respectively, while MFR was 0.250 ± 0.013. The wide variations on these values obtained underlie the impact of the factors under study on the process. The second order polynomial models determined produced satisfactory fittings of the experimental data with regard to R25 (R^2 = 0.97, $p < 0.0001$), R100 (R^2 = 0.95, $p < 0.0001$) and MFR (R^2 = 0.96 $p < 0.0001$). However, the lack of fit of R25 and R100 models did not allow good estimations of the predicted responses probably due to the absence of variability in the replicate values obtained (center points) of these independent variables in the model that provide an estimate of pure error. Nevertheless, the individual effects exerted by sugar, nitrogen, temperature or yeast co-inoculation on wine

fermentations have been identified. To our knowledge, this is the first study reporting their cumulative effect and their relative contribution to fermentation dynamics using mixed-cultures fermentation.

The beginning of alcoholic fermentation as seen by the time needed to consume 25% of sugars present in the media (R25) was greatly influenced by temperature ($p<0.0001$), nitrogen ($p<0.0001$), and sugar ($p=0.0003$) concentrations. On the contrary, co-inoculation with *H. guilliermondii* alone did not ($p=0.2293$) influence this parameter. Nevertheless, the interactions of the inoculum level with nitrogen ($p=0.0002$), sugars ($p=0.0135$) and temperature ($p=0.0135$) had a significant effect on the start of fermentation (Figure 3.2).

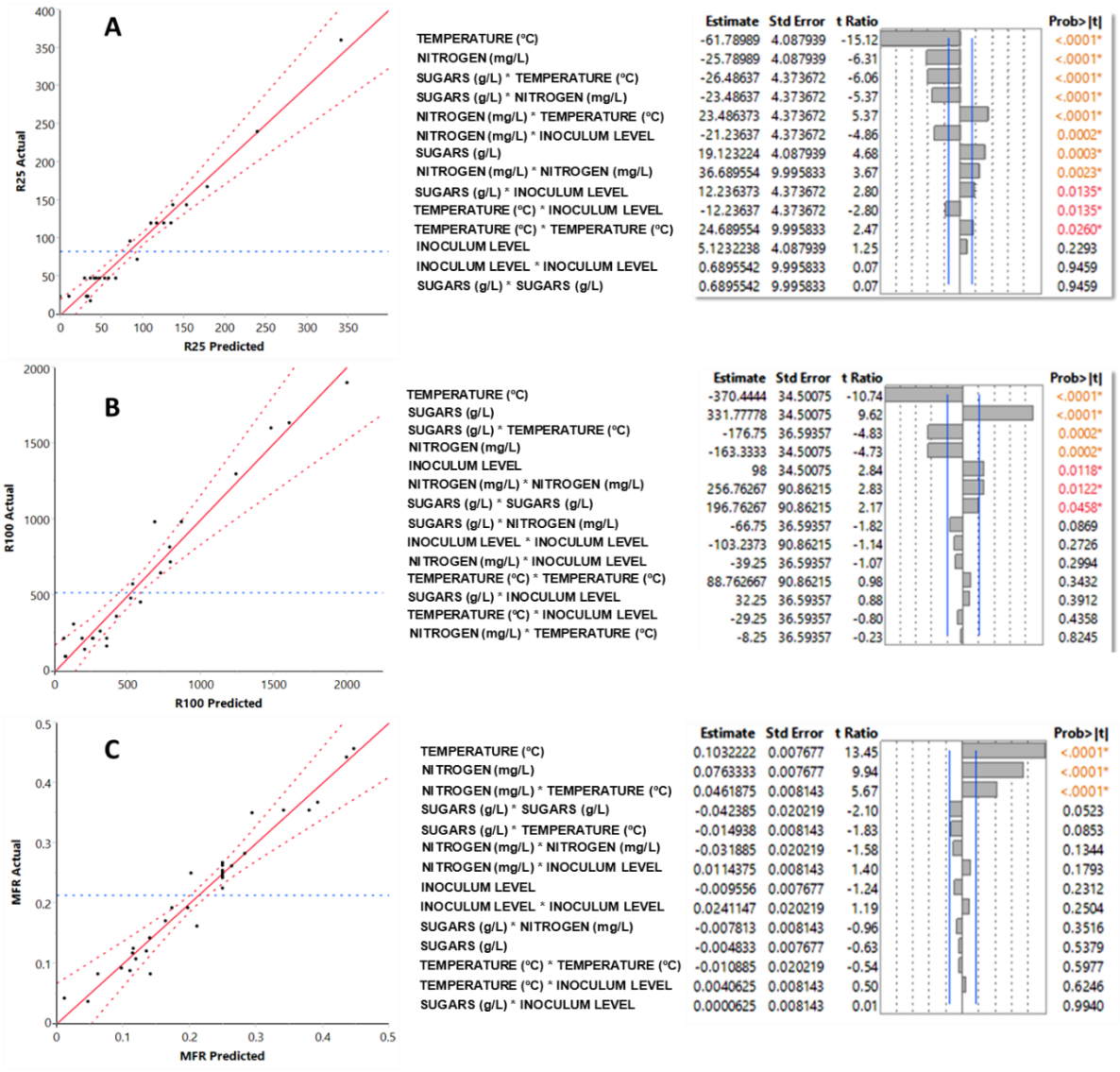


Figure 3.2. Plots of experimental/actual *versus* predicted values for R25 (A), R100 (B) and MFR (C) and the corresponding sorted parameters estimate.

On the other hand, R100 was directly influenced by all factors (Figure 3.2). The coefficients of linear terms showed that fermentation length was significantly affected by temperature ($p<0.0001$), followed by initial sugar concentration ($p<0.0001$), initial nitrogen concentration ($p=0.002$) and inoculum level ($p=0.0118$). In addition, the interaction between fermentation temperature and initial sugar concentration was highly significant ($p<0.0001$). Accordingly, as it can be seen in Table 3.1, the time needed to complete fermentation was longer in the trials conducted at the lower temperature (10 ºC). Also, the highest initial concentrations of sugar give rise to very long fermentations, where yeast cells were unable to consume all sugars (Table 3.1). On the contrary all fermentations with 225 and 150g/L of initial sugar concentration were all completed.

The observed negative effect of nitrogen on fermentation length is in line with previous observations by our and other groups, that reported an inverse correlation between nitrogen concentration and the fermentation length, maximum fermentation rate and final biomass (Mendes- Ferreira *et al*.,2004; Mendes- Ferreira *et al*.,2009).

Also it should be mentioned that the effects of the co- inoculation with *H. guilliermondii* were highly sensitive to extremes in temperature and nitrogen levels. For example, Figure 3.1 shows that for central point conditions, all fermentations were finished at 216h, with the presence of *H. guilliermondii* not interfering with the fermentation time, irrespective of the load of *H. guilliermondii* inoculum. (Figure. 3.1 C). This observation was somehow surprising, since previous experiments conducted in similar conditions of temperature, nitrogen conditions, within the range used in this study, and the same yeast strains (Lage *et al.*,2014) have clearly shown the negative effect of *H. guilliermondii* co-inoculation on the fermentative activity of *S. cerevisiae* UCD522. This conflicting result could be due to differences in culture media composition; while in this work a synthetic grape-juice medium was used, in the previous study the fermentations were conducted in a natural grape-juice. Indeed, Barbosa *et al*., (2015), have shown that the transcriptome of *S. cerevisiae* UCD522 is altered during mixed culture fermentation and that those changes appear to result from a cellular response to changes in nutrient availability in the fermenting must attributable to *H. guilliermondii* metabolic activity. The same observation was made for runs 16 and 17 (150 g/L of sugar, 500 mg/L YAN, 30 ºC), with no differences in R100 between both experiments (Figure. 3.1 D). In this case, it seems that in these extreme conditions of fermentation, with a short fermentation period (96 h) the presence of *H. guilliermondii* did not delay the completion of

fermentations, probably due to the high carbon/nitrogen ratio present in the medium which avoids the potential competition for nutrients by both strains.

However, when the level of sugar is raised to the upper extreme (Figure 3.1 E) the co-inoculated fermentations caused a delay on the completion of fermentation as compared with *S. cerevisiae* in single culture. The same negative effect was more markedly seen at lower temperatures (Figure 3.1 A, B). This may be due to the improved ability of *H. guilliermondii* to survive and persist in fermentation at lower temperatures as their susceptibility to ethanol is attenuated in these conditions (Fleet *et al*.,1988). Accordingly, yeast growth profiles presented in Figure 3.3 show that *H. guilliermondii* UTAD222 sustained higher number of viable cells in fermentation conducted at lower temperatures.

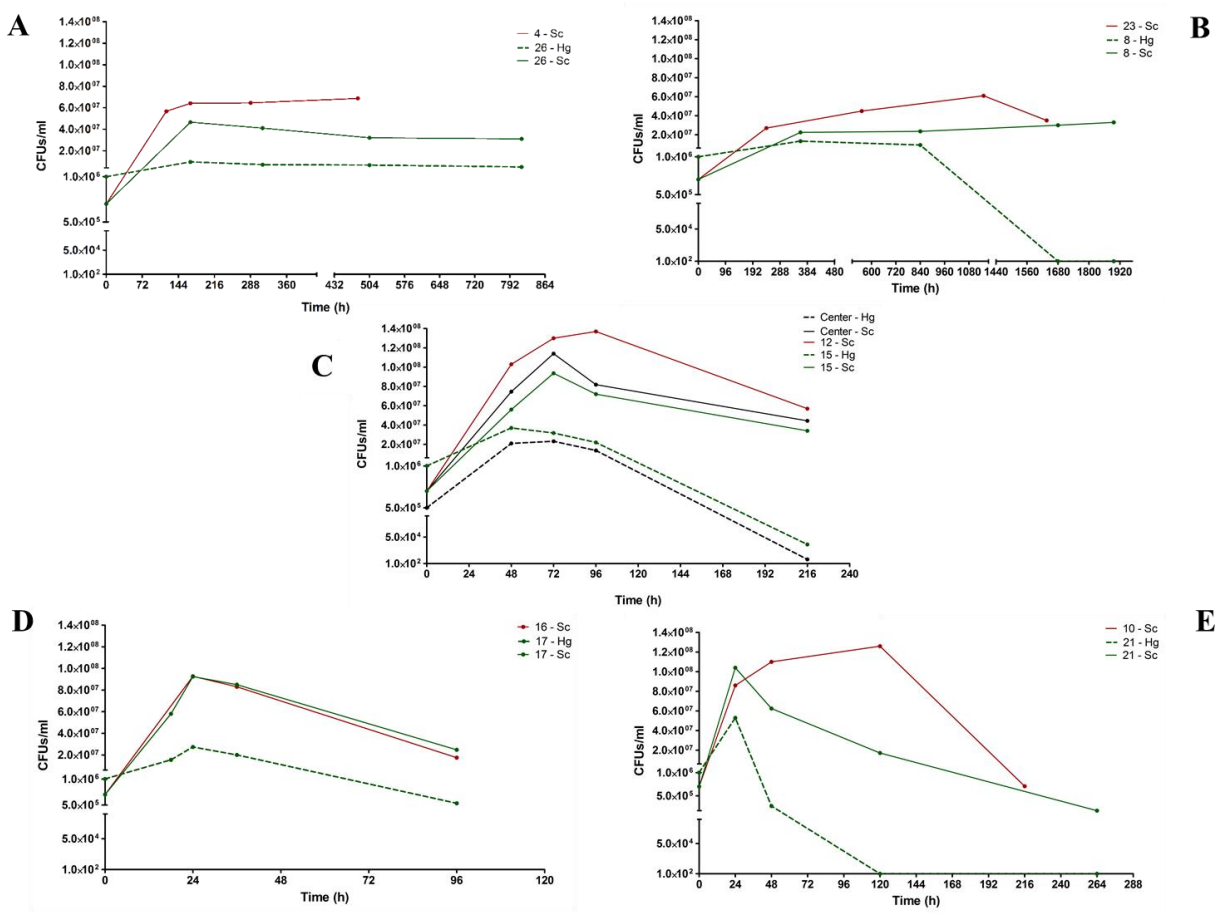


Figure 3.3 – Yeast growth profiles in selected experimental conditions, inoculated with *S. cerevisiae* UCD522 in single (red) or in mixed-culture (green). **A** (150 g/L of sugar, 100 mg/L YAN, 10 ºC); **B** (300 g/L of sugar, 100 mg/L YAN, 10 ºC); **C** (225 g/L of sugar, 300 mg/L YAN, 20 ºC) – Center conditions; **D** (150 g/L of sugar, 500 mg/L YAN, 30 ºC); **E** (300g/L of sugar, 500 mg/L YAN, 30 ºC).

The maximal fermentation rate was positively and significantly ($p<0.0001$) affected by initial nitrogen content, by temperature and by interaction of both parameters (Figure 3.2). The highest maximum fermentation rates were observed in the runs 16 and 17 (Table 3.1, Figure 3.1 D) conducted at the higher temperature and nitrogen concentration while sugars levels were at its lowest level. Indeed, it has been reported that yeast nitrogen requirements are reliant on the amount of sugars present, the higher the initial sugar concentration the more the nitrogen will be needed to complete sugar fermentations (Bisson *et al.*,2000). On the other hand, higher fermentation rates have been associated with high nitrogen conditions due to the higher final yeast cell biomass (Barbosa *et al*.,2014), which is in line with our results (Figures 3.1 and 3.2).

The presence of *H. guilliermondii*, slightly improved the fermentation efficiency, in these conditions. Interestingly in this experiment the effectiveness of temperature in decreasing *H. guilliermondii* viable yeast cell counts were not clear (Figure 3.3 D). In agreement, the *H. guilliermondii* strain used in this study have shown the ability to tolerate ethanol, up to 9% ethanol (Lage *et al*., 2014) The lowest maximum fermentation rate was determined for run 8 where extreme conditions were settled, that is the highest sugar concentration (300g/L) and the lowest yeast assimilable nitrogen level (100 mg/L) and fermentation temperature (10 ºC) and *H. guilliermondii* was inoculated at a higher rate than *S. cerevisiae.* After removing the insignificant variables by stepwise regression, the best-fit equation describing the main effects on maximal fermentation rate was the following

$$\mathbf{MFR}\ (g\ CO_2/h) = 0.245 + 0.076\ \mathbf{X_2} + 0.\ 103\ \mathbf{X_3} - 0.053\ \mathbf{X_2^2} + 0.046\ \mathbf{X_2}\ \mathbf{X_3}$$

As displayed in the equation, only two of the four factors were significant, nitrogen ($p<0.0001$) and temperature ($p<0.0001$), including its interaction ($p<0.001$) and the nitrogen quadratic effect ($p=0.003$). The p-value of lack of fit was not significant ($p = 0.098$), and the regression model was strongly significant ($p < 0.0001$, $R^2 = 0.916$, $adjR^2 = 0.0903$). The effects of both variables on MFR were better evaluated using response surface plots obtained while maintaining a constant value (i.e. the central point of CCD) to one variable at time. As shown in Figure 3.4, maximum fermentation rate occurred when temperature and yeast assimilable nitrogen concentration were at their highest levels. Indeed, the highest fermentation rate was attained in run 17 (500mg/L YAN, 30 ºC) and glucose content was at its lowest level (150g/L). In addition, it is observed that

at the lower fermentation temperature (10 ºC), highest MFR was attained at intermediate nitrogen levels, underlying the negative quadratic effect of this factor, as already seen by (Mendes-Ferreira *et al*.,2009).

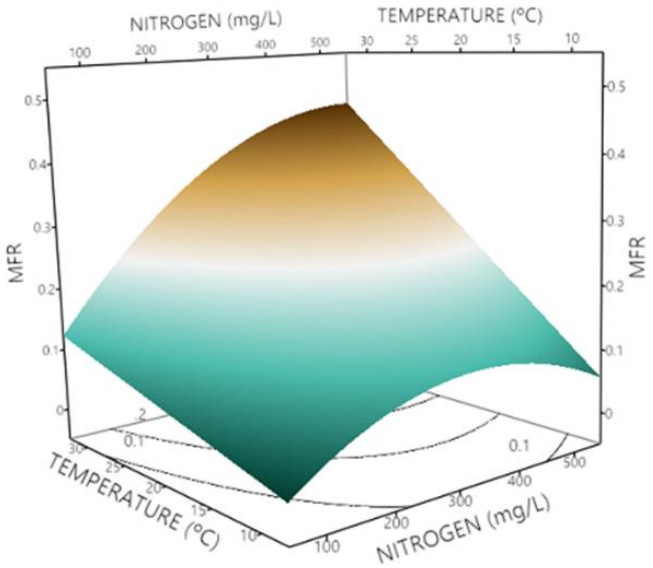


Figure 3.4. Response surface and contour plots showing MFR as function of the two independent variables with significant effect, while maintaining the others constant (central points).

III.2. Effect of fermentation conditions on the production of ethanol, glycerol, acetic and succinic acids

The primary products of alcoholic fermentation: ethanol, glycerol, acetic acid, and succinic acid make an important contribution to the aroma perception of wine (Styger *et al*. 2011). The ANOVA analysis of the second order polynomial models determined for these dependent variables were statistically significant at 95% confidence level producing adequate fittings of the experimental data with regard to ethanol (R^2 = 0.97, p < 0.0001), glycerol (R^2 = 0.97, p =0.0005), acetic acid (R^2 = 0.84, p =0.0005) and succinic acid (R^2 = 0.89 p < 0.0001). The statistical significance of the coefficients in the respective models are presented in Table 3.2. Nevertheless, the lack-of-fit tests were significant (p<0.05), for all these parameters, which indicated that the models were not adequate. Thus, experimental data were modelled using a stepwise regression with a backward selection procedure, which begins with all variables in the model and takes off one variable at a time, as long as the variable elimination adds significance to the model.

Table 3.2 – Regression coefficients significance level (p) of the linear effect, quadratic effect, and interaction effect, for ethanol, acetic acid, glycerol and succinic acid.

Compounds	Single effect				Quadratic effects				Interaction effects					
	S	N	T	I	SxS	NxN	TxT	IxI	SxN	SxT	NxT	SxI	NxI	TxI
Ethanol														
Acetic Acid														
Glycerol														
Succinic Acid														

Probability values (p) with red color gradient for positive effect, respectively dark red (P<0.001), red (P<0.01), light red (P<0.05), and blue color gradient for negative effect, respectively dark blue (P<0.001), blue (P<0.01), light blue (P<0.05). S- sugars, N- nitrogen, T- temperature and I- inoculum.

At each stage, the significance of variables is checked, and the system removes variables that become insignificant (P > 0.05). The coefficients of the best-fit equations describing the main, quadratic and interactive effects of each factor on ethanol, glycerol, acetic and succinic acids are reported in Table 3.3. The goodness-of-fit of the model obtained was again evaluated by multiple determination coefficients (R^2 and adjR^2) and significance of lack-of-fit (Table 3.3).

Table 3.3: Regression coefficients, R^2, R^2 adjusted of the quadratic models determined for ethanol, acetic acid, glycerol and succinic acid, following stepwise regression.

Parameter	Ethanol	Acetic acid	Glycerol	Succinic acid
β_0	12.84	0.68	6.04	0.45
β_1	2.98	0.18	1.43	0.29
β_2	0.41		-0.59	-0.52
β_3	-0.56	-0.07	0.76	
β_4		-0.07		
$\beta_1 1$	-1.19		1.24	
$\beta_2 2$			-0.38	0.61
$\beta_1 2$	0.43			-0.29
$\beta_1 3$	-0.6		0.22	
$\beta_2 3$			-1.17	
$\beta_3 4$		-0.05		
R^2	0.94	0.76	0.95	0.80
R^2 Adj	0.93	0.72	0.94	0.76
Lack-of-fit	0.052	0.09	0.38	0.08

Confirmation of the adequacy of the regression models for the four parameters was also reflected by the good agreement between experimental and predicted values of these response variables as shown in Figure 3.5 and Table 3.4.

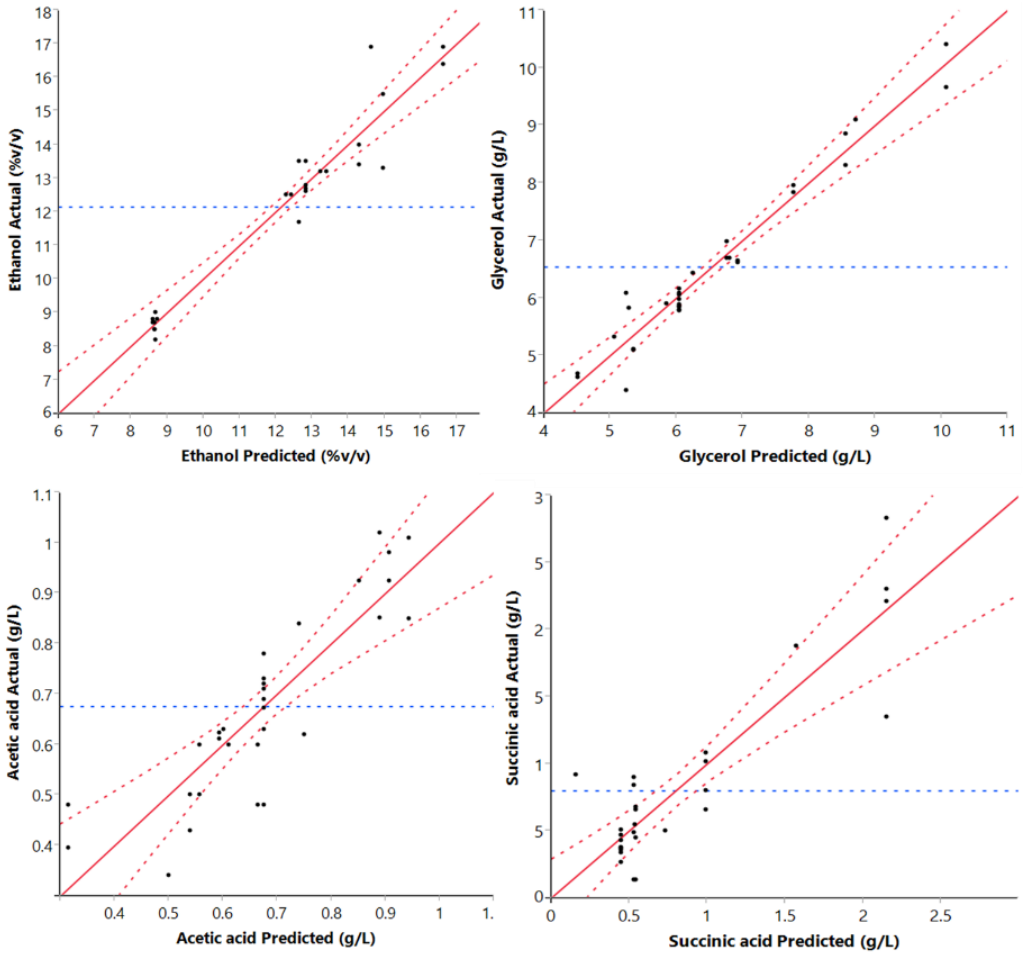


Figure 3.5. Plots of experimental/actual *versus* predicted values for ethanol glycerol, acetic and succinic acids.

Ethanol levels determined ranged from 8.2 to 16.9% (v/v), with a mean value of 12.8% ± 0.01 at the central point (runs 1, 5, 14, 19, 20, 28 and 30). The amount of ethanol produced was highly affected by the amount of sugar present in the media ($p<0.0001$), followed by temperature ($p=0.0025$), and nitrogen levels ($p=0.0224$). On the contrary, co-inoculation with *H. guilliermondii* alone did not ($p=0.1651$) influence this parameter. Additionally, the interactions of the sugar level with temperature ($p=0.0023$) and nitrogen levels ($P=0.0239$) had a significant effect on the amount of ethanol produced. The positive

effect of the levels of sugar on ethanol production was expected. However, it should be noted that all fermentations conducted with 300 g/L of sugars stopped prematurely leaving residual sugars in the medium. The effects of the fermentation temperature and initial nitrogen present on ethanol production are presented in response surface plots. As shown in Figure 3.6 (A), higher ethanol levels were obtained in the media with high levels of sugars at lower temperature fermentations.

Table 3.4 - Experimental/Real and predicted concentrations for fermentation metabolites: ethanol, acetic acid, glycerol and succinic acid.

Run	Ethanol (% v/v)	Predicted Ethanol (%v/v)	Acetic Acid (g/L)	Predicted Acetic Acid (g/L)	Glycerol (g/L)	Predicted Glycerol (g/L)	Succinic Acid (g/L)	Predicted Succinic Acid (g/L)
1	12.8	12.8	0.67	0.68	6.16	6.04	0.47	0.45
2	8.8	8.7	0.6	0.56	6.99	6.77	0.66	0.99
3	8.8	8.6	0.62	0.59	4.63	4.51	0.14	0.55
4	8.5	8.6	0.61	0.59	5.11	5.35	1.02	0.99
5	12.8	12.8	0.69	0.68	6.08	6.04	0.51	0.45
6	12.5	12.3	0.6	0.61	6.7	6.80	0.37	0.45
7	16.4	16.6	1.02	0.89	6.65	6.93	0.14	0.53
8	13.3	15.0	0.85	0.89	7.83	7.77	1.35	2.15
9	8.8	8.7	0.48	0.31	6.69	6.77	0.8	0.99
10	13.4	14.3	0.98	0.91	8.3	8.55	0.49	0.53
11	16.9	14.6	0.92	0.85	9.1	8.71	0.5	0.73
12	13.5	12.8	0.62	0.75	5.78	6.04	0.47	0.45
13	13.2	13.4	0.84	0.74	5.83	5.28	0.27	0.45
14	12.8	12.8	0.72	0.68	5.89	6.04	0.38	0.45
15	12.8	12.8	0.63	0.60	5.86	6.04	0.36	0.45
16	8.2	8.7	0.5	0.56	6.09	5.24	0.66	0.55
17	9	8.7	0.4	0.31	4.93	5.24	0.68	0.55
18	8.7	8.6	0.5	0.54	4.69	4.51	0.45	0.55
19	12.7	12.8	0.73	0.68	6.06	6.04	0.34	0.45
20	12.7	12.8	0.72	0.68	5.98	6.04	0.37	0.45
21	14	14.3	0.48	0.66	8.85	8.55	0.84	0.53
22	8.5	8.7	0.34	0.50	5.9	5.85	0.92	0.16
23	15.5	15.0	0.85	0.94	7.95	7.77	2.21	2.15
24	11.7	12.6	0.6	0.66	9.66	10.07	2.3	2.15
25	12.5	12.4	0.48	0.68	6.44	6.25	1.88	1.57
26	8.7	8.6	0.43	0.54	5.09	5.35	1.08	0.99
27	16.9	16.6	1.01	0.94	6.62	6.93	0.9	0.53
28	12.6	12.8	0.78	0.68	5.84	6.04	0.43	0.45
29	13.5	12.6	0.92	0.91	10.41	10.07	2.83	2.15
30	12.8	12.8	0.63	0.68	5.8	6.04	0.43	0.45
31	13.2	13.2	0.71	0.68	5.32	5.07	0.55	0.54

For instance, comparing the experimental runs 7 and 21, it was observed that the first, although displaying significantly longer fermentation duration (1600 and 264 h, respectively), yielded higher ethanol levels (16.4 and 14%, respectively). If for one side, this difference could be due to losses of ethanol due to evaporation at high temperatures, on the other hand this result is in line with known effect of temperature in enhancing the toxic effect of ethanol causing yeast cells inactivation or even death (Torija *et al*.,2001). A decreased in *S. cerevisiae* viability was detected in fermentations conducted at 30ºC (Figure 3.3).

Furthermore, the response surface plots presented in Figure 3.6 A, show that higher ethanol levels were obtained in the media with higher levels of initial nitrogen. The reason of this observation may be due to the fact that prolonged availability of nitrogen in the media stimulate and sustain yeast maximum specific growth rate (Henschke *et al*.,1993) and increase the storage of nitrogen compounds in the vacuoles, important for maintenance of yeast metabolic activity in the latter fermentation stages, where high ethanol levels unable efficient nitrogen transport (Bisson *et al*.,1999).

For all combinations tested, glycerol concentration ranged from 4.63 g/L to 10.41 g/L. The coefficients of the adjusted model obtained for glycerol production, as a function of the significant variables, are presented in Table 3.3. The amount of glycerol produced was highly affected by the amount of sugar present in the media ($p<0.0001$), followed by nitrogen levels ($p<0.001$) and fermentation temperature ($p<0.0001$). It should be mentioned that, in agreement with the positive and negative value of each parameter sugars level and fermentation temperature had a direct effect, while the initial nitrogen concentration was inversely associated with glycerol production Additionally, sugar level had a significant quadratic effect ($p<0.0001$) on the amount of glycerol produced, as well as it interaction with temperature ($p=0.0252$). In this line, maximum levels of glycerol were achieved in run 29 (300 g/L of sugars, 100 mg/L YAN, 30ºC), whereas the lowest levels were obtained in run 3 (150 g/L of sugars, 500 mg/L YAN, 10ºC).

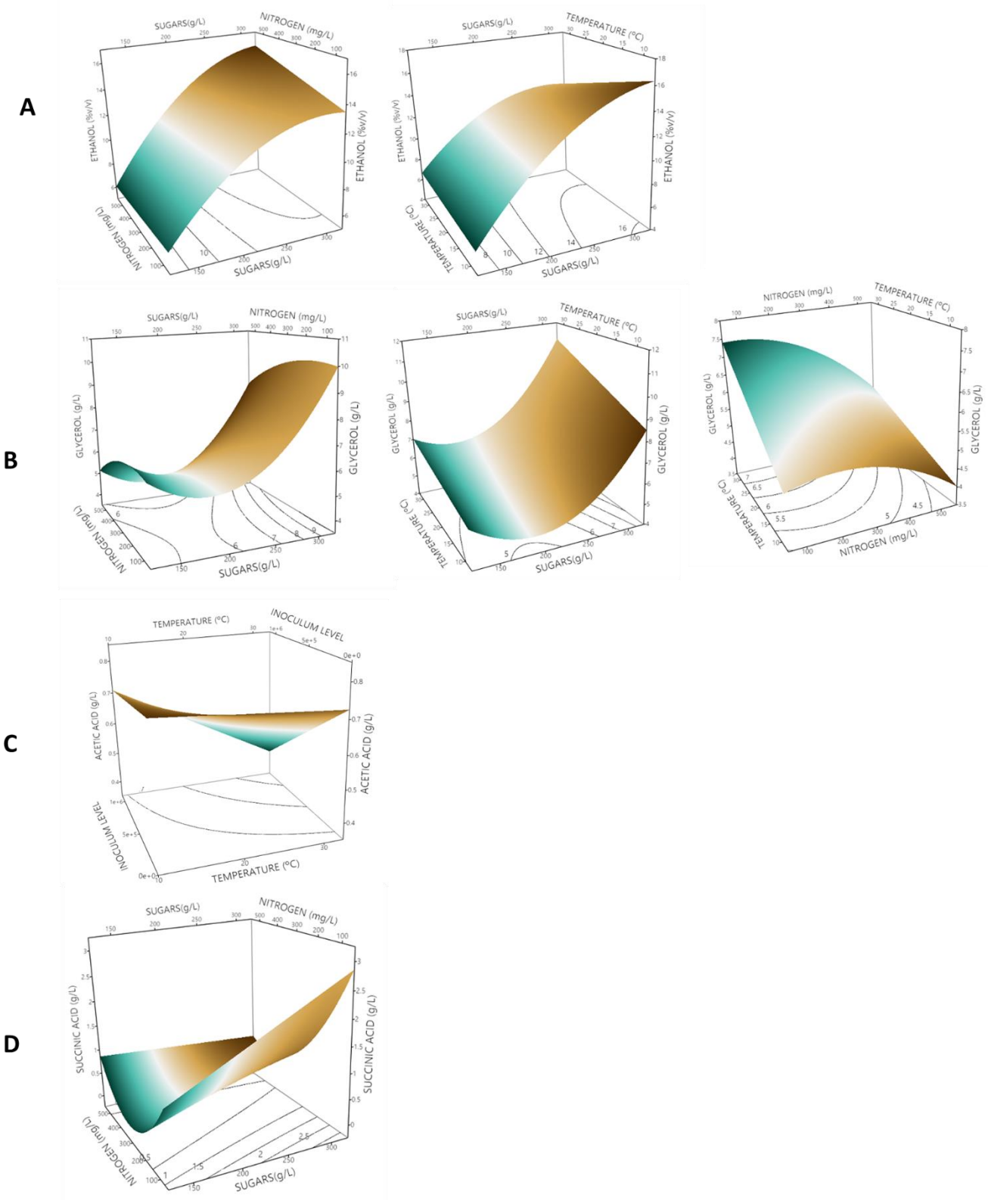


Figure 3.6. Response surface and contour plots showing ethanol (A), glycerol (B), acetic acid (C) and succinic acid (D) as function of two independent variables maintaining the others constant (central points).

The higher glycerol production in higher sugar fermentations was expected because it is known its major involvement in yeast cell redox homeostasis during fermentation, as well as in the metabolic stress response to osmolarity. However, we could not find a reason for the observed lower levels of glycerol in the central sugar condition. In the response surface plots presented in Figure 3.6B, it is presented the negative effect of nitrogen levels on glycerol production, being more evident in the highest sugar levels. This result is in line with those obtained by Remize *et al*.,(2000) that studied 19 industrial wine strains and found that the initial nitrogen concentration in the must had little effect on glycerol production although specific glycerol production was lower at higher nitrogen concentrations. Also, our results are in line with previous observations that increasing fermentation temperature results in greater glycerol production in must (reviewed in Scanes *et al*.,1998). In a study conducted with several *S. cerevisiae* wine yeasts, found that the concentration of glycerol increased by 1 g/L when the temperature was changed from 15° to 25°C. Again, and contrary to our previous observations (Barbosa *et al*.,2015) according to our model, co-inoculation with *H. guilliermondii* did not influence the production of glycerol ($p=0.1658$). One possible explanation could be the highly significant effect of the other factors under study, as well as their interactions, that may have masked the effect of co-inoculation.

Acetic acid levels determined along the 31 experiments ranged from 0.34 to 1.02 g/L, with a mean value of 0.71 ± 0.05 at the central point (runs 1, 5, 14, 19, 20, 28 and 30). Thus, the concentrations of acetic acid determined in the present study were lower than the legal limit established by Regulation (EC) No. 1493/1999, Annex VB-1b of 1.2 g/L). The amount of acetic acid produced was highly affected by the amount of sugar present in the media ($p<0.0001$), followed by inoculum level ($p=0.0035$), and temperature ($p=0.0086$). The direct relationship between acetic acid formation and sugar concentration has been clearly shown by Erasmus *et al*.,(2004), while using a range of sugar amounts of 20-50%, using seven commercial wine yeast strains. Our results show that co-inoculation with *H. guilliermondii* did not affect this relationship at least at the lowest temperature tested. In this line, the highest levels of acetic acid were achieved under the extreme conditions of 300 g/L of sugars, 500 mg/L YAN, 10ºC irrespective of the type of inoculum. Nevertheless, higher production of acetic acid was detected in wines obtained by single cultures of *S. cerevisiae* when compared to those obtained by mixed cultures, contrasting with the recognized ability *Hanseniaspora* spp. to produce unwanted

levels of acetic acid (Viana *et al*.,2008). However, these results confirm that co-inoculation with this *H. guilliermondii* strain does not contribute to a significant increase in the wine's volatile acidity compared with those obtained from the *S. cerevisiae* UCD522 single-culture (Lage *et al*.,2014). As it can be seen in the response surface plot presented in Figure 3.6 C, the effect of the inoculum was dependent on the temperature of fermentation. A significant decrease in acetic acid levels was observed in trial conducted at 3 ºC, directly linked to the inoculation with the *non-Saccharomyces* strain. This finding suggests that, at least at the highest temperatures, the presence of *H. guilliermondii* has a repressor effect on *S. cerevisiae* to produce acetic acid. Accordingly, it was being reported that the impact of *H. guilliermondii* on the production of wine compounds could result from its influence on the metabolic behavior of *S. cerevisiae* UCD522 (Barbosa *et al.*,2015).

Regarding succinic acid levels, they varied from 0.14 to 2.83 g/L, with mean values at the center points of 0.418 ± 0.06. Nitrogen levels had significant linear ($p<0.001$) and quadratic ($p<0.0001$) effects on the final concentration of succinic acid. In addition, sugar concentration ($p=0.0008$) and its interaction with nitrogen ($p=0.0011$) were found to interfere with succinic acid production. As shown in Figure 3.6 D, maximum succinic acid levels occurred when sugar concentration were at its highest levels. In addition, it is observed that the lower succinic acid levels were attained at intermediate nitrogen levels, underlying the positive quadratic effect of this factor which is, however, attenuated at higher sugar concentrations. Similar results have been obtained by Rollero *et al.*,(2015), where the production of succinic acid was highest at low assimilable nitrogen content and at high temperature. In our study, temperature and co-inoculation with *H. guilliermondii* did not significantly affected this parameter. Contrarily, Heerde *et al*.,(1978) have observed a stimulating effect of nitrogen on succinic acid production up to concentrations of about 500 mg/L in the presence of a 100 g/L fermentable sugar.

III.3. Effect of fermentation conditions on the production of aroma compounds

There is a widely recognized consumer demand for wines with distinct and diverse sensorial characteristics. Some aroma compounds arise from the must with minor or no

modifications varietal or primary aroma, while others that form the so called fermentation *bouquet*, are products of yeast metabolism, in particular from sugar and nitrogen metabolisms. Nutrients, namely sugars and nitrogen, and temperature are important factors that control wine fermentation (Reviewed in Mendes-Ferreira *et al*.,2011) and impact many aspects of yeast metabolism, including the production of fermentation volatile aroma compounds that contribute significantly to the organoleptic qualities of wines (Swiegers *et al*.,2005).

In this line, the combined effects of nitrogen and sugar levels, fermentation temperature and inoculum level of *H. guilliermondii* on the production of aroma compounds were also assessed. A variety of yeast-synthesized volatile aroma compounds (total of seventeen) belonging to fatty acids, higher alcohols and ethyl and acetate esters, were detected and quantified at the end of fermentations (Tables 3.6, 3.7, 3.8 and 3.9). The results obtained were used to determine the regression coefficients of the second-order multi regression models (Table 3.5). Again, the quality of the fit of the polynomial model equations obtained for each compound was assessed by whole-model and lack-of-fit p-value and R^2 and adjR^2 (Table 3.5). The reliability of the fitted models was overall very good, showing non-significant p-values at a 0.05 threshold, except for 2-phenylethanol and both fatty acids. A non-significant lack-of-fit test was obtained for the majority of the compounds, except for ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl dodecanoate, phenylethyl acetate, 1-butanol, and octanoic acid. Also lower values were obtained for R^2 and adjR^2 which varied between 0.84-0.58 and 0.65-0.21, respectively. These results, which did not allow good estimations of the predicted responses, could be due to experimental errors inherent to the biological nature of yeast fermentations or to analytical errors that are intrinsic to volatile aroma compounds analysis, (such as their volatility) or to the interference of other factor(s) that were not accounted in our experimental model in the production of these compounds. Nevertheless, in the following section we will attempt to describe the main effects of the independent variables under study, focusing on particular families and compounds.

Table 3.5. Effects of the studied factors described by the model on aroma compounds production.

Compounds	Single effect				Quadratic effects				Interaction effects						Model *p*-value	R^2	AdjR^2	Lack-of-fit *p*-value
	S	N	T	I	SxS	NxN	TxT	IxI	SxN	SxT	NxT	SxI	NxI	TxI				
Ethyl butanoate															0.0003	0.76	0.56	0.07
Ethyl hexanoate															0.0019	0.75	0.53	0.00
Ethyl pentanoate															<0.0001	0.67	0.37	0.11
Ethyl octanoate															0.0045	0.71	0.46	0.00
Ethyl decanoate															0.0300	0.68	0.41	0.02
Ethyl dodecanoate															0.0280	0.76	0.55	0.04
Ethyl acetate															0.0014	0.84	0.65	0.14
Σ Ethyl Esters															0.0015	0.81	0.65	0.13
Isoamyl acetate															0.0196	0.72	0.48	0.27
Phenylethyl acetate															0.0014	0.82	0.65	0.00
Σ Acetate Esters															0.0170	0.73	0.49	0.21
1-Propanol															0.0033	0.81	0.63	0.43
1-Butanol															0.0041	0.80	0.61	0.00
2-methyl-1-butanol															0.0081	0.76	0.55	0.25
3-methyl-1-butanol															0.0300	0.71	0.44	0.43
2-Phenylethanol															0.1491	0.62	0.26	0.39
Methionol															0.0470	0.70	0.41	0.73
Σ Higher Alcohols															0.0330	0.69	0.43	0.26
Octanoic acid															0.0600	0.66	0.37	0.03
Decanoic acid															0.1886	0.58	0.21	0.91
Σ Fatty Acids															0.0540	0.67	0.38	0.09

Probability values (p) with red color gradient for positive effect, respectively dark red (P<0.001), red (P<0.01), light red (P<0.05), and blue color gradient for negative effect, respectively dark blue (P<0.001), blue (P<0.01), light blue (P<0.05). S-sugars, N-nitrogen, T-temperature and I-Inoculum level.

Table 3.6. Estimated coefficients fitted to a second-order polynomial equation to study the effects of the independent variables on factors for each aroma compound and corresponding families

Compounds	β_0	β_1	β_2	β_3	β_4	$\beta_1 1$	$\beta_2 2$	$\beta_3 3$	$\beta_4 4$	$\beta_1 2$	$\beta_1 3$	$\beta_1 4$	$\beta_2 3$	$\beta_2 4$	$\beta_3 4$
Ethyl butanoate	0,2500		0,0400	-0,0400		-0,1200									
Ethyl hexanoate	0,1800		0,0078			-0,0200									
Ethyl pentanoate	0,0300	0,0006													
Ethyl octanoate	0,4900		0,0900			-0,3200									
Ethyl decanoate	0,4900					-0,2800									
Ethyl dodecanoate	0,1600					-0,0200			-0,0100						
Ethyl acetate	69,2600				32,2200		25,8800							-11,4400	
Σ Ethyl Esters	70,8600						26,1200							-11,3900	
Isoamyl acetate	2,4800		0,4800			-1,5000									
Phenylethyl acetate	0,7300		-0,6400	0,4900	0,6200		0,9800						-0,4800	-0,6000	0,5100
Σ Acetate Esters	3,2300				0,8000			-1,6200							
1-Propanol	30,3400		10,8100			-13,4000									
1-Butanol	21,8000		-7,0100	11,4600	5,6200										
2-methyl-1-butanol	27,7900	-5,4200	-8,2100	4,1900									1,0400		
3-methyl-1-butanol	106,4000	-19,5700	-33,9200										-2,8800		
2-Phenylethanol	6,6800		-2,3400												
Methionol	5,1700	1,4700	1,3100							1,5900	-1,9600				
Σ Higher Alcohols	200,0300	-26,9700	-43,1100										-2,5900		
Octanoic acid	15,0800	-2,2900													
Decanoic acid	5,8000							-2,0400							
Σ Fatty Acids	20,8800	-2,7900													

As can be seen in Table 3.5, nitrogen concentration was the factor that had the greatest effect on the production of volatile compounds, more precisely in twelve out of the seventeen compounds analyzed, which is in line with the recognized pivotal role that nitrogen has on the modulation of aroma compounds formation in wine (Henschke *et al*.,2005; Mendes-Ferreira *et al*.,2011). Its simple effect was positive in the production of compounds such as two ethyl esters, isoamyl acetate, 1-propanol, methionol and octanoic acid, that is, the final concentration of these volatile compounds increased with the initial nitrogen content. On the contrary, the effect was negative for the majority of higher alcohols, phenylethyl acetate and ethyl butanoate. The levels of sugar content presented a linear moderate positive effect on ethyl pentanoate and methionol, and a strong negative linear effect on higher alcohols production. Temperature and inoculum level displayed only positive linear effects on the synthesis of esters and some higher alcohols, being the effect of inoculum level on ethyl acetate and of temperature on 1-butanol those with highest significance.

III.3.1 Effect of fermentation conditions on the Ethyl Esters concentrations

Ethyl esters of fatty acids (ethyl hexanoate, ethyl octanoate, ethyl decanoate) are, together with acetate esters, qualitatively one of the most important flavours in wine, due to the desirable fruity taste that impart to wines (Pretorius *et al*.,2009; Saerens *et al*.,2010; Ugliano *et al*.,2009; Bisson *et al*.,2010). Exception is ethyl acetate that gives an unpleasant solvent or nail-polish character when present in excess. Wines containing more than 90 mg/L of ethyl acetate or 200 mg/L of total esters are considered defective (Bisson *et al*.,2010; Lambrechts *et al*.,2000). In this study, the concentration of total ethyl esters presented a range of values from 20. to 123.96 mg/L, in runs 4 (150 g/L of sugars, 100 mg/L YAN, 10ºC) and 24 (300 g/L of sugars, 100 mg/L YAN, 30ºC), respectively (Tables 3.1 and 3.6), being nitrogen the factor with the highest significant influence, either with quadratic effect ($p=0.0462$) or with interaction with inoculum level ($p=0.0326$)). Regarding ethyl acetate formation, the minimum (17.55 mg/L) and maximum (137.75 mg/L) levels were obtained in run 2 (150 g/L of sugars, 100 mg/L YAN, 30ºC) and run 26 (150 g/L of sugars, 100 mg/L YAN, 10ºC), respectively. These experiments differed

in the inoculum level and in the temperature at which the fermentations were conducted (Table 3.5), being the highest level detected when high rates of *H. guilliermondii* inoculum and lower temperatures were used. Indeed, a recent study (Barbosa *et al*.,2015) using a transcriptomic based approach showed that the expression of genes of *S. cerevisiae* involved in the biosynthesis of these compounds is higher in when in co-culture with *H. guilliermondii*, suggesting that the presence of this *non-Saccharomyces* strain affects the metabolism of *S. cerevisiae* and contributes to a greater production of ethyl esters. Moreover, also sugar content and temperature displayed simple positive effects on the production of ethyl pentanoate and ethyl butanoate, respectively, in agreement with results obtained by others (Molina *et al*.,2007).

Ethyl acetate was by far the most important ester quantified in the fermentations (Table 3.7). It is widely accepted that an increase in initial nitrogen content is associated with an increase in ester production (Ugliano *et al*.,2010; Hernandez-Orte *et al*.,2006; Mendes-Ferreira *et al*. 2009; Lage *et al*. 2014). The good fitting of the model in ethyl acetate response, (R^2 = 0.84, AdjR^2 = 0.65 and a lack-of-fit p-value = 0.14) showed that the most significant effects modulating this nail polish ester, as seen for the total esters formation were, once again, quadratic effect of nitrogen (p=0.0472) and its interaction with inoculum level (p=0.0312). Most importantly, the inoculum level effect was the factor that has the most impact (p<0.001) being clearly associated with biosynthesis of this ester, in line with the several studies that point *non-Saccharomyces* yeast strains as proficient in esters production (Manzanares *et al*.,2011; Rojas *et al*.,2003; Lage *et al*.,2014; Barbosa *et al*.,2015;Viana *et al*.,2008). As illustrated in the response surface plots (Figure 3.7), it was possible to verify a strong positive effect of inoculum factor, underlining that the presence of *H. guilliermondii* promotes the increase on the ethyl acetate. Indeed, lower levels of these compounds were found in the central nitrogen conditions and in absence of *H.guilliermondii*.

Table 3.7. Ethyl Esters concentrations (mg/L) in the final wines obtained in the 31 fermentations.

Run	Ethyl butanoate	Ethyl hexanoate	Ethyl pentanoate	Ethyl octanoate	Ethyl decanoate	Ethyl dodecanoate	Ethyl acetate	Ethyl Esters
1	0.31 ± 0.01	0.18 ± 0.00	0.03 ± 0.00	0.52 ± 0.01	0.52 ± 0.04	0.16 ± 0.01	54.01± 0.40	55.43 ± 0.50
2	0.07 ± 0.00	0.14 ± 0.00	0.02 ± 0.00	0.08 ± 0.00	0.12 ± 0.00	0.11 ± 0.00	17.55 ± 0.20	18.11 ± 0.25
3	0.17 ± 0.00	0.16 ± 0.00	0.02 ± 0.00	0.20 ± 0.00	0.16 ± 0.00	0.12 ± 0.00	28.40 ± 0.70	29.25 ± 0.67
4	0.08 ± 0.00	0.15 ± 0.00	0.02 ± 0.00	0.09 ± 0.01	0.12 ± 0.00	0.11 ± 0.00	19.77 ± 0.20	20.37 ± 0.20
5	0.30 ± 0.01	0.18 ± 0.00	0.03 ± 0.00	0.44 ± 0.02	0.40 ± 0.01	0.15 ± 0.00	49.92 ± 0.30	51.43 ± 0.38
6	0.14 ± 0.00	0.15 ± 0.00	0.03 ± 0.00	0.17 ± 0.01	0.21 ± 0.01	0.13 ± 0.00	36.02 ± 3.10	36.86 ± 3.14
7	0.37 ± 0.00	0.18 ± 0.00	0.03 ± 0.00	0.19± 0.00	0.16 ± 0.01	0.13 ± 0.00	73.05 ± 0.80	74.10 ± 0.79
8	0.12 ± 0.00	0.15 ± 0.00	0.03 ± 0.00	0.11 ± 0.00	0.13 ± 0.00	0.12 ± 0.00	102.95 ± 0.70	103.61 ± 0.68
9	0.04 ± 0.00	0.14 ± 0.00	0.03 ± 0.00	0.08 ± 0.00	0.13 ± 0.00	0.12 ± 0.00	105.56 ± 0.70	106.11 ± 0.75
10	0.12 ± 0.03	0.15 ± 0.00	0.03 ± 0.00	0.21 ± 0.03	0.26 ± 0.03	0.14 ± 0.00	31.83 ± 0.80	32.75 ± 0.92
11	0.18 ± 0.00	0.16 ± 0.00	0.03 ± 0.00	0.18 ± 0.03	0.20 ± 0.03	0.13 ± 0.00	81.34 ± 2.40	82.23 ± 2.46
12	0.27 ± 0.01	0.17 ± 0.00	0.03 ± 0.00	0.44 ± 0.03	0.37 ± 0.03	0.14 ± 0.00	61.68 ± 1.40	63.11 ± 1.51
13	0.34 ± 0.01	0.18 ± 0.00	0.03 ± 0.00	0.44± 0.01	0.32 ± 0.00	0.14 ± 0.00	76.86 ± 0.80	78.32 ± 0.81
14	0.21 ± 0.02	0.17 ± 0.00	0.03 ± 0.00	0.38 ± 0.00	0.40 ± 0.00	0.15 ± 0.00	80.71 ± 0.30	82.06 ± 0.33
15	0.30 ± 0.03	0.20 ± 0.00	0.03 ± 0.00	0.60 ± 0.01	0.59 ± 0.03	0.19 ± 0.01	79.87 ± 1.60	81.79 ± 1.68
16	0.08 ± 0.01	0.15 ± 0.00	0.02 ± 0.00	0.15 ± 0.00	0.16 ± 0.00	0.12 ± 0.00	32.13 ± 0.60	32.83 ± 0.60
17	0.10 ± 0.00	0.16 ± 0.00	0.03 ± 0.00	0.23 ± 0.01	0.22 ± 0.01	0.13 ± 0.00	61.40 ± 1.40	62.27 ± 1.42
18	0.14 ± 0.01	0.15 ± 0.00	0.02 ± 0.00	0.23 ± 0.01	0.19 ± 0.01	0.12 ± 0.00	95.74 ± 1.70	96.61 ± 1.77
19	0.27 ± 0.01	0.19 ± 0.00	0.03 ± 0.00	0.56 ± 0.01	0.55 ± 0.01	0.17 ± 0.00	69.52 ± 1.80	71.29 ± 1.82
20	0.22 ± 0.01	0.19 ± 0.00	0.03 ± 0.00	0.50 ± 0.02	0.49 ± 0.02	0.16 ± 0.00	61.32 ± 0.20	62.91 ± 0.23
21	0.13 ± 0.03	0.15 ± 0.00	0.03 ± 0.00	0.13 ± 0.00	0.18 ± 0.00	0.14 ± 0.00	69.68 ± 1.00	70.45 ± 1.07
22	0.08 ± 0.00	0.15 ± 0.00	0.02 ± 0.00	0.16 ± 0.00	0.19 ± 0.00	0.12 ± 0.00	15.85 ± 2.00	16.60 ± 1.97
23	0.14 ± 0.01	0.15 ± 0.00	0.03 ± 0.00	0.17 ± 0.01	0.17 ± 0.00	0.13 ± 0.00	35.31 ± 0.50	36.11 ± 0.50
24	0.06 ± 0.00	0.14 ± 0.00	0.03 ± 0.00	0.08 ± 0.00	0.12 ± 0.00	0.12 ± 0.00	123.39 ± 2.30	123.96 ± 2.27
25	0.07± 0.00	0.15 ± 0.00	0.02 ± 0.00	0.10 ± 0.00	0.12 ± 0.00	0.11 ± 0.00	94.03 ± 9.00	94.61 ±9.00
26	0.06 ± 0.00	0.15 ± 0.00	0.02 ± 0.00	0.09 ± 0.00	0.12 ± 0.00	0.11 ± 0.00	137.75 ± 2.70	138.33 ± 2.71
27	0.03 ± 0.00	0.14 ± 0.00	0.02 ± 0.00	0.07 ± 0.00	0.11 ± 0.00	0.11 ± 0.00	18.20 ± 3.50	18.70 ± 3.50
28	0.21 ± 0.01	0.18 ± 0.00	0.03 ± 0.00	0.58 ± 0.02	0.65 ± 0.02	0.17 ± 0.00	81.33 ± 6.40	83.15 ± 6.49
29	0.03 ± 0.00	0.14 ± 0.01	0.02 ± 0.00	0.07 ± 0.00	0.11 ± 0.00	0.11 ± 0.00	24.74 ± 1.10	25.25 ± 1.09
30	0.23 ± 0.01	0.17 ± 0.01	0.03 ± 0.00	0.46± 0.01	0.52 ± 0.01	0.16 ± 0.00	84.13 ± 0.00	85.72 ± 0.04
31	0.35 ± 0.02	0.21 ± 0.01	0.03 ± 0.00	1.12 ± 0.02	1.12 ± 0.04	0.18 ± 0.00	97.64 ± 0.90	100.66 ± 0.95
OT	0.40	0.08	1.3	0.06	0.51	0.64	22.50 - 63.50	[a]

[a](Moreira *et al.*,2010; Swiegers *et al.*,2005)

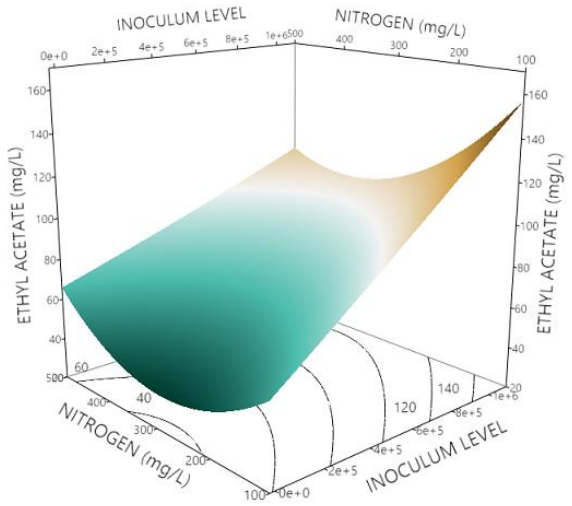


Figure 3.7. Response surface and contour plot of ethyl acetate production.

III 3.2. Effect of fermentation conditions on the acetate esters concentrations

Acetates of higher alcohols (phenylethyl acetate, isoamyl acetate) are one of the most important flavours in wine, because they contribute to a desirable fruity taste (Saerens *et al*.,2010; Ugliano *et al*.,2009; Bisson *et al*.,2010).

In this study, the concentration of total acetate esters varied within a range of 0.18 to 5.90 mg/L corresponding in run 27 (300 g/L of sugars, 500 mg/L YAN, 10ºC) fermented with *S. cerevisiae* in single culture and run 9 (150 g/L of sugars, 100 mg/L YAN, 30ºC), fermented with co-inoculation with the highest load of *H. guilliermondii*, respectively (Tables 3.1 and 3.7). In line with this observation, the factor which presented the most important impact in the total production of acetate esters was the level of inoculum (Table 3.5). This result corroborates the already known role of *non-Saccharomyces* yeasts in acetate esters production. In a study conducted by Rojas *et al*.,(2003), where *H. guilliermondii* and *Pichia anomala* were used in co-cultures with *S. cerevisiae*, an increase in acetate ester concentrations were found when compared to the pure culture of *S. cerevisiae*, which was later corroborated by other works (Viana *et al*.,2008; Lage *et al*.,2014). The response surface plots presented in (Figure 3.8) clearly show the significant quadratic effect of temperature, being the highest levels of acetate esters obtained at intermediate temperatures. In addition, although not significant we observed a quadratic effect of nitrogen concentration, with the lower levels being produced at the central points.

Analyzing the individual acetate esters produced, phenylethyl acetate production was positively affected by inoculum level (p=0.002) and temperature (p =0.011) and negatively by nitrogen (p=0.0016) in its linear and quadratic terms. The significant higher effect of nitrogen on this compound is widely acknowledged (Ugliano *et al*.,2010; Hernandez-Orte *et al.*,2006; Mendes-Ferreira *et al*.,2009). Also the effect of the inoculum on the formation of this compound was expected as high production of phenylethyl acetate seems be restricted to the genus *Hanseniaspora* (Viana *et al*.,2008).

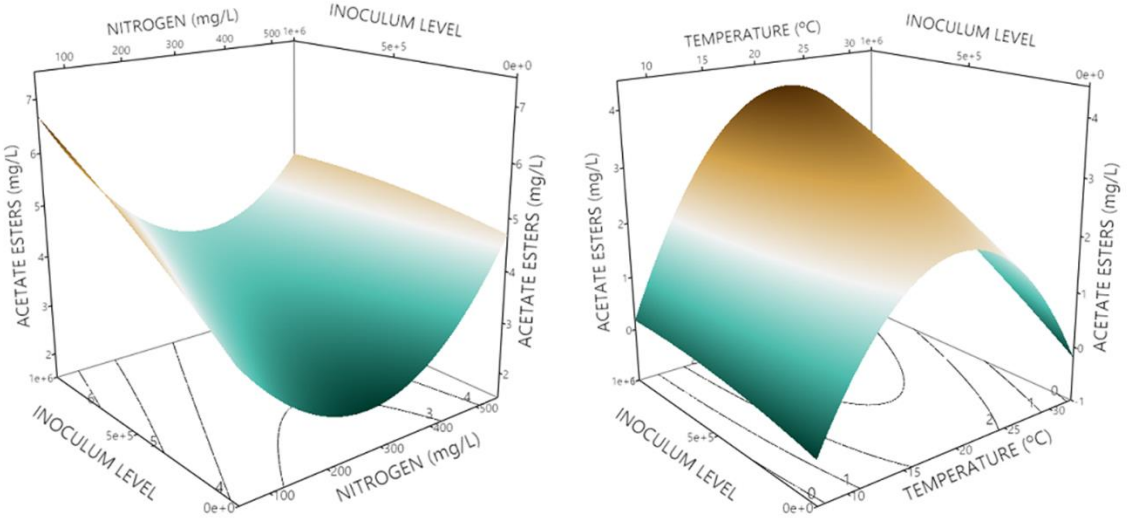
Table 3.8. Acetate Esters concentrations (mg/L) in the final wines obtained in the 31 fermentations.

Run	Isoamyl acetate	Phenylethyl acetate	Acetate esters
1	3.13 ± 0.09	0.39 ± 0.05	3.55 ± 0.14
2	0.36 ± 0.03	0.15 ± 0.02	0.53 ± 0.05
3	0.53 ± 0.05	0.11 ± 0.00	0.67 ± 0.05
4	0.23 ± 0.01	0.10 ± 0.00	0.36 ± 0.01
5	2.49 ± 0.01	0.32 ± 0.00	2.84 ± 0.01
6	1.12 ± 0.04	0.20 ± 0.01	1.34 ± 0.05
7	1.70 ± 0.23	0.23 ± 0.01	1.97 ± 0.24
8	0.38 ± 0.01	0.40 ± 0.02	0.80 ± 0.03
9	0.62 ± 0.03	5.26 ± 0.94	5.90 ± 0.97
10	0.65 ± 0.08	0.26 ± 0.06	0.93 ± 0.15
11	1.15 ± 0.02	0.20 ± 0.02	1.37 ± 0.04
12	2.74 ± 0.05	0.29 ± 0.02	3.05 ± 0.07
13	2.21 ± 0.06	0.20 ± 0.00	2.44 ± 0.07
14	1.59 ± 0.19	0.43 ± 0.01	2.04 ± 0.20
15	2.84 ± 0.37	0.83 ± 0.06	3.70 ± 0.43
16	1.05 ± 0.01	0.25 ± 0.03	1.33 ± 0.04
17	0.85 ± 0.01	0.42 ± 0.05	1.30 ± 0.06
18	0.70 ± 0.12	0.27 ± 0.01	0.99 ± 0.13
19	2.44 ± 0.04	0.54 ± 0.02	3.01 ± 0.06
20	1.82 ± 0.08	0.50 ± 0.01	2.34 ± 0.09
21	1.39 ± 0.11	0.34 ± 0.01	1.75 ± 0.12
22	0.96 ± 0.07	2.14 ± 0.11	3.12 ± 0.18
23	0.43 ± 0.01	0.16 ± 0.02	0.61 ± 0.03
24	0.44 ± 0.07	4.19 ± 0.08	4.65 ± 0.14
25	0.55 ± 0.00	3.12 ± 0.02	3.69 ± 0.03
26	0.30 ± 0.03	0.81 ± 0.06	1.13 ± 0.09
27	0.04 ± 0.01	0.11 ± 0.01	0.18 ± 0.02
28	1.89 ± 0.23	0.59 ± 0.03	2.51 ± 0.27
29	0.09 ± 0.02	0.09 ± 0.00	0.21 ± 0.02
30	3.54 ± 0.16	1.15 ± 0.04	4.71 ± 0.19
31	5.12 ± 0.57	0.70 ± 0.02	5.85 ± 0.59
OT	0.16[a]	1.80[a]	

[a](Moreira *et al.*,2010; Swiegers *et al.*,2005)

The concentration of isoamyl acetate, a volatile compound with aromatic importance due to its odor descriptor of banana and fruity, ranged from 0.04 mg/L (300 g/L of sugars, 500 mg/L YAN, 10ºC - run 27) to 5.12 mg/L (225 g/L of sugars, 500 mg/L YAN, 20ºC - run 31). The values exceeded its OT (odor threshold), (0.16 mg/L) in all experiments, except in runs 27 and 29. The factors with major impact on its production were nitrogen and sugars level, with the last displaying a quadratic effect (Figure 3.8), that is higher production of isoamyl acetate are produced in the center conditions of sugar.

A



B

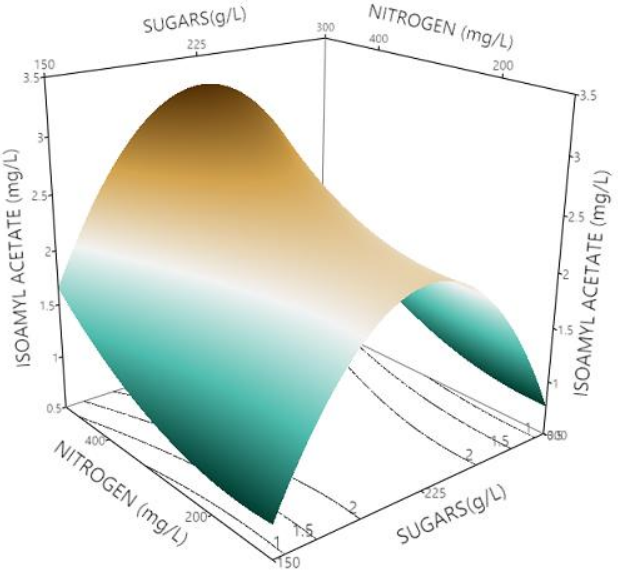


Figure 3.8. Response surface and contour plot of acetate esters (A) and isoamyl acetate (B) production.

III.3.3. Effect of fermentation conditions on the alcohols concentrations

Regarding the higher alcohols, the amounts varied from 4.10 to 380.89 mg/L. Levels up to 300 mg/L are found to contribute positively to wine sensory profile, while excessive amounts (higher than 400 mg/L) may detract wine quality (Bisson *et al*.,2010; Lambrechts *et al*.,2000). Nitrogen, temperature and sugar are factors that influence significantly the production of higher alcohols, as well as the interaction between nitrogen and temperature (Table 3.5 and Figure 3.9A).

Notably, the production of 1-propanol and methionol were positively affected by initial nitrogen concentration. Similar results have been obtained by Rollero *et al*.,(2015) for the first compound. As it can be seen in Figure 3.9B, higher levels of propanol are obtained in the highest nitrogen level with a quadratic effect of sugar concentration as seen by the curvature of the response surface, near middle points. Regarding methionol (Figure 3.9C), high nitrogen and sugars levels.

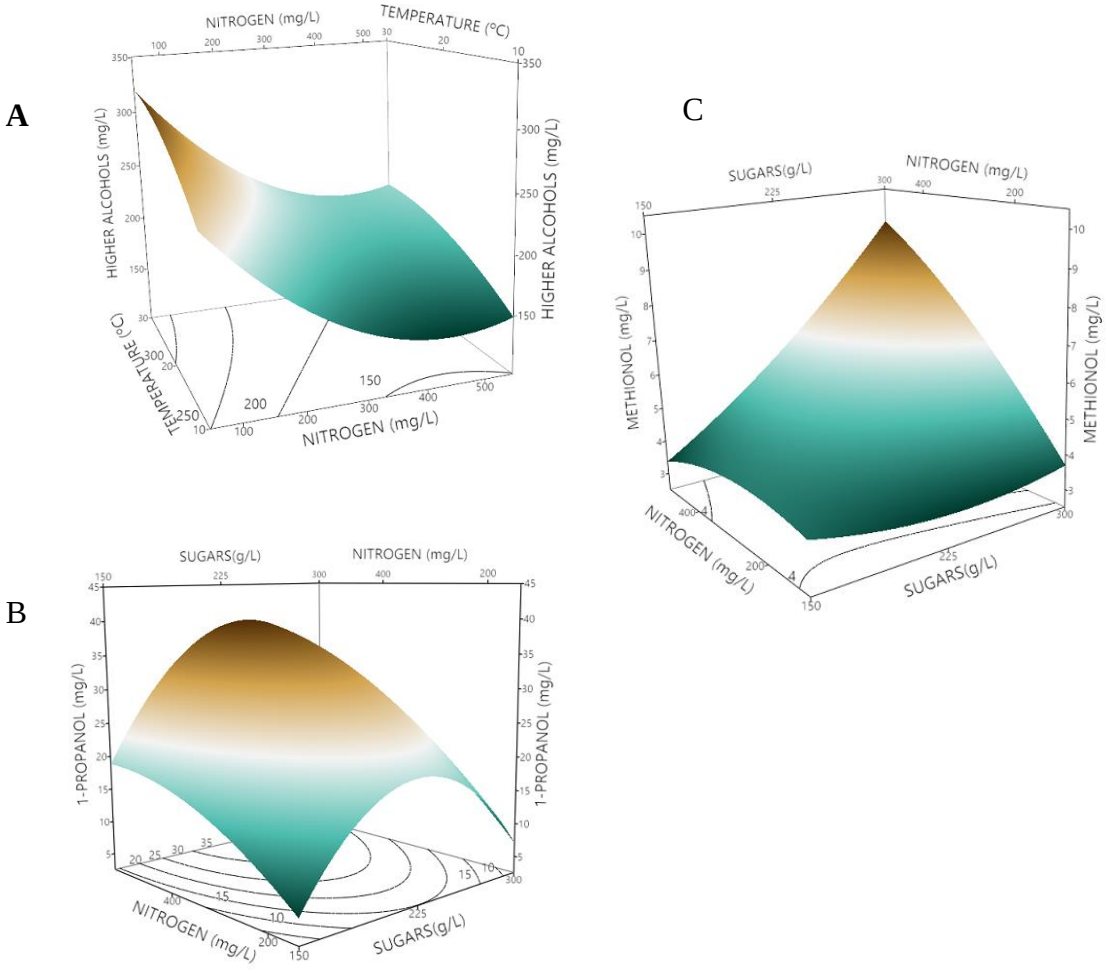


Figure 3.9. Response surface and contour plot alcohols (A), 1-propanol (B), and methionol (C) production.

Table 3.9. Higher alcohols concentrations (mg/L) in the final wines obtained in the 31 fermentations.

Run	1-propanol	1- butanol	2-methyl-1-butanol	3-methyl-1-butanol	Methionol	2-Phenylethanol	Alcohols
1	30.56 ± 0.30	20.94 ± 0.20	29.14 ± 0.20	118.46 ± 0.10	5.40 ± 0.14	9.54 ± 1.15	214.06 ± 2.09
2	10.78 ± 0.50	56.12 ± 0.7	59.11 ± 0.20	239.28 ± 0.40	7.19 ± 1.28	8.42 ± 1.60	380.89 ± 4.73
3	30.52 ± 0.70	12.46 ± 0.2	16.11 ± 0.00	49.21 ± 0.20	4.53 ± 0.09	2.05 ± 0.05	114.89 ± 1.31
4	11.72 ± 0.00	22.84 ± 0.0	37.27 ± 0.30	126.25 ± 0.30	2.94 ± 0.20	4.45 ± 0.14	205.47 ± 1.05
5	30.89 ± 0.10	17.80 ± 0.0	25.39 ± 0.20	102.09 ± 0.50	5.66 ± 1.75	9.04 ± 0.12	190.85 ± 2.63
6	34.84 ± 0.20	30.38 ± 0.6	29.57 ± 0.10	100.53 ± 0.40	6.29 ± 0.06	7.76 ± 0.54	209.36 ± 1.90
7	30.01 ± 0.10	20.52 ± 0.0	25.00 ± 0.10	83.45 ± 0.20	14.46 ± 0.93	5.57 ± 0.41	179.02 ± 1.77
8	8.79 ± 0.00	25.39 ± 0.8	35.53 ± 0.10	119.76 ± 0.30	3.22 ± 0.19	8.82 ± 0.37	201.52 ± 1.79
9	5.28 ± 0.20	67.46 ± 0.7	46.27 ± 0.00	155.24 ± 0.00	3.39± 0.90	6.74 ± 0.35	284.39 ± 2.19
10	32.49 ± 0.50	20.25 ± 0.7	18.36 ± 0.40	48.52 ± 0.70	4.28 ± 0.86	3.08 ± 0.81	126.98 ± 4.02
11	24.22 ± 0.20	21.57 ± 0.0	21.23 ± 0.10	89.35 ± 1.70	9.05 ± 1.60	5.04 ± 0.14	170.47 ± 3.75
12	26.35 ± 0.20	23.17 ± 0.3	31.11 ± 0.10	122.63 ± 0.20	7.24 ± 0.50	8.11 ± 0.71	218.62 ± 1.98
13	32.14 ± 0.70	17.75 ± 0.1	24.88 ± 0.00	94.18 ± 0.00	3.01 ± 0.02	4.49 ± 0.18	176.45 ± 0.99
14	38.40 ± 0.30	19.49 ± 0.0	24.39 ± 0.70	84.97 ± 0.70	3.09 ± 0.91	4.80 ± 0.22	175.15 ± 2.78
15	29.86 ± 0.40	19.04 ± 0.2	23.73 ± 0.00	86.69 ± 0.10	5.32 ± 0.16	6.61 ± 0.26	171.23 ± 1.13
16	14.20 ± 0.70	48.37 ± 0.2	45.02 ± 0.20	157.28 ± 0.50	4.95 ± 0.04	6.21 ± 0.70	276.03 ± 2.35
17	32.79 ± 0.70	27.34 ± 1.0	24.85 ± 0.40	45.71 ± 0.90	2.47 ± 0.26	2.07 ± 0.21	135.23 ± 3.42
18	13.36 ± 0.40	28.84 ± 2.0	29.88 ± 0.20	112.15 ± 0.00	0.65 ± 0.06	3.19 ± 0.13	188.06 ± 2.75
19	33.44 ± 0.00	18.60 ± 0.3	24.84 ± 0.20	89.57 ± 0.20	3.67 ± 0.29	5.68 ± 0.05	175.75 ± 1.04
20	35.61 ± 0.30	18.3 ± 0.3	22.39 ± 0.20	72.60 ± 0.30	3.15 ± 0.34	3.68 ± 0.04	155.71 ± 1.43
21	25.09 ± 0.20	40.2 ± 0.8	27.34 ± 0.00	93.23 ± 0.10	3.99 ± 1.51	5.65 ± 1.33	195.53 ± 4.11
22	6.64 ± 0.10	31.2 ± 0.3	35.51 ± 0.50	141.56 ± 1.80	3.07 ± 1.05	6.11 ± 0.68	224.11 ± 4.48
23	12.20 ± 0.50	18.0 ± 0.2	36.90 ± 0.20	121.33 ± 0.50	4.99 ± 1.07	14.19 ± 1.25	207.62 ± 3.75
24	5.12 ± 0.10	80.9 ± 0.2	44.66 ± 0.30	160.63 ± 1.00	3.47 ± 0.50	12.56 ± 0.05	307.37 ± 2.13
25	7.14 ± 0.10	45.4 ± 0.2	45.86 ± 0.30	173.11 ± 0.00	3.73 ± 0.02	11.39 ± 0.25	286.59 ± 0.88
26	7.42 ± 0.10	23.4 ± 0.2	32.74 ± 0.30	117.78 ± 0.00	1.40 ± 0.09	4.31 ± 0.25	187.03 ± 0.95
27	nd	nd	0.91 ± 0.20	2.96 ± 0.50	0.07 ± 0.00	0.17 ± 0.01	4.10 ± 0.67
28	36.01 ± 0.10	20.0 ± 0.1	24.10 ± 0.20	80.82 ± 0.20	2.83 ± 0.35	5.08 ± 0.45	168.88 ± 1.45
29	1.88 ± 0.10	18.5 ± 0.4	19.37 ± 0.20	73.04 ± 0.20	0.83 ± 0.09	4.44 ± 0.04	118.06 ± 1.01
30	16.65 ± 0.60	27.2 ± 0.2	40.50 ± 0.20	167.23 ± 1.30	9.66 ± 0.69	21.56 ± 0.88	282.84 ± 3.85
31	43.82 ± 0.10	21.7 ± 0.2	22.53 ± 0.10	83.39 ± 0.60	6.59 ± 1.39	5.16 ± 1.02	183.19 ± 3.43
OT	306.00	150[a]	1.3[a]	30.00[a]	1.00[a]	200.00[a]	

[a] (Moreira *et al.*,2010; Swiegers *et al*.,2005)

For higher concentrations of nitrogen, a higher production of 1-propanol was associated, this effect was verified in the fermentations corresponding to 300 and 500 mg/L of nitrogen. In more detail, higher production was related to intermediate nitrogen conditions of 300 mg/L, because the yeasts are submitted in a medium with excess nitrogen, and it is not necessary to exhaust the nitrogen source in order to proceed with their growth and the production of aromatic compounds, since, the ideal concentration of nitrogen for *S. cerevisae* to yield a complete fermentation in a timely manner and to produce aromatic compounds is approximately 267 mg/L of nitrogen, as described by Barbosa *et al*.,(2012), who also indicates that excess nitrogen does not indicate that it will assume a higher production, because its behavior is the same as when it has nitrogen values approximate to 267 mg/L. All assays had lower 1-propanol values than their OTs (306.00 mg / L).

The 1-butanol was positively correlated with temperature and inoculum, and nitrogen content had a negative effect. Thus, fermentations with low nitrogen that it, for fermentations with low nitrogen levels conducted at elevated temperatures, there is greater production of 1-butanol in mixed cultures, than in single culture, for example, runs 2 and 9, 16 and 24. The mixed culture assays have higher 1-butanol values in runs 9 and 24 than in single culture in runs 2 and 9, not taking into account the other factors. All assays had lower 1-butanol values than their OTs (150.00 mg/L).

The final concentration of 2-methyl-1-butanol, was only affected by the initial nitrogen content ($P < 0.01$), at lower levels of nitrogen there was a higher yield, for example runs 2, 9, 24 and 25, which single culture as mixed culture, nitrogen at 500 mg/L concentrations presents low production, runs 3, 10, 27. For 3-methyl-1-butanol, it was shown that nitrogen and sugar were the only factors that affected significantly its production, both presenting negative effects. Thus, at lower levels of nitrogen there was a higher yield, for example, runs 2, 9, 24 and 25. These two compounds are very similar, up to their deleterious descriptor alcohol, nail polish. Nevertheless, all produced values of 2-methyl-1-butanol are lower than their OTs (30 mg/L), except run 27 which is well below the detection threshold value.

The methionol was positively correlated with sugars content ($P < 0.05$), suggesting that in trials with high levels of sugar, there was a higher production of methionol as well as with nitrogen content as refered previously. Generally, with some exceptions, all trials

presented methionol values higher than their OTs (1.00 mg / L), being its odor descriptor sweet or potato. In particular, 2-phenylethanol was considered to be one of the most important aromatic alcohols contributing to wine flavor for odor descriptor rose, honey.

In the present work, the production of this compound was only negatively affected by the nitrogen content (Table 3.5); however, it was expected that all factors studied are directly related to the formation of 2-phenylethanol, influencing its production depending on the combined conditions between the factors. According to some studies, it is verified that in single culture occurs greater production than in mixed culture. In the same way, it is confirmed that low concentrations of nitrogen promote its production, as we can observe in runs 23, 24 and 25.

In the majority of studies nitrogen applied in the vineyard decreased the higher alcohol concentration in wine compared to wine prepared from vines that received no nitrogen. The greater proportion of alcohols produced are synthesized from sugars. When amino acids are absent and ammonium is the sole source of nitrogen, alcohols are nevertheless produced, thought at lower concentrations, that is, when the nitrogen concentration of must is low, a direct relationship between initial nitrogen concentration and the total concentration of alcohols exists, whereas at moderate must nitrogen an inverse relationship with alcohols prevails. At high initial must nitrogen, the concentrations of total alcohols are at their lowest. In wines, higher alcohols are quantitatively dominant and important in the sensory properties and quality. Below 300 mg/L higher alcohols contribute positively to wine quality, while excessive amounts (higher than 400 mg/L) may detract quality.

III.3.4. Effect of fermentation conditions on the fatty acids concentrations

The final concentrations of octanoic acid are presented in Table 3.10. In the experimental central points octanoic acid values was higher than their corresponding odour thresholds of 10 mg/L.

Table 3.10. Fatty Acids (mg/L) concentrations in the final wines obtained in the 31 fermentations.

Run	Octanoic acid	Decanoic acid	Acids
1	14.18 ± 1.12	3.69 ±0.01	17.87 ± 1.12
2	5.33 ± 0.38	3.57 ± 0.00	8.90 ± 0.38
3	11.51 ± 0.20	3.75 ± 0.01	15.26 ± 0.21
4	8.13 ± 0.83	3.57 ± 0.00	11.70 ± 0.83
5	13.64 ± 2.50	3.77 ± 0.00	17.41 ± 2.51
6	8.14 ± 0.83	3.57 ± 0.00	11.72 ± 0.83
7	2.89 ± 0.00	3.57 ± 0.00	6.46 ± 0.00
8	3.06 ± 0.01	3.57 ± 0.00	6.64 ± 0.01
9	5.93 ± 0.15	3.84 ± 0.03	9.77 ± 0.18
10	6.62 ± 0.93	3.57 ± 0.00	10.19 ± 0.93
11	6.16 ± 0.36	4.10 ± 0.14	10.26 ± 0.50
12	11.44 ± 1.51	5.20 ± 0.21	16.64 ± 1.72
13	9.88 ± 1.07	4.34 ± 0.11	14.22 ±1.18
14	13.94 ± 0.12	6.47 ± 0.04	20.41 ± 0.16
15	23.89 ± 2.75	9.42 ± 0.92	33.31 ± 3.67
16	11.16 ± 1.03	4.92 ± 0.13	16.08 ± 1.15
17	10.90 ± 0.95	5.56 ± 0.20	16.46 ± 1.15
18	12.10 ± 0.76	5.34 ± 0.21	17.43 ± 0.98
19	17.91 ± 0.85	6.73 ± 0.64	24.64 ± 1.49
20	19.35 ± 0.17	7.57 ± 0.54	26.92 ± 0.70
21	3.95 ± 0.06	3.57 ± 0.00	7.52 ± 0.06
22	11.87 ± 0.13	6.11 ± 0.02	17.99 ± 0.15
23	5.15 ± 0.28	3.57 ± 0.00	8.72 ± 0.28
24	4.15 ± 0.16	3.57 ± 0.00	7.72 ± 0.16
25	2.99 ± 0.03	4.29 ± 0.00	7.28 ± 0.03
26	3.10 ± 0.02	5.46 ± 0.14	8.57 ± 0.16
27	3.33 ± 0.03	3.89 ± 0.03	7.22 ± 0.06
28	12.62 ± 4.12	7.34 ± 0.21	19.95 ± 4.32
29	3.42 ± 0.03	3.75 ± 0.02	7.17 ± 0.04
30	11.30 ± 1.07	3.93 ± 0.21	15.23 ± 1.27
31	32.22 ± 2.80	7.48 ± 0.63	39.70 ± 3.43
OT (mg/L)	10.00[a]	6.00[a]	

[a] (Moreira *et al.*,2010; Swiegers *et al*.,2005)

For decanoic acid, there was no significance linear effect of any of the factors however, the quadratic term of temperature affected negatively the production of this compound. On the other hand, a positive effect of nitrogen was seen for octanoic acid. The observation that lower levels of these sensory negative compounds, due to its fat and rancid odour, were detected where *H. guilliermondii* was present, does not compromise the future application of this *non-Saccharomyces* strain in mixed-starter culture fermentations.

III.4. References

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CHAPTER IV

Conclusions

IV. Conclusions

Winemaking industry has gained particular interest in the use of mixed starter cultures of *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts towards improving the complexity and enhancing the particular and specific characteristics of wines. In this line, a strain of *H. guilliermondii*, previously isolated in our laboratory from a fermenting grape-juice from Douro Region (Neto *et al*.,2005) has been selected to be used in mixed-wine fermentations. In a previous work conducted in a natural grape-juice it was found that, although the presence of *H. guilliermondii* negatively affected *S. cerevisiae* UCD522 growth and fermentation rate, its presence significantly alter the panoply of aroma compounds found at the end of the fermentation (Lage *et al*.,2014).

Based on these evidences, and with the aim to increase knowledge on this yeast consortium, in this study, we aimed to evaluate the combined effect of different conditions, on yeast growth and fermentative activity and on the production of sensory relevant wine compound. The influence of fermentation temperature (10ºC-30ºC), initial nitrogen (100-500 mg/L) and sugars concentration (150–300 g/L) and inoculum levels of *H. guillliermondii* (0 – $1x10^6$ CFU/ml) were evaluated using a central composite design. In this way, we could minimize the number of experiments required to evaluate the effects of these four factors as well as its putative interactions. A total of thirty-one fermentations were performed using a synthetic grape-juice medium in order to accurately control nutrient levels to be tested. ANOVA analysis indicated that all models were statistically significant at 95% confidence level (except for isoamyl alcohol) and a range of R^2 between 55 and 95 %. Lower R^2 were obtained for the volatile compounds (0.58 - 0.84) which could, at least partially, be attributable to higher experimental error inherent to the measure of the compounds. In addition, the lack of fit of some of the obtained models did not allow good estimations of the predicted responses. Models for some dependent variables had significant lack of fit suggesting that there may be some systematic variation unaccounted in the theorized model, or as it is the case of R25 and R100 could be due to the lack of variability in the replicate values obtained (center points) in the model that provide an estimate of the pure error. Overall, nitrogen was the main factor impacting either yeast growth and fermentative activity or production of volatile and non-volatile compounds. It was shown the potencial of *H.guilliermondii* to be use as and adjunct of *S.cerevisiae*, at least of UCD522 strain, since it did not compromised pivotal fermentation

parameters and given its effect on aroma composition, its presence results in a diversified aromatic profile of the wines.

Most of the models obtained were somehow difficult to describe and interpret, since they were largely influenced by the particular factors of the fermentation processes that are used in their establishment. More data as well as replicate experiments are necessary to evaluate the usefulness of the models obtained in this study, particularly for aroma compounds. Despite the mentioned constrains in the models obtained we were able to characterize the extent to which the initial nitrogen and sugar concentrations and temperature impact on mixed-culture fermentations with *H. guilliermondii* and on chemical composition of the final product and provide experimental information for future research.

In general terms, our results showed that inoculation with *H. guilliermondii* decreased the production of acetic acid, not contributing to a significant increase in the wine's volatile acidity, and increased the production acetate esters that are desirable due to their fruity taste. Nevertheless, the inoculation with *H. guilliermondii* might increase the production of ethyl acetate, which is undesirable due to its nail polish smell. Thus, more studies are needed in the future, such as sensory analysis and using real samples, in order to find a compromise with the aim of producing wines with high quality.