Nutritional requirements and survival of the red wine spoilage yeast *Brettanomyces bruxellensis*

by

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Declaration

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Summary

Brettanomyces bruxellensis is a red wine spoilage yeast that plagues the wine industry. It facilitates the formation of compounds such as volatile phenols, which impart negative aromas in red wines. Its ability to survive in wine through adaptations to various stressors (e.g. high ethanol and sulphur dioxide concentrations, low pH) have made it the subject of several studies. These studies aim to understand its biology (and in particular its nutrient requirements) and its survival mechanisms in general, as well as to detect and enumerate it accurately and to eliminate it. Nevertheless, literature on the subject is at certain times contradictory with regard to the nutritional needs of this yeast. The survival of this yeast for extended periods of time lead us to question how it is so well adapted to this deficient environment. In other words, what substrates does *B. bruxellensis* utilise to sustain growth or at least survival in such a nutrient-deficient medium where stronger fermenters (e.g. *Saccharomyces cerevisiae*) cannot survive?

This study investigates the carbon and nitrogen source assimilation of three *B. bruxellensis* strains in a defined and model synthetic wine medium. In addition, the growth kinetics were determined (as well as the consumption pattern of the various carbon and nitrogen sources). This was performed in conditions similar to bottle ageing (anaerobic) and barrel maturation (semi-anaerobic). Furthermore, the purpose of assimilating these sources is explored with a focus on growth or cell maintenance (i.e. survival with no growth). The data showed that carbon consumption followed a step wise pattern. At first, sugars were consumed, thereby leading to the production of ethanol and biomass concurrently. Upon complete consumption of the sugars, malic acid was consumed together with ethanol, but only when oxygen was present for the latter. These compounds were consumed slowly and resulted in the survival of the cells for a period of 45 days. After this period, the consumption of ethanol allowed for the extended functioning of the cell, however the assimilation of ethanol lead to an increasing degree of imbalance (more and more NADH was produced with little to no conversion to NAD^+) in the cell and eventually lead to a slow but steady decline of the population. The formation of 4-Ethylphenol (4-EP) was investigated in order to ascertain its ability to correct the redox potential of the *B. bruxellensis* cell. The results displayed the partial regeneration of NAD^+ during 4-EP formation, however, this metabolic pathway alone is not solely responsible.

Unlike for carbon sources, the data showed that the assimilation of nitrogen compounds were strain specific and certain strains required more nitrogen than the others. The sources of prime importance were ammonia and arginine and were assimilated during the exponential growth phase (i.e. during sugar consumption). During the stationary phase, proline was assimilated regardless of the presence/absence of oxygen, possibly to counteract stressors in the cell and ensure survival of the population.

This study contributes to an improved understanding of how *B. bruxellensis* survives in wine and is able to maintain cell function for extended periods of time. This leads to a better understanding of the spoilage yeast *B. bruxellensis* and will allow for the production of wine in an integrated manner to avoid the proliferation of this microorganism.

Opsomming

Brettanomyces bruxellensis is ’n gis wat rooiwyn bederf en daardeur die wynbedryf teister. Dit fasiliteer die vorming van verbindings soos vlugtige fenole, wat negatiewe aromas aan rooiwyn oordra. Sy vermoë om in wyn te oorleef deur aanpassings tot verskillende stressors (bv. hoë konsentrasies van etanol en swaeldioksied, lae pH) het daartoe gelei dat dit reeds die onderwerp van verskeie studies was wat gepoog het om die biologie daarvan te verstaan (veral sy voeidingsvereistes) sowel as sy oorlewingsmeganismes in die algemeen, en ook hoe om dit akkuraat te bespeur en te enumerate en dit te elimineer. Nietemin is die literatuur oor die onderwerp by tye teenstrydig met betrekking tot die voedingsbehoeftes van hierdie gis. Die oorlewing van die gis vir lang tydperke noop die vraag hoe dit so goed aangepas is tot hierdie deficient omgewings. Met ander woorde, watter substrate gebruik *B. bruxellensis* om groei of ten minste oorlewing in so ’n voedingsarme medium te onderhou waar sterker gisters fermenters (bv. *Saccharomyces cerevisiae*) nie kan oorleef nie?

Hierdie studie het die koolstof en stikstof assimilasie van drie *B. bruxellensis* rasse in ’n gedefinieerde en model sintetiese wynmedium ondersoek. Daarbenewens is die groeikinetika in elke medium bepaal, sowel as die verbruikspatroon van die verskillende koolstof- en stikstofbronne wat verskaf is. Dit is ook ondersoek onder omstandighede wat eenders is aan bottelveroudering (anaërobies) en vatveroudering (semi-anaërobies). Verder is die doel van die assimilering van hierdie bronne ondersoek met ’n fokus op groei of selonderhoud (m.a.w. oorlewing sonder groei). Die data het getoon dat koolstofverbruik ’n stapsgewyse patroon gevolg het. Aanvanklik is suikers verbruik, wat sodoende gelei het tot die gelyktydige produksie van etanol en biomassa. Sodra die suikers volledige verbruik is, is appelsuur tesame met etanol verbruik, maar laasgenoemde slegs wanneer suurstof daarvoor teenwoordig was. Hierdie verbindings is stadig verbruik en het gelei tot die oorlewing vna selle vir ’n tydperk van 45 dae. Hierna het die verbruik van etanol die verlengde funksionering vna die sel toegelaat, hoewel die assimilering van etanol gelei het tot ’n toenemende mate van wanbalans (toenemend NADH is produseer met min of geen omskakeling na NAD^+) in die sel en uiteindelik tot ’n stadige maar bestendige afname in die bevolking. Die vorming van 4-etielfenol (4-EP) is ondersoek om die vermoë daarvan om die redokspotensiaal van die *B. bruxellensis* sel te korrigeer, te bepaal. Die resultate het gedeeltelike regenerasie van NAD^+ tydens 4-EP-vorming vertoon, maar hierdie metaboliese pad is egter nie alleen daarvoor verantwoordelik nie.

In teenstelling met koolstofbronne toon die data dat die assimilering van stikstofverbindings rasspesifiek is en dat sekere rasse meer stikstof as ander benodig. Die belangrikste bronne was ammoniak en arginien, wat tydens die eksponensiële groeifase geassimileer is (m.a.w. tydens suikerverbruik). Tydens die stasionêre fase is prolien geassimileer, ongeag die teenwoordigheid/afwesigheid van suurstof, moontlik om stressors in die sel teen te werk en die oorlewing van die bevolking te verseker.

Hierdie studie maak ’n bydrae tot ’n verbeterde begrip van hoe *B. bruxellensis* in wyn oorleef en die vermoë het om selfunksionering vir verlengde tydperke te onderhou. Dit lei tot ’n beter begrip van die *B. bruxellensis* bederfgis en sal die produksie van wyn in ’n geïntegreerde wyse moontlik maak om sodoende die vermenigvuldiging van hierdie mikro-organisme te verhoed.

This thesis is dedicated to
my family for their unwavering encouragement, support and love.

“Quickly, bring me a beaker of wine, so that I may wet my mind and say something clever.”
Aristophanes

Biographical sketch

Brendan Daniel Smith was born on 24 July 1992, in Cape Town. He matriculated from Table View High School in 2010 and in 2011 he began studying at Stellenbosch University. It was here that he discovered his passion for wine. He completed his undergraduate degree in Viticulture and Oenology in 2014. In 2015, Brendan commenced his MSc of Agriculture in Oenology at the Stellenbosch University.

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"Accept what life offers you and try to drink from every cup. All wines should be tasted; some should only be sipped, but with others, drink the whole bottle." Paulo Coelho.

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"We all need something to help us unwind at the end of the day. You might have a glass of wine, or a joint to silence your silly brainbox of its witterings but there has to be some form of punctuation, or life just seems utterly relentless." Russel Brand

Last, but most certainly not least to my family, thank you for encouraging me in all of my pursuits and inspiring me to follow my dreams. I am especially grateful to my parents, who supported me emotionally and financially. I always know that you believe in me and want the best for me. No matter what lies ahead I can always count on you. You have been the most unyielding foundation in my life from which I have been able to grow and flourish as an individual. To my mother, **Gayleigh Smith** your tireless hard work and achievements have never gone unnoticed. This tenacity to strive for the best motivates me to do the best possible job in whatever I might undertake. Your encouragement for me to follow my dreams and succeed have helped me get to where I am today. For that I will always be grateful. To my father, **Michael Smith** your continued support and example will always be cherished. You have guided me since my formative years and always assisted whenever I need help. You have shown me how great a man can be and I will endeavour to live up to the example you have displayed. To **Gérard Smith,** it is fantastic that I can not only call you a brother but also a friend.

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Preface

This thesis is presented as a compilation of 4 chapters. Each chapter is introduced separately and is written according to the style of Food Microbiology in which Chapter 2 was published.

Chapter 1 **General Introduction and project aims**

Chapter 2 **Literature review**
Brettanomyces bruxellensis, a survivalist prepared for the wine apocalypse and other beverages

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The nutrient consumption pattern of the spoilage yeast *Brettanomyces bruxellensis* reveals an adaptation for survival in wine

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Chapter 1

Introduction and project aims

1. General introduction and project aims

1.1. Introduction

The characteristics of the genus *Brettanomyces* were first described in 1904, by Claussen, and its name was coined following its isolation from the secondary fermentation of English beers (Claussen, 1904). The first taxonomic investigation of this yeast genus was conducted in 1940 and describes more specifically the species *Brettanomyces bruxellensis* (Custers, 1940). Initially this yeast was exclusively isolated from beer products. However, in 1933, the first isolate was found in French grape must (Van der Walt and van der Kerken, 1958) and later in other wines worldwide. In recent years, most authors agree that this yeast has received special attention due to its ability to spoil wine through the production of ill-smelling volatile phenols (Henick-Kling et al., 2000; Suárez et al., 2007; Romano et al., 2008; Oelofse et al., 2008). It is intriguing that to date, so little is known regarding the mechanisms of survival and growth of this yeast in nutrient deficient media such as wine despite decades of research. The ability of *B. bruxellensis* to survive and grow in wine is somehow fascinating. Indeed, in wine, most of the nutrients present in the initial grape juice are consumed by the microorganisms responsible for the transformation of grape juice to wine. *B. bruxellensis* seems to have remarkably adapted to this peculiar environment and to various conditions including the absence of oxygen. Indeed, the species *B. bruxellensis* is isolated commonly from red wine in barrels as well as bottles (Loureiro and Malfeito-Ferreira, 2006). These vessels allow for varying degrees of oxygen to enter the wine matrix. Indeed, bottle-ageing can be considered a mostly anaerobic process, as little oxygen if any enters the matrix, whereas more, but yet limited, oxygen can come into contact with the wine when stored in a barrel (du Toit et al., 2006).

B. bruxellensis has been reported to utilise a vast number of carbon and nitrogen sources from the medium (Conterno et al., 2006). These nutrients range from simple sugars to more complex compounds found in the wine environment. However, it is not known under what environmental conditions (e.g. aerobic or anaerobic conditions) this occurs as literature is conflicting. Furthermore, certain studies report the secretion of extracellular hydrolytic enzymes (e.g. α-glucosidase, β-glucosidase and glucanase activity) in order to liberate monomers/oligomers (e.g. monosaccharides) from more complex polymers (e.g. polysaccharides). Indeed, wine contains many complex compounds that cannot be utilised by other yeast. Therefore, this may confer an advantage over other yeasts in the wine medium (Mansfield et al., 2002; Shantha Kumara et al., 1993). This may be another strategy employed by *B. bruxellensis* to utilise nutrients released from other yeast species (i.e. autolysis) in a deficient medium such as wine. Furthermore, the scavenging nature of this yeast occurs over lengthy periods of time as consumption of carbon sources is prolonged. *B. bruxellensis* has also adapted other mechanisms for maintaining its intracellular redox balance as cell viability is observed over decades, even though it does not produce key-metabolites such as those known to be involved in redox balancing e.g. glycerol (Coulon et al., 2010; Blomqvist et al., 2010; Galafassi et

al., 2010). The production of other metabolites has therefore been investigated. In particular, the ability of *B. bruxellensis* to produce the volatile phenols 4-ethylphenol and 4-ethylguaiacol has been proposed as a corrective mechanism of any imbalance occurring in the yeast cell, but this hypothesis has not been experimentally verified yet (Duckitt, 2012; Curtin et al., 2013).

B. bruxellensis has also been characterised to exhibit a high degree of genetic variability that consequently results in differences with regard to growth rate, biomass formation and ethanol production as well as certain physiological characteristics such as the formation of pseudomycelia by certain strains of this yeast (Curtin et al. 2007; Hellborg and Piškur, 2009; Blomqvist et al., 2010; Louw et al., 2016). This diversity presents challenges regarding the study of this yeast, as in previous studies, different strains have been utilised. However, these strain-dependent characteristics may explain variations amongst numerous studies (Oelofse et al., 2008; Harris et al., 2009; Romano et al., 2009). Furthermore, it could possibly explain certain contradictions that have occurred in past literature as reviewed by Smith and Divol (2016). Certain discrepancies may be noted between studies that investigated carbon and nitrogen sources assimilated by *B. bruxellensis*. Regarding ethanol and glycerol for instance, certain studies observed the assimilation of these compounds whereas others did not (Conterno et al., 2006; Vigentini et al., 2008; Crauwels et al., 2015). The same was noted regarding nitrogen assimilation in *B. bruxellensis*, as reviewed by Smith and Divol (2016). Therefore, to date, it is still not known how or why *B. bruxellensis* survives in/spoils wine. Consequently, it is difficult to prevent the growth of this yeast, which is a major problem for the wine industry.

1.2. Rationale and project aims

This study aims to determine which nutrients are consumed by different strains of *B. bruxellensis* in a chemically defined wine-like medium. In addition to this, the study endeavours to investigate the metabolites produced and their functional outcome (e.g. for growth or cell maintenance). In particular, this study focuses on addressing many discrepancies regarding the nutrient requirements of the spoilage red wine yeast *B. bruxellensis* under different environmental conditions (i.e. semi-aerobic or anaerobic conditions). The ultimate goal of this study is to determine the metabolic mechanisms by which *B. bruxellensis* is able to survive in the wine matrix. A broader understanding of this yeast will thereby come to light with regard to its nutrient needs and prolonged survival. Furthermore, the knowledge can be utilised by the wine industry in order to prevent growth of *B. bruxellensis* and subsequent spoilage, or even combat this yeast in a more efficient/effective manner

The main project aims are as follows:

1. To investigate the carbon and nitrogen sources that can be utilised by *B. bruxellensis* using plate assays and in a wine-like medium with further attention placed on the chronological order of consumption of these various nutrients.

2. To investigate the effect of oxygen as well as strain variation on the utilisation of these carbon and nitrogen sources.
3. To determine the metabolic means by which *B. bruxellensis* maintains its intracellular redox balance in the cell, with specific focus on volatile phenol production.

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Chapter 2

Literature review

Brettanomyces bruxellensis, a survivalist prepared for the wine apocalypse and other beverages

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Brettanomyces bruxellensis, a survivalist prepared for the wine apocalypse and other beverages

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ABSTRACT

Brettanomyces bruxellensis is a common red wine spoilage yeast. Yet, in addition to wine, it has been isolated from other ecological niches that are just as nutritionally deficient as wine. *B. bruxellensis* can therefore be regarded as a survivor, well adapted to colonise harsh environments not often inhabited by other yeasts. This review is focused on the nutritional requirements of *B. bruxellensis* and the relevance thereof for its adaptation to the different matrices within which it occurs. Furthermore, the environmental conditions necessary (e.g. aerobic or anaerobic conditions) for the assimilation of the carbon or nitrogenous sources are discussed in this review. From literature, several confusing inconsistencies, regarding nutritional sources necessary for *B. bruxellensis* survival, in these specialist ecological niches are evidenced. The main focus of this review is wine but other products and niches that *B. bruxellensis* inhabits namely beer, cider, fruit juices and bioethanol production plants are also considered. This review highlights the lack of knowledge regarding *B. bruxellensis* when considering its nutritional requirements in comparison to *Saccharomyces cerevisiae*. However, there is a large enough body of evidence showing that the nutritional needs of *B. bruxellensis* are meagre, explaining its ability to colonise harsh environments.

Contents

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0740-0020/

1. Introduction

Wine is the final product of multiple biotic and abiotic interactions that occur in a complex medium (i.e. grape juice). The former are facilitated by microorganisms of which some are beneficial and others detrimental to wine quality. Certain abiotic factors, which include temperature and the initial chemical composition, play a major role in the composition of the resulting wine. The aforementioned detrimental microorganisms are referred to as spoilage microorganisms. Amongst these, yeasts of the genus *Brettanomyces*, or their teleomorphs known as *Dekkera*, have been found to negatively alter the chemical composition of wine by producing by-products detrimental to the organoleptic properties of the final product (Loureiro and Malfeito-Ferreira, 2003). Of this genus, the species *Brettanomyces bruxellensis* is a spoilage microorganism of wine. *B. bruxellensis* however, not only occurs in wine but also in many other alcoholic beverages, where it is not considered as a spoilage yeast e.g. some speciality beers and cider (Davenport, 1976; Loureiro and Malfeito-Ferreira, 2006). These yeasts can persist through the harsh conditions that occur during the winemaking process, such as rising ethanol concentrations and increasing additions of sulphur dioxide; they have in recent years become a major oenological concern worldwide.

Generally, many other species of yeasts, that naturally occur on grapes in the vineyard, are present at the onset of alcoholic fermentation but are readily eliminated due to: (i) the increase in ethanol concentration, (ii) the release of toxic compounds (e.g. killer toxins, weak acids, phenolic compounds and sulphur dioxide) by the dominant yeast *Saccharomyces cerevisiae*, (iii) competition for space and nutrients and (iv) low oxygen conditions (Pretorius et al., 1999; Holm Hansen et al., 2001; Nissen and Arneborg, 2003; Pérez-Nevado et al., 2006). *B. bruxellensis* however, is commonly isolated in wine and is well suited to surviving on all surfaces in and around the winery: winery walls, presses, fermentation tanks as well as within the wood of barrels used for maturation (Fugelsang, 1997). These environments are opportune for the colonisation of *B. bruxellensis* while the must is fermenting but even more so when the wine is ageing in barrel. In addition, the formation of biofilms by *B. bruxellensis* makes disinfection challenging, as biofilms are relatively resistant to chemical cleaning agents and sanitisers (Oelofse et al., 2008). Generally, grape juice is an environment inhabited by many yeasts, including *S. cerevisiae* the dominant yeast during alcoholic fermentation as well as other non-*Saccharomyces* yeasts. Amongst these yeasts, *B. bruxellensis* can also persist in this medium (Renouf et al., 2006). The ability however, of *B. bruxellensis* to reproduce and grow in wine, as opposed to grape juice, may have resulted in its adaptation to surviving in low nutritional environments.

The aromatic profile of wines is negatively influenced by the yeast *B. bruxellensis* and tends to be characterised by mousy, medicinal, wet wool, burnt plastic or horse sweat smells/scents. These off-aromas are said to be a part of the “Brett” character in wine (Licker et al., 1999). This is potentially problematic for winemakers, as this can result in serious economic losses (Loureiro and Malfeito-Ferreira, 2003; Fugelsang, 1997). The contamination of wines by *B. bruxellensis* has increased in recent years due to winemaking techniques changing to favour the production of wines that contain more residual sugar and that may be unsulphited, unfiltered, aged on the lees or aged longer in barrels. All of these factors are considered favourable to *B. bruxellensis* growth (de Orduña, 2010; Alston et al., 2011).

Although there has been a lot of research on factors influencing the growth of *B. bruxellensis*, it is surprisingly still uncertain which nutrients (and the concentrations thereof) are required for this microorganism to proliferate in inhospitable environments such as wine. It can be assumed from its ability to proliferate in a nutrient-poor medium that unlike *S. cerevisiae*, *B. bruxellensis* displays low nutrient requirements. In this review, the many ecological niches of *B. bruxellensis* will be reviewed with a primary focus on the nutritional composition of the products or niches concerned, also taking into account the adaptations of this yeast allowing it to survive in these media with varying compositions. Formerly, the history and sources of isolation of *B. bruxellensis* will be briefly discussed as this organism has been isolated from many different industries and matrices and has been referred to by various names since its discovery. In addition, the more common niches, products and matrices that *B. bruxellensis* is isolated from, will be discussed with regard to the potential nutrients available to the yeast. The general carbon and nitrogen sources utilised by *B. bruxellensis*, and its ability to utilise the nutrient sources from the different matrices it is isolated from, will be thoroughly reviewed. Overall, this review aims to provide a systematic and argumentative summary of *B. bruxellensis*' nutritional requirements in an attempt to characterise the biological adaptations connected to surviving in a harsh medium.

2. History of *Brettanomyces*

Brettanomyces was given its name due to its close connection to the British brewing industry, as it was first isolated in the secondary fermentation of English beers. Indeed, once the initial fermentation by *Saccharomyces* was completed, it was noted by Claussen, that a secondary slow fermentation occurred. Later, he identified the yeast dominating this secondary fermentation and named it *Brettanomyces* (Claussen, 1904). Derived from the Greek "*Brettano*" meaning British and "*myces*" meaning fungus (Licker et al., 1999). The initial species names for *Brettanomyces* confirm that it was originally isolated from beer. In fact, the species *lambicus* was isolated from Belgium lambic beers, *carlbergensis* was first isolated at the Carlsberg brewery in 1904 by N. Hjelte Claussen, *bruxellensis* is named after the Senne valley near Brussels, Belgium and finally *claussenii* was isolated from strong English stock beer (Claussen, 1904). Following the discovery of this yeast by Claussen, it was not until the 1920's, when more isolates were isolated from lambic beers, that *Brettanomyces* was proposed as a genus (Kufferath and van Laer, 1921). In 1933, it was isolated from German grape must and referred to as *Mycotorula intermedia* (Krumbholz and Tauschanoff, 1933). *Brettanomyces vini* was then isolated in wines originating from France in the 1950's (Barret et al., 1955; Peynaud and Domercq, 1956). Wineries and the equipment utilised have been investigated for the occurrence of *B. bruxellensis*. These investigations have revealed the presence of *Brettanomyces* yeasts in air samples, on cellar walls, drains, pumps, transfer lines and other equipment that can be difficult to sanitise (Peynaud, 1959; Van der Walt, 1984; Neva et al., 1998; Fugelsang, 1998; Connel et al., 2002).

Due to their formation of acetic acid aerobically, as well as their negative impact on the organoleptic properties of the resulting wine, these yeasts were identified as spoilage yeasts capable of deteriorating wine quality (Peynaud and Domercq, 1956). In the late 1950's and early 1960's, two species of *Brettanomyces* namely *Brettanomyces intermedius* and *Brettanomyces schanderlii* were associated with haze formation in South African wines (Van der Walt and van Kerken, 1958; 1959, 1961; Van Zyl, 1962). From the 1940's to the 1980's, the occurrence of *Brettanomyces* has also been reported by various authors in wines from Italy, Australia and New Zealand (Sponholz, 1993). Towards the end of the 1970's, some members of the *Brettanomyces* genus were also isolated from the cider industry. These species were isolated from water used in the washing of the apples as well as from the apple conveying channels (Davenport, 1976). More recently, *B. bruxellensis* has been isolated

from cider fermentations as well as from maturing cider (Beech, 1993; Michel et al., 1988; Morrissey et al., 2004). Prevalence of *B. bruxellensis* in the liquor industry is rather uncommon, but it has been isolated during all stages of fermentation leading to tequila (Lachance, 1995). In the bioethanol industry, *B. bruxellensis* has been isolated from alcoholic fermentations intended for distillation on numerous occasions (De Miniac, 1989; Uscanga et al., 2000; Passoth et al., 2007; Basílio et al., 2008). Finally, it was isolated in both Brazilian and Swedish ethanol production plants, where different substrates are utilised e.g. molasses and starch (Guerra, 1998; Souza-Liberal et al., 2005, 2007; Leite et al., 2013).

The species of *Brettanomyces*, *Brettanomyces lambicus*, *B. intermedius*, *B. vini*, *B. schanderlii*, *Brettanomyces claussenii*, *and M. intermedia* as well as many others have been reclassified as *B. bruxellensis* and these previously used names are now listed as synonyms (Barnett et al., 1990). Today, only five species are acknowledged: *Brettanomyces custersianus*, *Brettanomyces naardenensis*, *Brettanomyces nanus*, *Brettanomyces anomalus* and *Brettanomyces bruxellensis* (Smith, 1998a, 1998b). Of these five species, *B. anomalus* and *B. bruxellensis* have a teleomorph state known as *Dekkera* and are mainly found in wine, the latter being the predominant species in this matrix. Morphologically, *B. anomalus* is typically differentiated from *B. bruxellensis* by the presence of non-septate filaments. The name for the teleomorph state *Dekkera* comes from Dr. N.M. Stelling-Dekker, a renowned taxonomist, however most researchers refer to this genus under the umbrella term "*Brettanomyces/Dekkera*" (Van der Walt, 1964) because of the difficulties in distinguishing between the two forms, as well as the technical challenges regarding the sporulation of this species (Henschke et al., 2007; Curtin and Pretorius, 2014; Daniel et al., 2014). The former three *Brettanomyces* species *B. custersianus*, *B. naardenensis* and *B. nanus* are included and mentioned in taxonomic manuals but are seldom isolated. These species have generally been isolated from industrial niches. *B. naardenensis* however, has not been isolated from fermentation environments, although its beverage niche can still be considered industrial (Kolfschoten and Yarrow, 1970). These three species have been isolated from manufacturing equipment in beer brewing, olives, lemonade, soft drinks and bottled beer (Kurtzman and Fell, 1998; Smith, 1998a; Barnett et al., 1990). Only a brief history has been outlined in this review to summarise the sources of isolation of the different *Brettanomyces* species. The reader is invited to read a more detailed historic background on *B. bruxellensis* in an article published by Steensels et al. (2015). This review will focus on *B. bruxellensis* (including all synonyms from old literature), the most commonly encountered species in fermented beverages/solutions.

3. The ecological niches associated with *B. bruxellensis*

As mentioned above, *B. bruxellensis* has been isolated from many ecological niches. These various products and environments display common features/characteristics. Indeed, they are characterised by high alcohol concentrations, with the exception of soft drinks, fruit juices and the initial onset of alcoholic fermentations, and tend to be harsh matrices for microorganisms to live in as they contain limited nutrients, including low residual sugar concentrations and nitrogen sources, depending on the product. In each of these varying ecological niches, *B. bruxellensis* has been identified in a beneficial or a spoilage capacity. For example, *B. bruxellensis* can be beneficial in certain beer types but is usually considered detrimental in wine products.

Wine, one of principal environments from which *B. bruxellensis* is commonly isolated, is considered a harsh medium. In fact, it is known to exhibit a low pH (3–4), to contain yeast-derived ethanol, and a high sulphite concentration, a powerful antimicrobial agent, normally added by the winemaker. *B. bruxellensis* can potentially occur in wine, originating from various sources: as a contaminant from the starter culture used, it may naturally occur on grape skins, it could be introduced by insects, or may exploit a primary habitat in the cellar itself (Fugelsang, 1997). Most common *B. bruxellensis* contaminations occur from unsanitary wine making practices, use or purchase of wine barrels, new or previously used barrels from other vintners that are contaminated with this yeast (Henick-Kling et al., 2000). Additionally, as *B. bruxellensis* is generally able to utilize cellulose as well as other wood substrates, it is difficult to get rid of the microbe once it is established in a cellar environment. Thus, one defence against this yeast would be to limit or avoid potential sources of contamination. In order to reduce the risk of contamination precautionary measures such as SO_2 addition can be implemented, this can be done just before fermentation or upon completion of the alcoholic and malolactic fermentations.

In order to combat *B. bruxellensis* spoilage, several methods have been described in order to detect these yeast cells, to further control this yeast early on in the fermentation (Stender et al., 2001; Cecchini et al., 2013). With this in mind, other authors report the benefits of this "Brett" character in certain wine styles, as the influence of minimal amounts of these aromas may enhance the complexity as well as impart a mature character on younger wines (Fugelsang, 1997). The acceptability of these *Brettanomyces*-induced aromas depends on the flavour intensity, the winemakers' personal preference and consumer expectations (Wedral et al., 2010). Spoilage is mostly connected to the production of volatile phenols such as 4-ethylphenol (4 EP), 4-ethylguaiacol (4 EG), 4-ethylcatechol (4 EC) and the vinyl derivatives, 4-vinylphenol (4 VP), 4-vinylguaiacol (4 VG) and 4-vinylcatechol (4 VC). The formation of these aromas (4 EP, 4 EG and 4 EC) stems from the catabolism of the hydroxycinnamic acids, *p*-coumaric acid, ferulic acid and caffeic acid, respectively. In red wine, these compounds are only considered as being negative when these metabolites are present at high concentrations. Additionally, the "Brett" character may arise from a combination of the compounds, including 4 EP, 4 EG, acetic acid and the 'mousy off flavour' component (*N*-heterocyclic compounds) (Romano et al., 2008). According to a report by Romano et al. (2008), this "Brett" character is also the result of an interaction among strain, wine pH and sugar content as well as the winemaking stage at which the spoilage occurs. At lower levels these metabolites could contribute to the complexity of a young red wine by enhancing the aromatic profile and imparting notes of leather, smoke, spice and meat or game, which is considered acceptable by consumers (Chatonnet et al., 1992, 1993; Loureiro and Malfeito-Ferreira, 2003). However, in many wine-producing regions around the world this character is regarded more as a wine fault or defect in higher concentrations. Up to 90% of recorded alterations in premium red wines are associated with these "Brett" characters. Regardless of these sensory considerations, the metabolic benefit of the degradation of hydroxycinnamic acid for *B. bruxellensis* is unclear. It has been suggested that it could be connected to the redox balance since the second step of this enzymatic reaction is a reduction (Fugelsang, 1997; Duckitt, 2012), but this is yet to be demonstrated. Moreover, in a report by Dweck et al. (2015), the production of ethylphenols allow for the detection of hydroxycinnamic acids by the vinegar fly, *Drosophila melanogaster*. Therefore, the production of these compounds by *B. bruxellensis* may enhance its own distribution by these flies as *B. bruxellensis* has been isolated from the breeding ground of *Drosophila* spp. (Loureiro and Malfeito-Ferreira, 2006). The aroma profile resulting from a *B. bruxellensis* contamination in wine is generally regarded as a fault, although, in old world wine regions, this character in small amounts may add to an aged aroma profile or be considered a part of the terroir of that area (Verachtert, 1992; Wedral et al.,

2010).

Wine is furthermore a nutrient poor growth medium with many limiting factors for both yeasts and bacteria. These conditions are the result of a combination of factors: the initial chemical composition of grape juice, alcoholic and malolactic fermentations and interventions from the winemakers. Negligible amounts of sugar and assimilable nitrogen compounds are available after alcoholic and malolactic fermentations as most would have been utilised by *Saccharomyces cerevisiae* and *Oenococcus oeni*. In addition, cellar temperatures of wineries tend to be low (Guzzo and Desroche, 2009), ranging between 9 and 20 °C during wine storage as reviewed by Barbaresi et al. (2015).

Many of the other products that *B. bruxellensis* has been isolated from tend to exhibit similar environmental conditions to those of wine. Certain products such as tequila, cider and beer will have varying ethanol concentrations. The fermentation process conducted in these industries also tend to be similar to that occurring during vinification however, the substrates utilised initially differ from industry to industry. With regard to beer, malt, hops, barley and other grains are utilised. This gives rise to cellulose and hemicelluloses which further provide the sugars necessary for fermentation to occur (Wunderlich and Back, 2009).

While *Saccharomyces* is the dominant genus in both wine and beer fermentations, *Brettanomyces* species play an important role in the production of certain specific beer products. An example of these beers are Belgian beer styles such as Lambic (Gueuze), fruit lambic (Kriek) and faro as well as other beers such as Flanders red ales and Orval. Typically like wine cellars, *B. bruxellensis* can be found in the barrels that are utilised for beer maturation. During maturation, *B. bruxellensis* facilitates a secondary slow fermentation and generally contributes positively, unlike in wine, to the flavour profile of the final product. Inoculation with this yeast results in the development of these secondary aromas representative of Lambic beer style, coupled with this change in aroma there is a drop in pH of the beer. Sometimes, this process is conducted with the lactic acid bacterium *Pediococcus*. These two microorganisms metabolise dextrins, not utilised by *Saccharomyces*, develop the acidity and are involved in the bio flavouring of these beers (Shantha Kumara et al., 1993; De Cort et al., 1994).

Of the *Brettanomyces* species, *B. bruxellensis* tends to dominate these secondary fermentations once *Saccharomyces* has completed the primary alcoholic fermentation. There are many complex alterations to the flavour profile that occur and the effect on the resulting beer is 2 fold. An example of such an alteration is a cell-bound esterase that changes the ester profile of the beer (Spaepen and Verachtert, 1982). In beer, *Brettanomyces* yeasts and *Pediococcus* bacteria are the main microorganisms responsible for volatile phenol production (Spaepen et al., 1981; Thurston and Tubb, 1981; Martens et al., 1997). However, other beer bacteria can also produce vinyl phenols, from ferulic acid and *p*-coumaric acid (Lindsay and Priest, 1975). Furthermore, beers inoculated with *B. bruxellensis* tend to give rise to a sour and earthy aroma profile, as there are higher concentrations of ethyl acetate and lactate in certain beers (Van Oevelen et al., 1976).

In bioethanol production, the substrates utilised are sugarcane and molasses which are hydrolysed into simple sugars to allow for fermentation (Sheoran et al., 1998). Recently however, the general negative stigma attached to *B. bruxellensis* has changed in the bioethanol industry. As mentioned above, it has been isolated in bioethanol plants, where it can potentially outperform even the strongest fermenter, *S. cerevisiae*, by being more energy efficient regarding its conversion of available resources to ethanol.

B. bruxellensis has been isolated previously from tequila and cider fermentations (Lachance, 1995; Morrissey et al., 2004). It is unclear in literature whether *B. bruxellensis* is beneficial or detrimental to cider production. Furthermore, *B. bruxellensis* is more common in the ageing of cider once the alcoholic fermentation is complete, similar to its trend in wine. Some cideries support populations of other *Brettanomyces* spp. One such yeast is *B. anomalus* which has also been found to cause volatile phenol production and subsequent spoilage in cider products (Morrissey et al., 2004). Only strict microbial control of the cider making process will prevent this yeast from being present in the finished product. In tequila and cider production, agave and apple are fermented, respectively. Upon completion of the initial fermentation, tequila is distilled and in some instances cider can also be distilled. Distilled apple cider is known as Calvados, which is an apple brandy that is named after a French département, in the Normandy region (Rodríguez Madrera et al., 2006). Cider can undergo a secondary fermentation depending on the wishes of the producer. The second fermentation occurs in the bottle and utilises the sugars remaining from the first fermentation or supplemented from which additional alcohol is produced (Buglass, 2011). The dominant sugars in these raw materials are generally glucose and fructose and in certain instances sucrose (Wang et al., 2004; Patzold and Bruckner, 2005). The specific products and environmental characteristics will be discussed in detail later in the chapter.

Physical and chemical conditions, notably pH, and limited availability of sugar and assimilable nitrogen sources are limiting factors for viability and growth of yeast cells. A lag phase after alcoholic fermentation where no growth of *B. bruxellensis* occurs is indeed typically noted. This could potentially be due to stress factors caused by the alcoholic environment, the presence of ethanol in high concentrations, sulphites and a low pH, could result in the lag phase after the initial alcoholic fermentations (Britz and Tracey, 1990). During this lag phase, it is believed that *B. bruxellensis* slowly adapts its metabolism to the environmental conditions. Indeed, *B. bruxellensis* possesses numerous adaptations that allow it to survive in these harsh mediums. These mechanisms will be described in the next sections. After this lag phase, it nevertheless manages to grow where no other yeast can. This evolutionary trait along with other traits allow *B. bruxellensis* to proliferate in various environments across various industries.

A summary of these products and niches is outlined in Table 1 but for a more comprehensive list of all the products and niches that *Brettanomyces* as a genus has been isolated from, see a review on *Brettanomyces* by Loureiro and Malfeito-Ferreira (2006). In the next paragraph, the chemical composition of the different matrices that *B. bruxellensis* has been isolated from will be detailed in an attempt to better comprehend the nutritional requirements of this yeast.

4. Chemical characterisation of *B. bruxellensis*' habitats and the role of *B. bruxellensis* therein

The major ecological niches and products that host *B. bruxellensis* will be discussed in detail below. A specific focus will be placed on the nutrient and chemical composition of these different media, a summary of which is outlined in Table 2, and the accommodation of these media for the growth of *B. bruxellensis*.

4.1. Wine

Regarding residual sugar remaining after alcoholic fermentation, the concentrations are very low in dry wines where *B. bruxellensis* is commonly isolated (Chatonnet et al., 1995; Fugelsang and Zoecklein, 2003). Glucose tends to be preferred by *S. cerevisiae* thus fructose may be more common in wine, with trace amounts of glucose being recovered. Some sucrose residues may be present, although most of it would potentially have been utilised by the

Table 1
A summary of the products or industrial niches that *B. bruxellensis* has been isolated from, and a description of its positive or negative impacts in these media.

Product/ Environment	Frequency of prevalence	Role		Reference
		Positive	Negative	
Wine				
Red	Often	Complexity	Phenolic taint	(Fugelsang and Zoecklein, 2003) (Chatonnet et al., 1995)
White			Medicinal characters	(Dias et al., 2003)
Sparkling			Turbidity	(Malfeito-Ferreira et al., 1997)
Sherry			Spoilage organism	(Ibeas et al., 1996; Esteve-Zarzoso et al., 2001)
Beer	Often	High gravity beer such as Lambic beer	Spoilage in low gravity beers	(Gilliland, 1961; Shantha Kumara and Verachtert, 1991)
Bio-ethanol	Often	Strong fermenter	Contaminant	(Loureiro and Malfeito-Ferreira, 2006; Souza-Liberal et al., 2007; Passoth et al., 2007; Beckner et al., 2011)
Cider (French, Irish)	Occasionally	Isolated in the fermentation		(Morrissey et al., 2004)
Tequila	Rarely	Isolated in the fermentation		(Lachance, 1995)
Kombucha	Rarely	Natural occurrence		(Teoh et al., 2004)
Fermented milk	Rarely	Natural occurrence		(Gadaga et al., 2000)
Yoghurt	Rarely		Spoilage organism	(Kosse et al., 1997)
Soft drinks and fruit juices	Rarely		Spoilage organism	(Wareing and Davenport, 2008)
Pickles	Rarely		Spoilage organism	(Jimenez et al., 1999)

Table 2
Chemical characterisation of the various ecological habitats exploited by *B. bruxellensis* with regards to pH as well as ethanol, sugars, nitrogenous compounds and vitamins (average values).

Product/ Environment	pH	Ethanol	Sugars	Nitrogen sources	Vitamins
Wine Red (Ribéreau-Gayon et al., 2000) White	3.3–3.6 3.0–3.4	12–16%v/v 11–15% v/v	In grape must, glucose and fructose are the major sugars, which make up a total concentration of 17–22% w/v. In a dry wine, sugars are less than 0.2% w/v mostly made up of pentoses.	Grape must contains a relatively high concentration of nitrogen compounds (0.01–0.1% soluble N). These constituents include the ammonium cation (3–10% of total nitrogen), amino acids (25–30%), polypeptides (25–40%) and proteins (5–10%). In wine, these sources are mostly depleted leaving only trace amounts.	Grape must contains ample vitamins but fermentation depletes most of them. Vitamins left in wine (White: 0.000025–0.000095% w/v, red: 0.00004–0.000064% w/v) include riboflavin, nicotinamide, pantothenic acid, pyridoxine and biotin
Lambic Beer (Preedy, 2009; Buglass, 2011)	3.4–3.9	4–7% v/v	At the end of fermentation, < 0.101% w/v sugars are present and consist of mainly unfermented α-glucans and trace amounts of sucrose, maltose and maltotriose.	Nitrogen component in beer ranges between 0.03 and 0.1% w/v and comprises amino acids, peptides, amines and heterocyclic compounds.	Vitamins occur in trace amounts in beer at 0.0005–0.001% w/v and are comprised of aminobenzoic acid, biotin, folic acid, nicotinic acid, riboflavin pantothenic acid and thiamin.
Cider (Buglass, 2011)	3.1–3.5	2–8.5% v/v	In initial cider must, mainly sucrose, fructose and glucose are present at an initial total concentration of 11–17%. The final cider contains <0.09% w/v in dry cider and between 0.101–0.105% w/v in semi-sweet ciders. The remaining sugar is either non fermented sugar or sugar syrup (sucrose) added before bottling.	Total nitrogen component in cider is between 0.0018 and 0.0063% w/v and is comprised of trace amounts of amino acids, peptides and amines.	Vitamins in cider include thiamin, nicotinic acid, pantothenate and riboflavin.
Bio-ethanol (Walker, 2010)	3.8–5.0	Ethanol concentrations can reach 10–20% v/v after fermentation, and up to 95–96% v/v after distillation.	In bio-ethanol production, sucrose, glucose and fructose are obtained by means of hydrolysis using starch-based cereal crops, juice or molasses from sugar crops. These concentrations range between 7 and 21% w/v, based on the source used.	A total nitrogen content of 0.008–0.03% w/v is generally acquired from the substrates, but sources of nitrogen including urea, yeast extract, peptone and ammonium sulphate can be added to bioethanol fermentations.	Certain vitamins are available during the production of bio-ethanol from the substrates used including; biotin, choline, pantothenic acid riboflavin, and thiamin.
Tequila	3.9–5.2	Ethanol levels between 4 and 9% v/v are found at the end of fermentation, and up to 60% v/v after distillation.	Initial sugar concentrations range from between 4 and 10% w/v composed of fructose and glucose. Lower levels of sugars are found after fermentation, with close to 0% w/v in the distilled product.	No literature is available regarding the nitrogen and vitamin components in tequila production.	
Soft drinks and fruit juices (Ashurst, 1998; Rodriguez-Saona et al., 2001)	2.3–4.0	0% v/v	Sugar concentrations between 4 and 17% w/v is found in soft drinks and fruit juices	Very little nitrogen sources are added to soft drinks. They usually occur in trace amounts e.g. pectin and amino acids. Fruit juices tend to contain higher concentrations of nitrogenous compounds between 0.05 and 0.5% w/v of pectin and proteins.	Soft drinks are typically devoid of vitamins. Fruit juices however, tend to contain 0.005% w/v, mainly comprised of ascorbic acid.

fermenting yeasts by initially being enzymatically hydrolyzed to glucose and fructose and being assimilated. The wine medium in addition contains trace amounts of pentoses, namely arabinose, rhamnose, ribose and xylose, present in the grape juice and may remain in the wine (Kunkee and Eschnauer, 2003). Indeed, the pentoses are not utilised by *S. cerevisiae* during the fermentation, but possibly may become carbon sources for the lactic acid bacteria, and other spoilage microorganisms, e.g. *B. bruxellensis* which carry out secondary fermentations. The wine medium also contains between 0.04 and 0.13 mg/l of galactose and mannose which are potential carbon sources for many microorganisms. The ethanol may reach levels of between 12 and 14% v/v in dry wines depending on the sugar level at harvest. There are also trace amounts of methanol. In addition, there are fusel alcohols between 5 and 773 mg/l and the organic compound glycerol between 3 and 14 g/l (Kunkee and Eschnauer, 2003).

The two major organic acids associated with wine are L-tartaric acid and L-malic acid at nearly equal concentrations in the grape berry. In red wine, malic acid is biologically unstable and is decarboxylated to lactic acid by lactic acid bacteria, but trace amounts may remain even after the completion of malolactic fermentation. Malic acid degradation only occurs in certain white wines inoculated with lactic acid bacteria. Although detrimental to wine quality at high concentration, acetic acid is also present as one of the major by-products of both alcoholic and malolactic fermentations. Typically, tartaric acid is most prevalent in wine at 3–11 g/l with malic, citric and acetic acids found at lower concentrations between 0 and 6 g/l (Kunkee and Eschnauer, 2003).

The concentration and diversity of nitrogenous compounds in grape juice have the ultimate influence on the fermentations and have a crucial effect on the wine yeast. During alcoholic fermentation, most of the nitrogenous components are utilised by the dominant yeast but a small release of these compounds can occur at the end of alcoholic fermentation due to autolysis. Subsequently, these compounds are removed from the juice, and eventually from the wine once the lees containing the microorganisms have settled and been removed. With regard to potential nitrogenous compounds in the wine matrix, there are proteins, amino acids, amides, ammonia and trace amounts of nitrates and nitrites, usually within the range of 0 and 2 mg/l, higher concentrations can occur when grapes have a higher nitrogen content or when higher concentrations of di-ammonium phosphate (DAP) is added. There is very little literature regarding YAN levels recorded in wine however on average, it seems to equate to a YAN of 0.06 mg N/l if no additions of DAP were added at the winery (Lehtonen, 1996; McWilliam and Ough, 1974). This average yeast assimilable nitrogen (YAN) range is equal to the addition of the free amino nitrogen (FAN) fraction (0.05 mg N/l) and the ammonia/ammonium fraction (0.01 mg N/l). The main amino acid remaining in wine at the end of fermentation is proline but it is not included in the YAN pool, since it is poorly assimilated by *S. cerevisiae*. This could potentially be a good source of nitrogen under aerobic conditions but also under fermentative (anaerobic) conditions, should the yeast be able to assimilate proline in the absence of oxygen. Similar to the nitrogenous components, most of the vitamins contained in the grape juice are taken up by the yeast during fermentation. The vitamins most commonly occurring after fermentation are biotin, choline, inositol and thiamin at concentrations ranging from 1 μg/l to 730 mg/l (Kunkee and Eschnauer, 2003).

Additionally, it has been found that *B. bruxellensis* strains demonstrate glycosidic activity which will be discussed later. Products resulting from the hydrolysis of grape glycosides include monoterpenes, norisoprenoids, C6 alcohols, anthocyanins and benzene derivatives. The release of aglycons may occur enzymatically by the action of glycosidases, observed in *B. bruxellensis*, or via acid hydrolysis (Günata et al., 1985). This could bring about a release of sugar compounds from these complex molecules that could be utilised by *B. bruxellensis*.

4.2. Beer

In beer, the main component added is water which comprises up to 94% of the final product. This is an important aspect as it contains various minerals which imparts an important flavour to the finished product. After water, barley provides the essential constituents utilised by the necessary microorganisms in beer production. These components are: nitrogenous compounds, lipids, carbohydrates and vitamins. Nitrogenous compounds in beer are in the range of 0.3–1.0 g/l and comprise amino acids, peptides, amines and heterocyclic compounds. Some beers, especially unfiltered beers, can contain up to 2000 mg/l of total nitrogen. An average FAN of 80 mg N/l was recorded in beer (Abernathy et al., 2009).

Carbohydrates present in beer are fermented to ethanol by yeast but some carbohydrates are still present at the end of the process and their content is estimated at a range between about 1.0% and 6.0% w/v (Preedy, 2009). The carbohydrates remaining in beer are mainly composed of the unfermentable α-glucans (dextrins not assimilated by *S. cerevisiae*) that can potentially be assimilated by *B. bruxellensis* and other polysaccharide compounds, should it possess α-glucanase activity.

Barley and malt are rich sources of several vitamins that are solubilized into wort during the brewing process. These vitamins help to ensure a regular yeast response during the fermentation process. In particular, the B vitamins are crucial as growth factors for *Saccharomyces*, especially biotin, inositol and panthotenic acid.

4.3. Bio-ethanol production

Recently, an interesting new environment has been outlined where *B. bruxellensis* has increasingly been isolated. It is the industrial process used to produce bioethanol on a bulk scale. In this process, various plant wastes (e.g. sugar cane, sugar beet and molasses) contain complex mixtures of carbohydrate polymers from the cell walls known as lignin, cellulose and hemi cellulose. In order to produce sugars from the biomass, the cells are pre-treated with acids or enzymes in order to reduce the feedstock and degrade the plant structure. The cellulose and the hemicellulose portions are hydrolysed by enzymes or diluted acids into sucrose sugar that can then be utilised by *B. bruxellensis* (Souza-Liberal et al., 2007; Beckner et al., 2011). *B. bruxellensis* has been discovered as well as utilised in alcoholic fermentations that are intended for distillation (Uscanga et al., 2000). This process is normally solely facilitated by *S. cerevisiae* (Souza-Liberal et al., 2007; Passoth et al., 2007; Beckner et al., 2011). Due to the yeast's ability to tolerate low pH conditions, high ethanol, a high stress tolerance and a nutrient-effective metabolism, *B. bruxellensis* is very well adapted to the harsh conditions that occur in the bioethanol fermentation tanks (Blomqvist, 2011). Previously, the yeast species *B. bruxellensis* was mostly regarded as undesirable spoilage organism in these bio-ethanol fermentations. However, a *B. bruxellensis* strain was recently discovered as being the sole producing microorganism in a continuous industrial alcohol plant based on the use of starch, thereby making it an industrially relevant yeast.

This indeed suggests that *B. bruxellensis* can in fact be a favourable contributor to bioethanol fermentations. *B. bruxellensis* can also outcompete *S. cerevisiae* in a starch based media, which supports the idea that *B. bruxellensis* could be a favourable contributor to bio-ethanol fermentations (Passoth et al., 2007). Contrary to this, a spontaneous increase of *B. bruxellensis* cells was reported in an industrial bio-ethanol process in Brazil, which,

similar to the Swedish industrial ethanol plant, runs continuously with cell recycling (Souza-Liberal et al., 2007). In this process, the ethanol yield and the productivity of the system were reduced when the number of *B. bruxellensis* cells increased (Blomqvist et al., 2010). These discoveries suggests that during the high ethanol fermentations occurring in bioethanol plants utilising the same resources as *S. cerevisiae*, *B. bruxellensis* outcompeted the inoculated *S. cerevisiae* strain, without affecting the ethanol yield. This is remarkable as it has previously been reported that *S. cerevisiae* is the species best adapted to the conditions of ethanol fermentation.

B. bruxellensis is also well adapted to cope with a high number of lactic acid bacteria and develops better in complex culture media such as plant juices and molasses, this could be due to other vitamins and cofactors which could positively influence the yeasts growth (Uscanga et al., 2007; Passoth et al., 2007). Thus there is a real possibility for the potential use of this yeast as a starter culture in bioethanol fermentation sites. Nevertheless, certain characteristics of *B. bruxellensis* could jeopardise its use in this field. A few reports have outlined the slow growth and the effect of temperature on growth and ethanol production in *B. bruxellensis* to be a limiting factor in bioethanol production (Abbott et al., 2005; Brandam et al., 2008). Another shortfall of *B. bruxellensis* is its ability to utilise ethanol as a carbon source and will do so if other sources are not available (Dias et al., 2003). *B. bruxellensis* originally a contaminant in bioethanol production came to dominate the initial fermentation, in which case the productivity of the fermenter did not decrease. This may suggest that *Brettanomyces* spp. may possibly be ethanol producing organisms of industrial interest as opposed to only contaminants in bioethanol production (Beckner et al., 2011).

4.4. Other

4.4.1. Fruit juices and soft drinks

Characteristically, soft drinks, fruit juices and other products in the beverage industry tend to contain high concentrations of acids, with a low pH. These products contain high concentrations of sugars ranging between 1 and 8% w/v of sucrose, glucose and fructose (See Table 2). Trace amounts of vitamins and minerals can be found, mainly comprised of ascorbic acid. Finally the nitrogen segment is comprised of only proteins and pectins which can be utilised by microorganisms. A potential consequence of these matrices is that they are suitable environments for the growth of unwanted yeasts. *Brettanomyces* spp. are among the species of yeasts commonly isolated in these products and can be associated with the spoilage of soft drinks and fruit juice products (Jimenez et al., 1999).

4.4.2. Cider

Regarding the nutrients present in cider apples and the resulting cider, almost all the sugars of cider apples are either monosaccharides or disaccharides, all of which can be assimilated by yeast in the alcoholic fermentation (See Table 2). Therefore, very little residual sugar is left in fully fermented ciders (dry ciders) however, this is not true in off-dry or sweeter ciders which contain higher concentrations of residual sugars. The major sugars in apple must are fructose, sucrose and glucose. Apple musts tend to have original densities in the region of 1.050–1.060; this will give rise to ciders recording 6–7.5% v/v ethanol if fermentation is allowed to proceed to completion. Apple juices contain low levels of amino acids and proteins, the dominant amino acids being asparagine, aspartic acid and glutamic acid. Thus, compared with beer wort, apple must is rather poor in yeast nutrients (Buglass, 2011).

5. The nutritional requirements of *B. bruxellensis*

Like *S. cerevisiae*, *B. bruxellensis* is ethanol tolerant, facultatively anaerobic, Crabtree positive and petite positive, allowing the yeast to produce offspring without mitochondrial DNA (Hellborg and Piskur, 2009). It can therefore ferment preferentially in the presence of high glucose in oxygenated conditions (Kurtzman and Fell, 1998; Piškur et al., 2006). However, unlike *S. cerevisiae*, *B. bruxellensis* can appear in situations in which nutrients are scarce (as reviewed in the previous paragraphs), thus they can be termed as nutritionally undemanding yeasts (Suárez et al., 2007). In addition, the Custers effect (also called the negative Pasteur Effect) displayed in *Brettanomyces* yeasts is specific to this genus. It stimulates ethanol and acetic acid production under aerobic conditions (Scheffers and Wiken, 1969). It has also been found that *B. bruxellensis* is resistant to large changes in pH and temperature and it may also have a more energy efficient metabolism under oxygen limited conditions as opposed to *S. cerevisiae* (Blomqvist et al., 2010). Much is still unclear regarding the growth of *B. bruxellensis*, as it efficiently produces high amounts of biomass from limited amounts of nutrients (Uscanga et al., 2000). Presently, it is unclear what nitrogen and carbon sources *B. bruxellensis* utilises in a nutrient deficient environment.

5.1. *Brettanomyces bruxellensis'* genome reveals a potential adaptation to growing under nutrient limited conditions

In some studies, a correlation has been suggested between genotype groups of *B. bruxellensis* and their initial source of isolation, most probably a result of the yeast's ability to adapt to a specific ecological niche (Conterno et al., 2006; Crauwels et al., 2014, 2015). Although further research using more isolates is required to draw strong conclusions, the genome of *B. bruxellensis* is indeed diverse and consists of 4–9 chromosomes, which vary from 1 to 6 Mb. Through the full sequencing of the genome of a few strains, it has recently been found that there is significant strain variation, which is at least in part due to the fact that certain strains have triploid genomes while others have diploid genomes. Additionally, certain genomes contain approximately 1% polymorphic sites (Hellborg and Piskur, 2009; Curtin et al., 2012; Borneman et al., 2014; Crauwels et al., 2014). The total genome size can range from 20 to 30 Mb depending on the strain in question (Woolfit et al., 2007).

The genomes of five wine strains of *B. bruxellensis* have been sequenced to date; these strains comprise three Australian strains AWRI1499, AWRI1608, AWRI1613, CBS2499 from France and finally LAMAP2480 from Chile (Curtin et al., 2012; Piškur et al., 2012; Borneman et al., 2014; Valdes et al., 2014). A Belgium beer strain, ST05.12/22, has also recently been sequenced (Crauwels et al., 2014). Additionally, 3 more beer strains from Belgian beer were recently sequenced (ST05.12/26 (MUCL 49865), ST05.12/48 and ST05.12/53) as well as a dry ginger ale strain (CBS 6055) (Crauwels et al., 2015). If the genomes of the five wine strains are compared, it can be seen that the triploid genome discovered in AWRI1499 is also found in the AWRI1608 strain. However, the other wine strains are diploid in nature. A similar trend is found in the sequenced beer strains where of the four, three strains are diploid and one is triploid. It is thought that this variation in ploidy occurrence originates from two hybridisation events. In addition, ploidy variation, gene conversion and rearrangement as well as genomic insertions, deletions and duplications could be some of the key molecular mechanisms that have allowed *B. bruxellensis* to diverge from the common distant ancestor shared with *S. cerevisiae* (Piškur et al., 2012; Borneman et al., 2014). These insertions can explain how orthologous genes have been identified in *B. bruxellensis* originating from other ascomycetes; the same genes however, are not

present in *S. cerevisiae* (Woolfit et al., 2007).

Woolfit et al. (2007) revealed that the genome of *B. bruxellensis* is enriched in genes involved in nitrogen and lipid transport and metabolism and the authors suggested a potential connection with its ability to grow in a low-nutrient medium or the high ethanol niches. Indeed, certain strains identified by Crauwels et al. (2015) were able to utilise sodium nitrate. In contrast, certain Australian wine strains and three Belgian beer strains have lost the ability to utilise nitrates (Borneman et al., 2014; Crauwels et al., 2015), an ability characterising the *Brettanomyces* genus, among other yeast. Strains of *B. bruxellensis* possibly lost this ability as they grew accustomed to and eventually adopted a low nitrate environment. The ability of *B. bruxellensis* to grow in the aforementioned niches was further confirmed by Curtin et al. (2012) who noticed an extensive number of genes encoding amino acid permeases in *B. bruxellensis*' genome. Recently, using a comparative genomic approach, Crauwels et al. (2014) noticed that wine strains of *B. bruxellensis* possessed 20 genes involved in carbon and nitrogen metabolism that were absent from the beer strain. Furthermore, a high number of sugar transport-encoding genes identified in the strain AWRI1499 may highlight *B. bruxellensis*' ability to outcompete *S. cerevisiae* under limiting conditions. However, this would only be possible if the affinity of these transporters with their select substrates differed to those of *S. cerevisiae*. Glucose and hexose transporter genes as well as a high affinity glucose transporter were identified in the aforementioned strain (Tiukova et al., 2013). This would further indicate an evolution-based adaptation of specific strains to live in a low-nutrient medium. Furthermore, in a study by de Souza Liberal et al. (2012), two paralogs of phenylpyruvate decarboxylase-encoding genes (DbARO10-1 and DbARO10-2) were identified. These genes differ not only in their makeup, but also in their response to the type of carbon and nitrogen available in the medium. In another study, vinyl phenol reductase activity was assayed in *B. bruxellensis* strain AWRI 1499 which has a Cu/Zn superoxide dismutase in its genome, and was found to possess both vinyl phenol reductase and superoxide dismutase activities. Similarly, superoxide dismutases can be found in related microorganisms, such as *S. cerevisiae*, that do not display vinyl phenol reductase activity (Granato et al., 2015). These genes could play an important evolutionary role in *B. bruxellensis* regarding its enzymatic activities and broader adaptation to its different habitats.

5.2. Carbon and nitrogen utilisation

B. bruxellensis isolates have been reported as being able to grow using a variety of carbon and nitrogen sources, however certain sources are preferred and thus growth is easily facilitated (Smith, 1998a; Uscanga et al., 2000; Conterno et al., 2006; Rozpędowska et al., 2011; Crauwels et al., 2015). Tables 2 and 3 summarise the current knowledge on the assimilation of carbon and nitrogen sources by *B. bruxellensis*.

5.3. Carbon sources

As seen in Table 3, *B. bruxellensis*, dependent on strain variation regarding certain substrates, is able to utilise many carbon sources from various ecological niches including glucose, fructose, maltose, mannose, ethanol, acetic acid and glycerol (Dias et al., 2003; Conterno et al., 2006; Galafassi et al., 2011). This outlines the concept that *B. bruxellensis* is able to utilise or consume many substrates in order to grow. However, some publications oppose some of these former sources (Conterno et al., 2006; Vigentini et al., 2008; Galafassi et al., 2011). Firstly, with regard to ethanol as a sole carbon source, Vigentini et al. (2008) found that under semi-anaerobic conditions *B. bruxellensis* could not utilise ethanol. Conversely, both Dias et al. (2003) and Conterno et al. (2006) found that *B. bruxellensis* could utilise ethanol as a single carbon source. However, these authors did not specify whether the conditions used in their study were aerobic and/or anaerobic. Secondly, Conterno et al. (2006) found that there is variance between the isolates studied as certain isolates could utilise glycerol as a single carbon source while others could not. Vigentini et al. (2008) found that under semi anaerobic conditions *B. bruxellensis* could not utilise glycerol at all.

In *S. cerevisiae*, glycerol, ethanol, acetic acid, succinic acid and lactic acid can only be assimilated in the presence of oxygen. Indeed, in order for glycerol and succinate to be assimilated, $FADH_2$ is required and this cofactor can only be regenerated in the electron transport chain, which is dependent on oxygen to function. In order for *S. cerevisiae* to utilise lactate, an electron is required from the electron transport chain, as reported by Rosenfeld and Beauvoit (2003).

The metabolism of acetic acid, which can only occur under aerobic conditions, is required in order to utilise the acetyl–CoA synthetase which is the only source of cytosolic acetyl–CoA, an imperative building block of fatty acid biosynthesis (Van den Berg and Steensma, 1995; Flikweert et al., 1996). Finally, regarding ethanol, it can be converted to acetaldehyde by alcohol dehydrogenase using NAD^+ in *S. cerevisiae*. The utilisation of ethanol as carbon source therefore creates a need for NAD^+ regeneration which can be oxidized by the mitochondrial respiratory chain with oxygen as the terminal electron acceptor (de Vries and Marres, 1987; Bakker et al., 2000). Similarly to acetic acid, glycerol and succinic acid, it is therefore dependent on the presence of oxygen. Crabtree-positive yeasts tend to utilise many compounds which causes a number of products to be formed. One such product, ethanol, can in certain instances become a substrate and can be utilised if oxygen is present (Dickinson et al., 2003). This change in metabolism is known as the 'diauxic shift' where a carbon source preference changes upon depletion of glucose under aerobic conditions (Pronk et al., 1996).

Glycerol is mainly produced to counterbalance the surplus of NADH formed during biomass production, in order to correct the redox imbalance in the cell. The key enzyme in the pathway for glycerol formation is a NADH-dependent glycerol-3-phosphate dehydrogenase which converts dihydroxyacetone phosphate to glycerol-3-phosphate with the joint oxidation of NADH to NAD^+. The shift in redox balance (NADH:NAD^+ ratio) caused by increased formation of glycerol is typically corrected by using acetic acid as a redox sink to convert NAD^+ back to NADH (Michnick et al., 1997; Remize et al., 1999). Several yeasts such as *S. cerevisiae* can restore their redox balance in anaerobic conditions by producing glycerol but *B. bruxellensis* is unable to do this. Therefore, due to the Custers effect in *B. bruxellensis*, an imbalance in the redox potential of the cell will occur and this could possibly be due to the lack of glycerol 3-phosphate phosphatase activity. The genes involved in this process are indeed poorly expressed in *B. bruxellensis* (Wijsman et al., 1984; Tiukova et al., 2013). However, *B. bruxellensis* could utilise other biochemical pathways in order to correct the redox imbalance that occurs during sugar consumption. One such method could involve the formation of volatile phenols (Fig. 1), from hydroxycinammic acid precursors, which could regenerate $NAD(P)^+$ in the cell (Duckitt, 2012; Curtin et al., 2013), but this hypothesis has yet to be validated. Wine yeasts produce acetic acid by the oxidation of acetaldehyde to acetate by $NAD(P)^+$-dependent (acet)aldehyde dehydrogenases (Remize et al., 2000). Several studies have linked the production of acetic acid to increased glycerol production in *S. cerevisiae* (Valadi et al., 1998; Remize et al., 1999; Eglinton et al., 2002). As a Crabtree positive yeast, *B. bruxellensis* is expected to behave in a similar way to *S. cerevisiae*.

Table 3
Carbon sources utilised by *B. bruxellensis*.

Carbon sources	Uptake supporting growth	No growth change when present
Ethanol	*Brettanomyces bruxellensis* can metabolise ethanol (Dias et al., 2003; Conterno et al., 2006) Growth was species dependent (Smith, 1998a). Fairly good growth (Custers, 1940)	Ethanol was not utilised as a sole carbon source (Vigentini et al., 2008).
Glycerol	Growth was observed with some isolates (Conterno et al., 2006; Crauwels et al., 2015). Growth is possible (Smith, 1998a).	Most isolates did not utilise glycerol (Conterno et al., 2006; Vigentini et al., 2008).
Acetic Acid	Acetic acid as a single source of carbon provided a conversion rate of *p*-coumaric acid to 4-ethylphenol lower than 10% thus acetic acid was used however the conversion was not as strong as during the use of glucose as a sole carbon source (Dias et al., 2003)	
Glucose	Glucose supported the growth of *B. bruxellensis* (Van Zyl, 1962; Smith, 1998a; Galafassi et al., 2011; Crauwels et al., 2015). When glucose was the only energy and carbon source, the conversion rate of 4-ethylphenol was close to 90%. (Dias et al., 2003)	
Fructose	Most of the isolates could grow on the hexose monosaccharide fructose (Conterno et al., 2006; Crauwels et al., 2015)	
Galactose	Most of the isolates studied could grow on the hexose monosaccharide galactose (Conterno et al., 2006; Crauwels et al., 2015)	
Maltose	The growth rate and ethanol production were lower when utilising maltose (Blomqvist et al., 2010)	
Mannose	Mannose supported the growth of *B. bruxellensis* (Galafassi et al., 2011; Crauwels et al., 2015)	
Sugarcane molasses	A low fermentation rate was observed. However, a high growth-coupled production of acetic acid and ethanol was observed (Uscanga et al., 2007)	
Refined cane sugar		Refined cane sugar was not utilised by *B. bruxellensis* and a low growth coupled with the production of ethanol and acetic acid was observed (Uscanga et al., 2007)

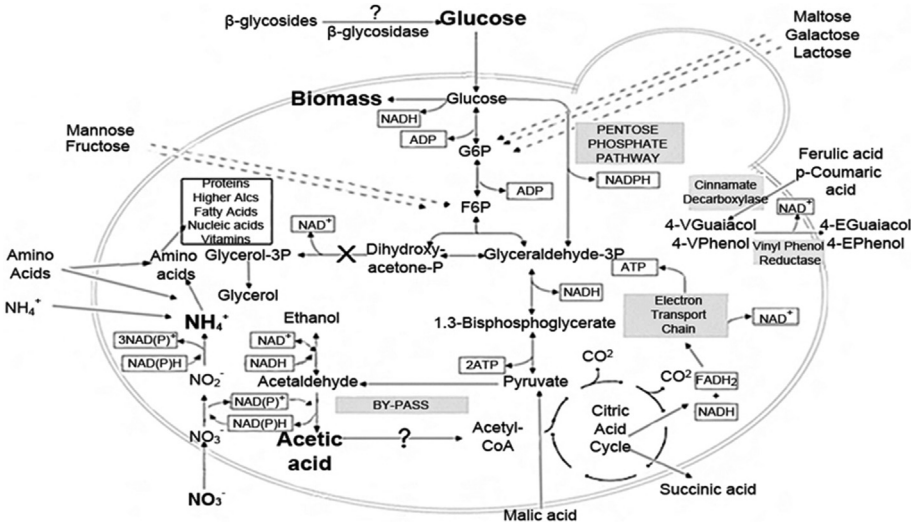

Fig. 1. A representation of the carbon and nitrogen utilisation pathways in *B. bruxellensis* under aerobic and anaerobic conditions. Adapted from (Galafassi et al., 2013). The question marks depict pathways that could potentially be utilised by *B. bruxellensis*, but are strain dependent. The X on the glycerol formation pathway indicates that only certain strains have a high enough gene expression to form glycerol. Nitrate assimilation abolishes the Custers effect, highlighted with a larger font, by allowing the cell to replenish the $NAD(P)^+$ pool through reduction of nitrate to ammonium. The Custers effect only occurs under anaerobic conditions when NAD^+ is unavailable. Under aerobic conditions the coenzyme is reoxidised through the respiratory pathway (Carrascosa et al., 1981).

Furthermore, it has not been recorded in literature under what conditions these corrective pathways are activated or which are preferred in *B. bruxellensis*. Results regarding the aforementioned carbon sources in literature are unclear, sometimes inconclusive and in most cases the authors did not specify the conditions in which the experiments were carried out with regard to oxygen availability or lack thereof. Thus, it is critical that in future studies detailed experimental conditions be laid out in order for the research performed to be clear and coherent.

B. bruxellensis also has the potential to indirectly utilise various carbon sources by expressing β-glycosidase activity (Fig. 1) allowing it to utilize dextrins and cellobiose as carbon sources as previously stated in other environmental niches. These adaptations can broaden the substrate range utilised for the bioethanol production industry. Both intracellular and extracellular α-glucosidase activities were found in *Brettanomyces lambicus* (now reclassified as *B. bruxellensis*) (Shantha Kumara et al., 1993). The glycosides can be either monoglycosides or disaccharide glycosides in which the glucose bound to the aglycon moiety is substituted by either α-L-arabinofuranoside, β-D-rhamnopyranoside or β-D-xylopyranoside

(Bloem et al., 2008). It has been found that *B. bruxellensis* has the ability to release glycosidically bound flavour compounds, for example terpenes, from natural grape glycosides; this could potentially increase the natural wine aroma that occurs (Mansfield et al., 2002), but the utilisation of these complex compounds may outline the competitive advantage that *B. bruxellensis* has over the other yeasts involved since it could use the glycosides released as carbon sources.

Other enzymatic activities, such as glucanase activity, are probably involved in the slowly fermenting spontaneous Belgian lambic beers, due to polysaccharide and disaccharide assimilation which is another metabolic trait of *Brettanomyces/Dekkera* yeasts that supports their ability to colonise specific products, where these substrates are the only carbon source (Ciani and Comitini, 2014). Positive results of β-glucosidase activity have been reported by authors studying beer with regard to dextrins (Shantha Kumara et al., 1993). This is strange as dextrins are alpha bound glucans so beta-glucosidase activity will not hydrolyse dextrins, this again displays *B. bruxellensis*' potential glucanase activity. In a recent study by Crauwels et al. (2015), certain beer strains of *B. bruxellensis* were found to possess only one β-glucosidase, in contrast the other strains investigated contained two beta-glucosidases. However, in the same study, strains from various ecological niches (e.g. wine, beer and soft drink) were able to metabolise dextrin (Crauwels et al., 2015). Furthermore, different phenotypic profiles for the use of β-glycosides were found within the set of tested isolates. Two different β-glucosidases were found in the isolates. It was therefore proposed by the authors that the strains may induce different flavour profiles (Crauwels et al., 2014, 2015) as this may be connected to the β-glucosidase expressed by the strain in question. The role of β-glucosidases are related to the development of flavour in beer and wine (Shoseyov et al., 1990; Daenen et al., 2004). Additionally, β-glucosidases play an important role in the fermentation of cellobiose by *B. bruxellensis* (Blomqvist et al., 2010). Over the long secondary fermentations, *B. bruxellensis* will consume almost all the sugars and dextrins in a beer, indicating glucanase activity. Most beers with any traces of this yeast will end up very dry as the yeast can survive on the residual sugars for months or even years in the fermenter or bottle, consuming all the trace sugars during this time.

Blondin et al. (1983) also studied β-glucosidase activity on cellobiose and the artificial substrate *p*-nitrophenyl-ß-D-glucopyranoside (pNPG). They described that *B. bruxellensis* displayed hydrolytic activity against cellobiose and a substituted aryl-glucosides, similar to those from *S. cerevisiae* reported by Duerksen and Halvorson (1958). However, the results for the pNPG substrate are not shown, as the article reports on the purification and properties of the β-glucosidase enzyme, thus no clear conclusion can be drawn from this. Furthermore, the β-glucosidase activity was also tested using the substrate pNPG, and all 14 strains tested showed positive results (Mansfield et al., 2002). In an article published by McMahon et al. (1999), seven of 10 strains displayed β-glycosidase activity using *p*-nitrophenyl-ß-D-glucopyranoside as a substrate. There are also reports of β-glucosidase-encoding genes isolated in strain CBS 11270 (Tiukova, 2014). There is no evidence in literature that supports wine trials, or trials on wine substrates, of any kind, but those undertaken by Mansfield et al. (2002) that were subsequently performed on Viognier juice using the strains that tested positive for the activity. However, the strains were not active on Viognier glycosides, although the specific glycosides utilised in the study were not stated. The use of Viognier glycosides from a white cultivar is curious as *B. bruxellensis* is more commonly isolated in red wines, which does not contain much terpenes, but also comprises glycosidically-bound compounds, such as grape-derived glucose-bound anthocyanins and yeast-derived mannose-bound proteins.

These could be a potential source of carbon for the *B. bruxellensis* and thus could explain how *B. bruxellensis* is able to survive in red wine. In beer, dextrins such as maltotetraose and maltopentaose tend to be present in the final product. These polysaccharides are the residual sugars present after the main fermentation of beer. *B. bruxellensis* produces α-glucosidase, enabling the hydrolysis of these complex sugars into less complex units that can be easily assimilated by the yeast (Shantha Kumara et al., 1993; Shantha Kumara and Verachtert, 1991; Steensels et al., 2015). In bioethanol production, cellobiose, a disaccharide present in second-generation bioethanol plants as substrates, from the hydrolysis of lignocellulose and wood also common in the wine industry, can be degraded by β-glucosidase enzymes, produced by *B. bruxellensis* (Blondin et al., 1983; Moon et al., 2001; Steensels et al., 2015). Thus, *B. bruxellensis* displays both α-and β-glucosidase activity that can be utilised to metabolise differing compounds in order to utilise the degraded compounds. At this time, several studies have shown that in general yeasts involved in the fermentation processes have varying degrees of β-glucosidase activity. The highest degree of this activity has been found in non-*Saccharomyces* yeast strains, including *B. bruxellensis*, as opposed to *S. cerevisiae* (Rosi et al., 1994; McMahon et al., 1999; Riccio et al., 1999; Manzanares et al., 2000). This may outline a niche group of substrates that *B. bruxellensis* could be able to utilise in a more efficient manner than *S. cerevisiae*. Furthermore, genes encoding β-glucosidases have been identified in AWRI 1499 and CBS 2499 strains. However, only 7 out of 26 isolates scored positive for the β-glucosidase-encoding gene in a study conducted by Crauwels et al. (2014). Therefore should a *B. bruxellensis* strain have the necessary genes, it could confer this strain a notable advantage an environment containing glycosidically bound compounds, by enzymatically releasing fermentable sugar from these glycosidic compounds.

5.4. Nitrogen

Knowledge of the utilisation of nitrogen by *B. bruxellensis* or preferential utilisation of individual nitrogen sources is limited (de Barros pita et al., 2013; Childs et al., 2015). It is nevertheless known that *B. bruxellensis* utilises many sources of nitrogen and has a low nitrogen requirement in aerobic conditions (Uscanga et al., 2000; Conterno et al., 2006; Morneau et al., 2011). However, under strict anaerobic conditions, the strain CBS 11270 was unable to grow in a medium without amino acids. In other studies, the common factor that allowed for anaerobic growth of *B. bruxellensis* is that the medium used either contained yeast extract or casamino acids (Rozpędowska et al., 2011; Uscanga et al., 2003; Ciani and Ferraro, 1997). As outlined in Table 4, a study by Blomqvist (2011) revealed that the proteinogenic amino acids lysine, histidine, arginine, asparagine, aspartic acid, glutamic acid and alanine all promoted the growth of *B. bruxellensis* under anaerobic conditions. Early studies also mentioned in Table 4 suggest that ammonium, nitrates and certain amino acids can all be utilised by *B. bruxellensis* (Conterno et al., 2006; Blomqvist, 2011; Blomqvist et al., 2012; de Barros pita et al., 2013; Galafassi et al., 2013). However, contrary to the above studies, Gaunt et al. (1988) recorded that ammonium has no effect on the growth of *B. bruxellensis* and Uscanga et al. (2000) stated that an ammonium sulphate concentration above 2 g/L can potentially be inhibiting to *B. bruxellensis*. In certain yeast species, *B. bruxellensis* included, nitrate is converted to ammonium by two successive reductions catalysed by nitrate reductase and nitrite reductase, respectively, as seen in Fig. 1.

B. bruxellensis has a superior advantage in lignocellulose media, common in bioethanol plants, which are rich in nitrate. In a review by Schifferdecker et al. (2014), it is hypothesized that NADPH and

Table 4
Nitrogen sources utilised by *B. bruxellensis*.

Nitrogen sources	Uptake supporting growth	No growth change when present
Ammonium	All isolates grew on ammonium, as a nitrogen source (Conterno et al., 2006; de Barros pita et al., 2013). Positive use of ammonium sulphate as a nitrogen source (Crauwels et al., 2014; Galafassi et al., 2013) Ammonium sulphate assimilation tests positive (Custers, 1940)	If the ammonium sulphate concentration is above 2 g/L, an inhibitory effect on *B. bruxellensis* growth is observed. (Uscanga et al., 2000). No effect on the rate of glycolysis (Gaunt et al., 1988). No growth was observed in medium containing ammonium sulphate under complete anaerobic conditions (Blomqvist et al., 2012).
Amino acids	Amino acids enable *D. bruxellensis* to grow (de Barros Pita et al., 2013). Amino acids aid anaerobic growth (Blomqvist et al., 2012; Rozpędowska et al., 2011).	
Proline	Proline may serve as a sole nitrogen source for *B. bruxellensis* if enough oxygen is available (Conterno et al., 2006; Blomqvist et al., 2012; Crauwels et al., 2015).	Proline supported only a slow and delayed growth (de Barros Pita et al., 2013). Proline did not cause an increase in rate of growth (Van Zyl, 1962; Blomqvist, 2011).
Arginine	All isolates grew on arginine (Conterno et al., 2006; Crauwels et al., 2015).	
Other amino acids	Lysine, histidine, arginine, asparagine, aspartic acid, glutamic acid and alanine all promoted the growth of *B. bruxellensis* under anaerobic conditions (Blomqvist, 2011). In a phenotypic microarray screening *B. bruxellensis* was able to oxidise alanine, asparagine, aspartic acid, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine and valine (Crauwels et al., 2015).	Cysteine and tryptophan did not stimulate growth under anaerobic conditions (Blomqvist, 2011).
Nitrates	Positive growth using nitrates (Conterno et al., 2006; Galafassi et al., 2013; de Barros pita et al., 2013; Crauwels et al., 2014) Potassium nitrate assimilation is possible (Custers, 1940).	No growth was observed in medium containing sodium nitrate under complete anaerobic conditions (Blomqvist et al., 2012; Borneman et al., 2014).
Nitrite	The nitrite reductase activity is present in some strains of *B. bruxellensis* (Crauwels et al., 2014) Some strains utilise nitrite in the auxanographic method (Van der Walt, 1963). Growth is possible by utilisation of Potassium nitrite, but very weak growth (Custers, 1940)	Assimilation of nitrite did not occur in the liquid medium (Van der Walt, 1963).

NADH, which are available under anaerobic conditions, could potentially neutralize the redox imbalance. Both of the aforementioned co-factors could play the role of an electron donor for nitrate reductase, which is active under anaerobic conditions. The expression of the genes encoding these enzymes is induced by nitrate and nitrite and repressed by ammonium as well as by other factors involved in the utilisation of secondary nitrogen sources (Rossi et al., 2005; Siverio, 2002).

Furthermore, *B. bruxellensis* has certain other adaptations benefiting it in this environment, such as its ability to co-consume nitrate and other nitrogen sources. The amounts of nitrates and nitrites are minimal in wine, whereas ammonium is a lot more abundant in must and is utilised readily by yeasts. However, when considering the low concentrations of nitrates in wine, it is necessary to recall that the nitrogen concentration needed for growth in *B. bruxellensis* is low. It has been found that the assimilation of nitrates abolishes the Custers effect in *B. bruxellensis* this may allow for better fermentative metabolism (Galafassi et al., 2013). Contrary to this finding however, Borneman et al. (2014) found that two Australian wine strains were unable to utilise nitrates. Therefore, nitrates may not be important in the growth of *B. bruxellensis* in wine. *S. cerevisiae*, the principal yeast used for wine fermentation, preferentially uses many simple nitrogen sources such as free alpha amino nitrogen compounds, present in the form of primary amino acids and leaves only minor concentrations of other nitrogen compounds (Cooper, 1982; Bisson, 1991; Monteiro and Bisson, 1991; Henschke and Jiranek, 1993; Jiranek et al., 1995). Additionally, *S. cerevisiae* is unable to use nitrates and nitrites and in certain conditions the amino acids proline and hydroxyproline which are not metabolised under winemaking conditions (Duteurtre et al., 1971; Ingledew et al., 1987; Suresh et al., 1999). Thus, certain amino acids are available at the end of alcoholic fermentation, and others even increase by release during autolysis, namely: proline, leucine, tryptophan and gamma aminobutyric acid (Lehtonen, 1996; Valero et al., 2003).

These nitrogen sources that exist after the initial fermentation either by autolysis or due to only partial assimilation by *S. cerevisiae* could potentially be assimilated by *B. bruxellensis*. With regard to the amino acid proline, two studies by Van Zyl (1962) and Blomqvist (2011) present data that indicates that proline does not have an effect on growth of *B. bruxellensis*. Contradictory to the latter study, Conterno et al. (2006) and Crauwels et al. (2015) showed that *B. bruxellensis* can utilise amino acids, mainly proline and arginine. However, the authors did not state under which conditions (i.e. aerobic or anaerobic) this growth was observed in either of the studies. In addition, the diversity of *B. bruxellensis* strains investigated could explain some contradictory results that occur in literature. Indeed, in *S. cerevisiae*, the catabolism of proline requires the presence of oxygen (Ingledew et al., 1987). It could also play a role in *B. bruxellensis*, as de Barros Pita et al. (2013) demonstrated that proline promotes only a slow and delayed growth in *B. bruxellensis* contradicting the studies mentioned above, however, once again, the conditions of the study are not outlined in full. The consumption or production of certain amino acids may be in part related to the maintenance of the redox potential of the cell. Thus, the amino acids released into the wine during/at the end of alcoholic and malolactic fermentations are compounds that could potentially be utilised by *B. bruxellensis*, thereby promoting growth and subsequent spoilage by this yeast.

In summary, the different pathways related to carbon source metabolism as well as certain nitrogen source utilisation, identified in *B. bruxellensis* are illustrated in Fig. 1. Considering the challenges faced by this yeast to rectify its redox balance through the consumption of certain substrates, the status of the redox co-factors is mentioned where applicable. It is also necessary to note that vitamins are typically beneficial for the growth of *B. bruxellensis* (Oelofse et al., 2008).

The carbon and nitrogen components listed above have been identified as those making up the majority of sources available in the different ecological niches that *B. bruxellensis* exploits.

6. Conclusion

B. bruxellensis has been isolated from many products and

environmental niches. These environments can be divided into those that are poor and generally not conducive for yeast growth, for example bottled wine or beer, as nutrients are scarce (Gilliland, 1961; Shantha Kumara and Verachtert, 1991; Ibeas et al., 1996; Esteve-Zarzoso et al., 2001) and those that are rich in nutrients, such as bioethanol plants, fruit juices and cider (Morrissey et al., 2004; Souza-Liberal et al., 2007; Passoth et al., 2007; Wareing and Davenport, 2008; Beckner et al., 2011). Furthermore, these environments are composed of various complex nutrients that cannot be utilised by other yeasts e.g. dextrins, xylose and cellobiose. However, *B. bruxellensis* is well suited to these environments and even thrives in these select niches, which for the most part are uninhabited by other microorganisms, due to the bareness or desolation of these media. Recent genome sequencing has revealed genes allowing for the utilisation of a broad array of substrates by *B. bruxellensis*. However, it is still unclear how *B. bruxellensis* is so metabolically efficient regarding biomass production under these nutritionally deficient media. As in grape must fermentations, *B. bruxellensis* can be easily outcompeted by *S. cerevisiae*. However, *B. bruxellensis* is a survivor and is well adapted to the conditions post fermentation, conditions that other yeasts are not fit to survive in.

It is therefore necessary to study the composition of the various media. Firstly to identify the components in the media that allow for the growth of *B. bruxellensis*. The factors, that allow this yeast to be better adapted to these environments, could either be genetic (internal) or nutritional (external) in nature or both, which for the most part has been reported recently in literature. The genome of certain strains of *B. bruxellensis* outline certain adaptations that could explain these survival characteristics. However, this only partly explains the evolution of this yeast to thrive in these conditions. The mechanisms regarding the growth of *B. bruxellensis*, by efficiently producing high amounts of biomass from limited amounts of nutrients and how it can outcompete all other yeasts after alcoholic fermentation is not fully understood. It could for the most part be due to its ability to survive in nutritionally scarce environments however, it should be the focus of future studies. Future research could involve the investigation of a vast array of *B. bruxellensis* strains, in order to determine how each group of isolates evolved according to the specific environment that it was isolated from. Since vastly different substrates are found in the different ecological niches, the strains are expected to have genetically evolved to adapt to the different environments. Furthermore, this knowledge will give rise to practical solutions to managing *B. bruxellensis* in order to eradicate it where it is seen as negative (e.g. wine) or promote/favour it where it is seen as a positive contributor (e.g. bioethanol and beer).

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Chapter 3

Research results

The nutrient consumption pattern of the spoilage yeast *Brettanomyces bruxellensis* reveals an adaptation for survival in wine

The nutrient consumption pattern of the spoilage yeast *Brettanomyces bruxellensis* reveals an adaptation for survival in wine

Abstract:

The wine matrix contains limited carbon and nitrogen compounds to sustain microbial life. *Brettanomyces bruxellensis* is one of very few yeast species that has adapted to this environment. Proliferating in this medium is an astounding achievement considering the presence of several growth-inhibiting compounds and conditions in wine such as ethanol, sulphur dioxide and limited oxygen concentrations. Literature regarding the nutritional requirements of this yeast is poor, which is surprising given the observation that *B. bruxellensis* can produce biomass, which requires less nutrients than other yeasts. In this study, various carbon and nitrogen sources were screened in a synthetic wine medium, under anaerobic and semi-aerobic growth conditions, in order to determine which compounds *B. bruxellensis* assimilates. Slight differences were observed between strains but overall, *B. bruxellensis* produced biomass from limited nutrients consumed in a specific order regardless of the oxygen conditions. Upon initial consumption of the simple sugars in the synthetic wine medium, *B. bruxellensis* was able to remain viable, by concurrently utilising ethanol and malic acid. From a wide selection of nitrogen sources some were non-utilised/released while others were consumed at varying degrees but never completely, during the growth period. The latter included arginine and proline, the most abundant nitrogenous compounds in wine. A formation of volatile phenols occurred during the consumption of the sugars, probably as a mechanism to help correct the redox imbalance. The study confirms that *B. bruxellensis* is able to survive using limited amount of nutrients, making this yeast a challenge for winemakers. Apprehending the nutritional requirements of *B. bruxellensis* is nevertheless a step forward in our understanding of its biology.

3.1 Introduction

The production of premium quality wine involves the appropriate management of desirable yeasts, for the most part *Saccharomyces cerevisiae* as well as certain non-*Saccharomyces* yeasts. These yeasts may be inoculated in order to reliably produce wine that is appealing to the consumer. However, microorganisms do not always yield positive organoleptic properties in wine. Indeed, certain microbes, referred to as spoilage microorganisms, produce negative aromas; one such example is the yeast *Brettanomyces bruxellensis,* which is often considered by various authors to be one of the most prevalent microbial issues that winemakers face today (Loureiro and Malfeito-Ferreira 2006; Suárez et al., 2007).

The alteration of the organoleptic properties of wine by *B. bruxellensis* tends to occur during barrel ageing prior to bottling but this yeast may also occur in the bottle if the wine has not been filter-

sterilised. *B. bruxellensis* is noted to cause film formation, cloudiness (Fugelsang et al., 1993; Fugelsang, 1997), loss of colour (Mansfield et al., 2002), production of volatile acidity (Fugelsang et al., 1993; Aguilar-Uscanga et al., 2003), production of 'mousy' off-flavours (tetrahydropyridines) (Heresztyn, 1986; Snowdon et al., 2006) and the formation of phenolic off-flavours (Chatonnet et al., 1995; Loureiro and Malfeito-Ferreira, 2003; Dias et al., 2003a). Of these negative attributes, volatile phenols are considered the most prevalent indicator of *B. bruxellensis* presence. Volatile phenols confer distinctive characters and/or off-flavours to wine (depending on concentration and consumer's liking), and are predominantly derived from hydroxycinnamic acid (HCA) precursors through enzymatic activity. The two-step nature of this pathway, with decarboxylation and reduction, eventually produces 4-ethylphenol (4-EP) and 4-ethylguaiacol (4-EG) from *p*-coumaric acid and ferulic acid, respectively (Chatonnet et al., 1992). The off-odours arising from volatile phenols have been described as 'animal' or horsey, 'medicinal', 'elastoplast' or band aid, 'sweaty leather', 'barnyard', 'spicy' and 'clove-like' and are detrimental to the aroma profile of wines at high concentrations (Heresztyn, 1986; Chatonnet et al., 1992; Chatonnet et al., 1995; Licker et al., 1998; Suárez et al., 2007). These aromas also suppress the natural fruity and flowery notes of red wine. It has been proposed that the formation of volatile phenols is a response to a metabolic need to regenerate $NAD(P)^+$ in the cell following redox imbalance created by the environmental conditions (Gerbaux and Vincent, 2001; Duckitt, 2012; Curtin et al., 2013).

B. bruxellensis has been isolated throughout the winemaking process from isolation in the vineyard to ageing wine in barrels as reviewed by Suárez et al. (2007), Oelofse et al. (2008) and Curtin et al. (2015). Indeed, this yeast may be found infrequently in grape must. Nevertheless, when present, *B. bruxellensis* manages to survive concurrently with the dominant fermentative yeast *S. cerevisiae* throughout alcoholic fermentation (AF) to ultimately contaminate the wine during ageing. Albertin et al. (2014) also demonstrated that strains of *B. bruxellensis* establish themselves in wineries over decades, spoiling wine year after year. It is only after the decline of other microbes at the end of AF that *B. bruxellensis* may dominate the wine matrix (Dias et al., 2003b; Renouf et al., 2006). Yet, microbial growth at this stage of winemaking is surprising as wine is nutritionally deficient in many regards. Genomic studies tend to suggest that this yeast species possesses the necessary machinery to use various substrates (Curtin et al., 2012a; Curtin et al., 2015). This could explain the perceived adaptation of survival in wine where little competition with other microbes occurs. *B. bruxellensis* is isolated from red wine predominantly and only occasionally from white wine (Loureiro and Malfeito-Ferreira, 2006). In fact, these environments tend to contain limited carbon and nitrogen sources since these have been depleted by the yeasts during alcoholic fermentation and when applicable also by lactic acid bacteria during malolactic fermentation. This suggests that *B. bruxellensis* has very low nutrient requirements and raises the question: how does it survive in such a nutrient-limited environment? It has been reported that *B. bruxellensis* requires a relatively low concentration of fermentable sugars <300 mg/L, much lower than levels recorded in most red wine, in order to proliferate (Chatonnet et al., 1995). In addition to this low carbon requirement, a

meagre 6 mg/l of yeast assimable nitrogen (YAN) allowed for the growth of *B. bruxellensis* from 10^5 to 10^7 cells/mL in a study conducted by Childs et al. (2015). Nevertheless, many discrepancies arise when reviewing previous studies on the nutritional preferences of *B. bruxellensis*, as highlighted by Smith and Divol (2016) (Chapter 2 of this thesis). These discrepancies could be due to the vast genetic (Curtin et al., 2007) and phenotypic (Conterno et al., 2006) diversity in *B. bruxellensis* strains as well as the growth conditions (e.g. anaerobic vs aerobic conditions) utilised in these studies. Finally, no study has so far investigated *B. bruxellensis*' nutrient requirements and preferences in a systematic manner.

In this study, the consumption of carbon and nitrogen sources typically present in wine (by opposition to grape juice) was monitored over time in a chemically defined synthetic wine medium for 3 strains of *B. bruxellensis*. A primary focus was placed on the sources that could potentially be consumed under anaerobic and semi-aerobic conditions. Redox balance was investigated in an attempt to assess whether the metabolites were produced in response to a specific need in NAD^+ or NADH. Ultimately this study was undertaken to determine the metabolic mechanisms by which *B. bruxellensis* is able to survive in the wine matrix. A broader understanding/knowledge of this yeast will thereby be improved with regard to its minimal nutritional needs. Furthermore, this knowledge could be utilised in order to prevent growth of *B. bruxellensis* and avoid subsequent wine spoilage. Thus allowing for the control of this yeast in a more efficient/effective manner in future.

3.2 Materials and methods

3.2.1 Yeast species utilised

The various yeast strains utilised in this study are listed in Table 3.1. The commercial *S. cerevisiae* strain Vin 13 and *S. polymorphus* var. *africanus* were utilised as outliers (i.e. non-*Brettanomyces* spp.) in the following experiments.

Table 3.1. Yeasts and strains utilised in this study

Yeast species	Strain/ Commercial name	Description	Collection/ Manufacturer
Saccharomyces cerevisiae	Vin 13	Commercial wine yeast	Anchor[a]
Schwanniomyces polymorphus var. *africanus*	8047	US Patent 3,939,279, feed for fish and crustaceans	CBS[b]
Brettanomyces bruxellensis	Y121	Isolated from South African red wine in 2004 (Oelofse, 2008)	IWBT[c]
	Y169	Isolated from South African wine in 2003	IWBT[c]
	1499	Isolated from Australian red wine (Curtin *et al*., 2012a)	AWRI[d]
	1649	Isolated from lambic beer	ISA[e]
	LO2E2	Isolated from French red wine (Serpaggi *et al*., 2012)	ITV[f]
	CB63	Isolated from red wine	ISVV[g]

[a]Anchor: Anchor© Bio-Technologies (Cape Town, South Africa) [b]CBS: Centraalbureau voor Schimmelcultures [c]IWBT: Institute for Wine Biotechnology (Stellenbosch University, South Africa) [d]AWRI: Australian Wine

Research Institute (Adelaide, Australia) [e]ISA: Instituto Superior de Agronomia (Lisbon, Portugal) [f]ITV: Institut Technique de la Vigne et du Vin (Beaune, France) [g]ISVV: Institut des Sciences de la Vigne et du Vin (France)

3.2.2 Carbon and nitrogen source screening plate assays

Selected carbon sources were utilised in order to carry out spot-plate studies. An agar base was prepared using Bacteriological Agar (Biolab Diagnostics, Wadeville, South Africa) and yeast nitrogen base (Biolab Diagnostics, Difco™) including anaerobic factors namely Tween 80 at 0.01 mL/L, ergosterol at 0.2 mg/L and supplemented alternatively with the following filter sterilised carbon sources from stock solutions: acetic acid at 0.1% v/v, ethanol at 3% v/v and glucose, glycerol and mannose at 2% w/v. The plates were incubated at 30°C, generally for 5-7 days, under anaerobic and aerobic conditions until colonies were fully formed. To attain an anaerobic environment, Anaerocult® chambers (Blanchardstown, Dublin) were utilised to store the plates. The same procedure that was utilised for the carbon sources was repeated for the following nitrogen sources at 200 mg N/L: ammonium, sodium nitrate and the amino acids arginine, leucine and proline. Glucose was added at 0.2% w/v to the nitrogen plates to provide a standard amount of carbon.

The procedure for plating the yeast strains was as follows. After preculture in YPD, a 10-fold dilution series of each strain culture was made from an $OD_{600\ nm}$ of 10 to 0.001. From these dilutions, 5 µL was spotted onto a matrix of dots (5x5); five strains were spotted per plate with five dilutions per strain (Figure 3.1). In the spot plates, *S. cerevisiae* and *S. polymorphus* var. *africanus* were used for comparative purposes. An arbitrary scale was devised in order to compare the growth of the strains, as explained in Figure 1. Strong growth on all spots was awarded 5 arbitrary units (AU) whereas a weak growth only of the lower dilution was awarded 1 AU. If no growth occurred, an “X” was used to indicate a lack of growth on the specific carbon or nitrogen source.

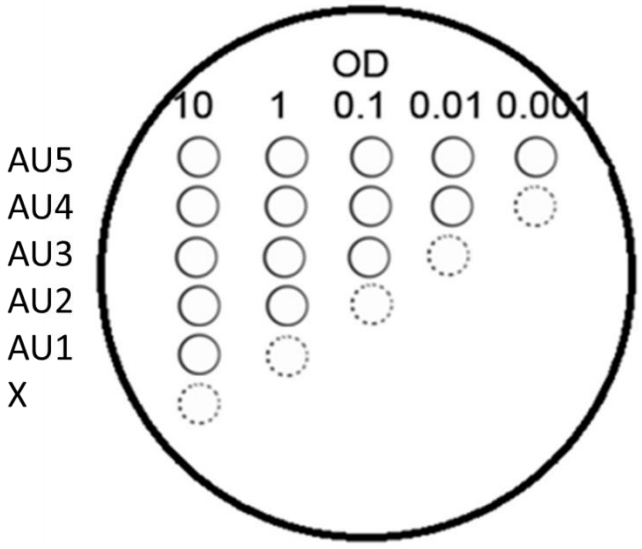

Figure 3.1: Layout for carbon and nitrogen spot plate screening. 5 AU indicates strong growth, whereas AU1 indicates weak growth and no growth is characterised by an X.

3.2.3 Preculture process, media and experimental conditions

Of the strains listed in Table 3.1, AWRI 1499, ISA 1649 and IWBT Y121 were initially grown on yeast peptone dextrose (YPD) agar. They were subsequently cultured in YPD broth (Biolab Diagnostics, Wadeville, South Africa) and incubated at 30°C with shaking. Before the inoculation of the *B. bruxellensis* strains into the synthetic wine medium (SWM) the strains underwent two preculture steps for activation and acclimatization. The first preculture was carried out in a 150-mL Erlenmeyer flask filled with 75 mL of liquid medium (PreLM1; Table 3.2). Initially, 5 mL YPD broth was inoculated with one single colony. Each PreLM1 flask was inoculated at an optical density ($OD_{600\ nm}$) of 0.1 and incubated at 30°C and stirred at 125 rpm for 48 h.

The second preculture was inoculated at an $OD_{600\ nm}$ of 0.1 from PreLM1 and incubated at 25°C and stirred at 125 rpm for 48 h (2 days). For this step, a 250-mL Erlenmeyer flask with 150 mL of pre liquid medium two (PreLM2; Table 3.2) was employed. This medium was similar to PreLM1 but it contained a lower sugar concentration with an additional 4% (v/v) ethanol, in order to adapt the yeast for the synthetic wine medium (SWM).

Table 3.2. The chemical compounds contained in the preculture media used before inoculation into the synthetic wine medium. The initial pH of all liquid cultures was adjusted to 3.4.

	PreLM1 (per litre)	**PreLM2 (per litre)**
Carbon sources	10 g glucose 10 g fructose	5 g glucose 5 g fructose 4% ethanol
Nitrogen sources	1 g yeast extract 2 g $(NH_4)_2SO_4$	1 g yeast extract 2 g $(NH_4)_2SO_4$
Salts	5 g KH_2PO_4 0.4 g $MgSO_4.7H_2O$	5 g KH_2PO_4 0.4 g $MgSO_4.7H_2O$
Organic acids	3 g tartaric acid 3 g malic acid 0.3 g citric acid	3 g tartaric acid 3 g malic acid 0.3 g citric acid

The PreLM2 preculture was used for the inoculation of the main experiment, once the strain reached mid-exponential phase. Each strain was inoculated at an $OD_{600\ nm}$ of 0.01. The main experiment was carried out in 500-mL Erlenmeyer flasks with an initial working volume of 400 mL of SWM (Table 3.3). The initial pH of all liquid cultures was adjusted to 3.4 with 10 M NaOH. All liquid media were sterilised by filtration through a 0.22-µm filter (Starlab Scientific, CA Membrane Filter). The oxygen concentration in the medium was adjusted to 7 mg/L for semi-anaerobic conditions and <1.5 mg/L for anaerobic conditions, by introducing nitrogen gas through the media into the medium. Furthermore, under semi-anaerobic conditions, two oxygen additions of 3 mg/L were applied to

simulate racking during the growth period (Day 14 and 31). All experiments were carried out in triplicate.

Table 3.3. The chemical concentrations of the compounds included in the composition of the synthetic wine medium (SWM) utilised for the growth of *B. bruxellensis* under wine-like conditions. The initial pH of the SWM was adjusted to 3.4.

	Compound	**Concentration (per litre)**
Primary carbon sources	Glucose	2.5 g
	Fructose	2.5 g
	Mannose	500 mg
Other potential carbon sources	Ethanol	10% (v/v)
	Acetic acid	400 mg
	Glycerol	8 g
Nitrogen sources	Yeast extract	500 mg
	$(NH_4)_2SO_4$	400 mg
	Amino acid stock solution (see below)	10 mL
	Sodium nitrate	12.5 mg
	Potassium nitrite	12.5 mg
Salts	KH_2PO_4	5 g
	$MgSO_4.7H_2O$,	400 mg
Organic acids	Tartaric acid	3 g
	Malic acid	3 g
	Citric acid	0.3 g
Volatile phenol precursor	*p*-coumaric acid	5 mg
Anaerobic factors	Tween 80	0.5 mL
	Ergosterol	10 mL

The amino acid fraction was comprised of 500 mg/L proline, 6 mg/L hydroxyproline, 24 mg/L arginine, 30 mg/L glutamic acid, 19 mg/L gamma-Aminobutryric acid, 14 mg/L ornithine, 33 mg/L alanine, 7 mg/L serine, 11 mg/L glycine, 5 mg/L valine, 9 mg/L leucine, 12 mg/L aspartic acid, 7 mg/L asparagine, 8 mg/L threonine, 4 mg/L isoleucine, 1 mg/L methionine, 14 mg/L lysine, 6 mg/L tyrosine, 10 mg/L phenylalanine, 7 mg/L histidine and 1 mg/L cysteine. The concentrations are an average taken from literature to resemble the levels found in wine (Etiévant et al., 1988; Lehtonen, 1996; Bouloumpasi et al., 2002). The amino acid fraction outlined in above, in addition to the ammonium sulphate added in the synthetic wine medium equate to a YAN concentration of 120 mg N/L.

Medium devoid of simple carbon sources

In an additional part of this study, the strain AWRI 1499 was tested in a similar medium to the SWM medium described above but no simple sugars were added to the medium. This additional experiment was conducted under semi-anaerobic conditions, in order to ascertain whether the strain could grow only utilising alternative carbon sources. When appropriate, *p*-coumaric acid was added to the medium to test whether 4-ethylphenol production was metabolically essential for the yeast to proliferate and survive in a wine-like medium.

3.2.4 Sampling, monitoring and analysis of experiment kinetics

All experiments were sampled in triplicate twice a week until the end of a predetermined time point (i.e. 45 or 68 days had elapsed since inoculation depending on the experiment). Samples were drawn from the side port of a modified Erlenmeyer flask to prevent unwanted oxygen supply. Thereafter, the $OD_{600\ nm}$ was measured and serial dilutions were performed, followed by the plating of the necessary dilutions. The samples were spun at 2,370 *g* for 5 min after which the supernatants were filtered through a 0.22-µm syringe filter (Starlab Scientific, Cape Town, South Africa) and stored at -20°C for further chemical analysis. The yeast cell populations were monitored by plating onto YPD agar during the experiments. Plates were incubated at 30°C, generally for 5-7 days, until colonies were fully formed.

Enzymatic assay analyses

The following kits and suppliers were utilised during this study; for residual glucose, fructose and mannose (Megazyme D-Mannose, D-Fructose and D-Glucose Code: K-Mangl 04/13, Wicklow, Ireland.), glucose (Roche, Basel, Switzerland) and ammonia (Megazyme K-AMIAR 07/14).

Quantification of stored glucose, glycogen and trehalose

At the ends of each preculture, intracellular glucose, glycogen and trehalose concentrations were determined in strain AWRI 1499. The cell pellet at each preculture stage was frozen on dry ice and stored at -80°C. For measurement of glycogen, the frozen samples were re-suspended with glass beads and breaking buffer. The samples were cooled on ice for 2 min. Furthermore, the samples were centrifuged, the supernatant was recovered and the stored glucose concentration was quantified and supernatant volume was recorded. Glycogen was precipitated by addition of 1 ml of ice-cold 95% (v/v) ethanol. The pellet was collected by centrifugation at 16,000 *g* at room temperature for 15 min. After two washes with 66% (v/v) ethanol, the pellet was dried, re-suspended in 400 mL of 50 mM sodium acetate and 5 mM $CaCl_2$ (pH 5.0), and digested with 30 mg of amyloglucosidase and 2 mg of α amylase at 56.5°C for 12 h. The glucose released was then determined using an enzymatic kit as described above (glucose (Roche)). The glycogen concentration was calculated and normalized to cell number. Trehalose was quantified using an enzymatic kit (Megazyme K-TREH 02/16) and freeze dried cells mentioned above and normalised to cell number.

Quantification of organic constituents by High Performance Liquid Chromatography

Organic acids (tartaric, malic, citric, acetic and succinic acids), glycerol and ethanol were quantified using HPLC. These compounds were separated on a Biorad Aminex HPX-87H (300 × 7.8 mm) column, at 55°C with 0.5 mM H_2SO_4 as mobile phase at a flow rate of 0.5 mL/min (Eyéghé-Bickong et al., 2012). Agilent RID and UV detectors were used concurrently in order to detect peaks and subsequently quantify each compound. Data analysis was carried out using the HPChemstation software package. All data is reported as the average of three replicates.

Quantification of amino acids by High Performance Liquid Chromatography

The quantification of all amino acids investigated in the study was performed by high performance liquid chromatography (HPLC), Agilent 1100 (Agilent Technologies, Waldbronn, Germany) by pre-column derivatisation and concurrent fluorescence detection based upon a method previously described (Henderson and Brooks, 2010). The following modifications were implemented to the derivatisation and injection stages. A Zorbax Eclipse plus C18 Rapid Resolution column (4.6 x 150 mm, 3.5 µm particle size; Agilent Technologies) was used following derivatisation of the amino acids. Derivatisation was performed using three different reagents: iodoacetic acid (Sigma Aldrich) for cysteine, o-phthaldialdehyde (OPA, Sigma Aldrich) for primary amino acids and fluorenylmethyloxycarbonyl chloride (Sigma Aldrich) for secondary amino acids. Internal standards, norvaline (Sigma Aldrich) and sarcosine (Sigma Aldrich) were spiked to each sample prior to derivatisation. One millilitre of each filtered sample was used for time point 0 h, the onset of stationary phase (Day 10) and for the final time point upon halting of the experiment (end point).

Quantification of 4-ethylphenol by Gas Chromatography Mass Spectrometry

Volatile phenols (e.g. 4-ethylphenol) released by the different strains were evaluated at the Central Analytical Facility, Stellenbosch University (unpublished method). 1 mL of sample was added to a 20 mL headspace vial, together with 20 µL of 100 µg/L anisole-d8 internal standard and 9 ml of 20% NaCl solution. The compounds were extracted, identified and quantified based on headspace solid-phase micro-extraction using DVB/CAR/PDMS (Divinylbenzene/Carboxen/Polydimethylsiloxane) fibres, with separation of the desorbed compounds being achieved using a polar FFAP column prior to their detection using tandem Gas Chromatography-Mass Spectrometry (GC-MS).

Analysis of Major volatile compounds via Gas Chromatography

The quantification of higher alcohols (Isobutanol, Isoamyl alcohol and 2-phenylethanol), acetate esters (2-Phenylethyl Acetate), fatty acids (Propionic acid, Iso-butyric acid, Butyric acid and Iso-valeric acid) and fatty acid ethyl esters (Ethyl phenylacetate) were carried out by gas chromatography. A flame ionization detector (GC-FID) was equipped. The Agilent GC System HP 6890 Series (Agilent, Little Falls, Wilmington, USA) was used as described by (Louw et al., 2009) with minor modifications. Five millilitres of each of the filtered samples was used with 100 µL of 4-methyl-2-pentanol (internal standard). One millilitre diethyl ether was added to the mixture. Thereafter the samples were then placed in an ultrasonic bath for 5 min to extract the volatile compounds. Finally, the samples were centrifuged at 1,300 *g* for 3 min. Sodium sulphate was added to remove any water from the non-polar layer. The samples that were analysed included day 0 and day 68 for AWRI 1499 with and without p-coumaric acid under both anaerobic and semi-anaerobic conditions.

Statistical analysis

All univariate statistical analyses were performed using Statistica 13 (Dell Inc., USA) to confirm the results obtained from different treatments on yeast growth and metabolite accumulation. An initial factorial ANOVA was used to analyse the data using a 95% confidence level. Furthermore, a Tukey's HSD post-hoc test or an Unequal N post-hoc test was performed when necessary.

3.3 Results

3.3.1. Initial screening for carbon and nitrogen source utilisation

Tables 3.4 and 3.5 summarise the results of the screening for single carbon and nitrogen sources utilisation. An arbitrary scale (Figure 3.1) was used in order to compare the growth performance on the different carbon and nitrogen sources between the strains under the 2 different conditions of oxygen supply (i.e. strict aerobic and anaerobic conditions). The plates were incubated for 7 days, regardless of the strain/species in question, for comparative purposes.

Table 3.4. Utilisation of carbon sources by various yeast strains on the spot plates under strict anaerobic and aerobic conditions using the arbitrary unit scale (see Figure 3.1). CBS 8047 and Vin 13 were utilised as non-*B. bruxellensis* strains for comparative purposes.

	Aerobic							
	CB 63	**IWBT Y121**	**IWBT Y169**	**LO2E2**	**AWRI 1499**	**ISA 1649**	**CBS 8047**	**Vin 13**
Glucose	5	5	5	5	4	3	5	5
Mannose	5	5	5	5	4	3	5	5
Glycerol	3	1	1	3	2	2	5	5
Acetic acid	2	1	1	2	2	2	3	5
Ethanol	3	2	2	2	2	2	5	5
Malic acid	3	3	3	3	2	3	5	2
	Anaerobic							
	CB 63	**IWBT Y121**	**IWBT Y169**	**LO2E2**	**AWRI 1499**	**ISA 1649**	**CBS 8047**	**Vin 13**
Glucose	3	4	3	4	2	3	X	5
Mannose	4	4	3	3	2	2	X	5
Glycerol	X	X	X	X	X	X	X	X
Acetic acid	X	X	X	X	X	X	X	X
Ethanol	X	X	X	X	X	X	X	X
Malic acid	X	1	1	1	1	1	X	1

From the carbon sources listed in Table 3.4, a clear difference between the results obtained under aerobic and anaerobic conditions was observed. Furthermore, the results varied depending on the strain and the carbon source in question. Under aerobic conditions, the sugars, namely glucose and mannose, allowed for stronger growth than the other carbon sources in all strains. However, strain variation was observed possibly due to slower growth kinetics over the set incubation period, as AWRI 1499 and ISA 1649 grew marginally weaker on the sugars. The strains were able to utilise malic acid under both aerobic and anaerobic conditions. However, a weaker level of growth was obtained under anaerobic conditions. All the *B. bruxellensis* strains tended to behave similarly when

utilising malic acid with the only exception being CB 63, which did not growth. Growth was also obtained in all strains utilising ethanol, glycerol and acetic acid however, the latter allowed for weaker growth. Under anaerobic conditions, the sugars allowed growth in all strains, although the growth was weaker when compared to that obtained under aerobic conditions. Ethanol, glycerol and acetic acid, did not allow for growth in any of the strains tested under anaerobic conditions.

S. polymorphus var. *africanus* strain CBS 8047 and *S. cerevisiae* strain Vin 13, grew strongly on all carbon sources under aerobic conditions, with the exception of acetic acid in CBS 8047 and malic acid in Vin 13 on which growth was marginally weaker. Under anaerobic conditions, CBS 8047 did not grow on any of the carbon sources. Under the same conditions, Vin 13 grew strongly utilising the sugars. Weak growth was obtained when Vin 13 utilised malic acid. Finally, no growth was observed on the ethanol, glycerol and acetic acid plates under anaerobic conditions.

Table 3.5. Utilisation of nitrogen sources by various strains on the spot plates under strict anaerobic and aerobic conditions using the arbitrary unit scale (see Figure 3.1) and an "X" indicating no growth obtained. CBS 8047 and Vin 13 were utilised as non-*B. bruxellensis* strains for comparative purposes.

	Aerobic							
	CB 63	**IWBT Y121**	**IWBT Y169**	**LO2E2**	**AWRI 1499**	**ISA 1649**	**CBS 8047**	**Vin 13**
Ammonium	2	1	1	1	2	1	5	3
Sodium Nitrate	4	2	2	4	2	3	5	1
Arginine	1	1	1	1	1	1	5	4
Leucine	4	1	1	4	2	3	5	5
Proline	4	1	1	4	2	3	5	5
	Anaerobic							
	CB 63	**IWBT Y121**	**IWBT Y169**	**LO2E2**	**AWRI 1499**	**ISA 1649**	**CBS 8047**	**Vin 13**
Ammonium	5	1	1	5	2	2	X	3
Sodium Nitrate	5	4	4	5	4	4	X	1
Arginine	5	1	1	5	2	4	X	4
Leucine	5	1	1	5	2	4	X	4
Proline	5	2	2	5	2	4	X	1

The nitrogen sources listed in Table 3.5 represent the potential sources found in the wine matrix. Ammonium is the dominant nitrogenous source that is present in wine after AF. The amino acids tested in this experiment represent those most abundant in wine (i.e. proline and arginine) and preferred by *S. cerevisiae* (i.e. leucine) (Crépin et al., 2012). Nitrate is also present in trace amounts but is said to confer an advantage over in *B. bruxellensis*, as *S. cerevisiae* cannot assimilate nitrate (de Barros et al., 2011). The nitrogen sources outlined in Table 3.5 display varying results under aerobic and anaerobic conditions. Furthermore, the results differ between strains. Under anaerobic conditions overall, stronger growth was observed when compared to the same strains under aerobic conditions. In general, no nitrogen source allowed for better growth above others, with the exception

of nitrate for IWBT Y121, Y169 and AWRI 1499. It is interesting to note that the *B. bruxellensis* strains all grew utilising proline under anaerobic conditions, when compared to CBS 8047 and Vin 13 which could not grow. The strains tested seemed to utilise nitrogen differentially with regard to growth, as some strains displayed stronger growth on certain nitrogen sources (e.g. CB63 and LO2E2) than others (IWBT Y121).

S. polymorphus var. *africanus* strain CBS 8047 grew strongly on all nitrogen sources under aerobic conditions. However, under anaerobic conditions, no growth was observed. Vin 13 grew strongly on the amino acids under aerobic growth. Similar growth was obtained utilising the amino acids under anaerobic conditions with the exception of proline which only allowed for very weak growth. Ammonium allowed for moderate growth under both aerobic and anaerobic conditions, however weaker than the growth obtained on the amino acids. Sodium nitrate elicited weak growth under both aerobic and anaerobic conditions.

For the following experiments, three strains were selected, namely AWRI 1499, ISA 1649 and IWBT Y121. These strains were chosen as ISA 1649 is the type strain of the *B. bruxellensis* species, AWRI 1499 is a well-researched strain (Harris et al., 2009a; Harris et al., 2009b; Curtin et al., 2012b; Crauwels et al., 2014; Granato et al., 2015) and IWBT Y121 is well-researched at Stellenbosch University (Duckitt, 2012; Willenburg and Divol, 2012; Louw, 2014; Mehlomakulu et al., 2014). These strains were isolated from different geographical regions (Table 3.1) with varying growth rates (Louw et al., 2016), and obtained differing results from the initial carbon and nitrogen plate screening (Tables 3.4 and 3.5). Therefore, these strains will help to identify possible strain variation within the species of *B. bruxellensis*.

3.3.2. Synthetic wine growth kinetics and carbon metabolism

In order to determine the utilisation of various carbon sources by three different *B. bruxellensis* strains in a synthetic wine-like medium under conditions mimicking bottle ageing (strict anaerobiosis) and tank ageing with regular rackings (semi-anaerobiosis). The growth in CFU/mL as well as the metabolite consumption and production of the two strains of *B. bruxellensis* was compared (Figure 3.2 A and B). The experiment in which no sugar was added to the initial medium is included in Figure 3.2 B (dashed lines).

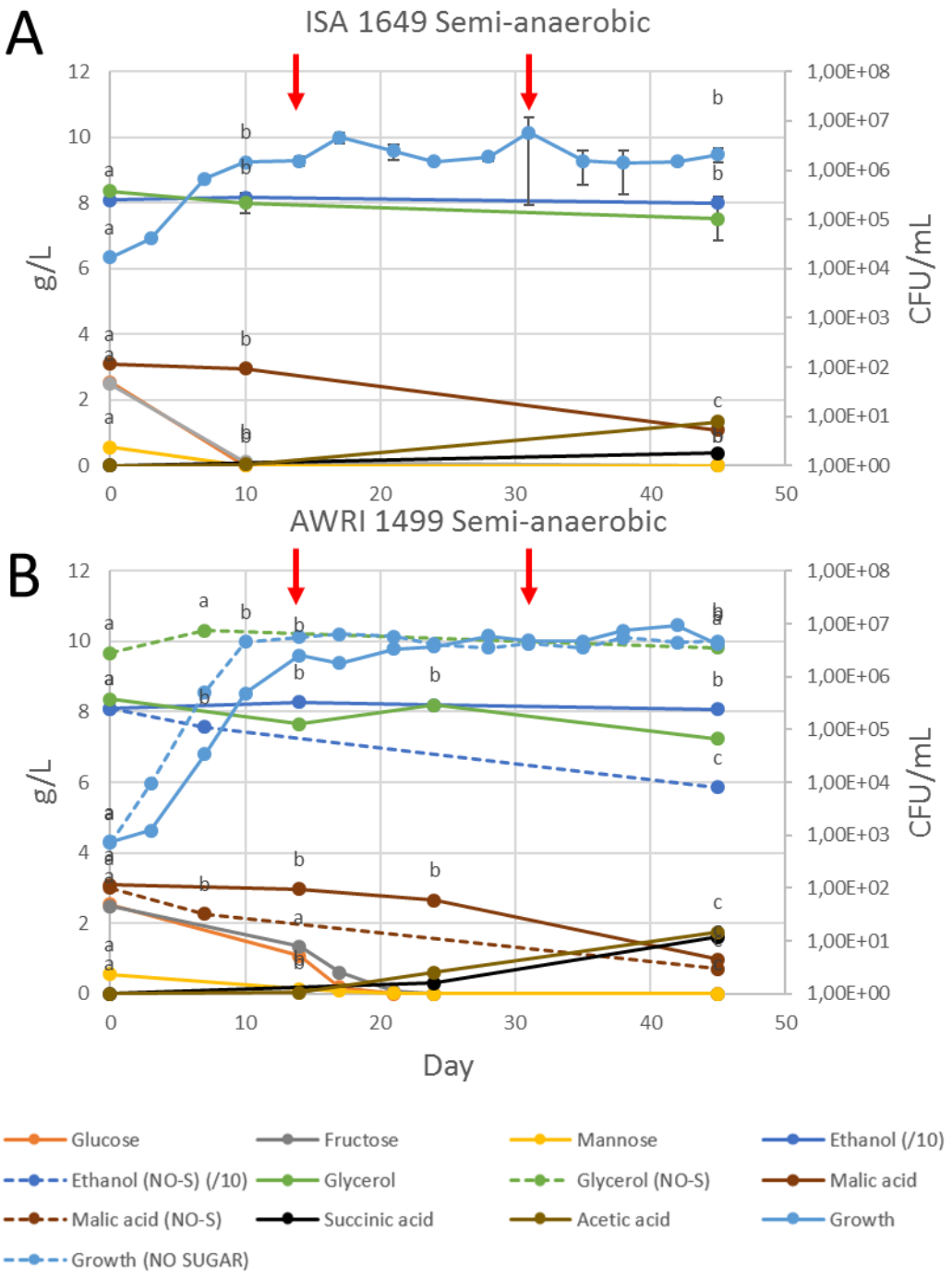

Figure 3.2: The growth kinetics of ISA 1649 (A) and AWRI 1499 (B) under semi anaerobic conditions over the defined growth period. The various metabolites produced or consumed are displayed on the primary vertical axis in g/l, whereas CFU/mL are displayed on the secondary vertical axis. The dotted lines in (B) indicate metabolites and growth that occurred in the experiment where no initial sugar was added to the media. Red arrows indicate times of oxygen supplementations. Values with the same letter for the same compound are statistically similar when compared with Tukey's HSD post-hoc test at 95 % confidence level.

The results obtained for ISA 1649 and IWBT Y121 (Appendix A: Figure S1) were very similar under both anaerobic and semi-anaerobic conditions with the exception of an insignificant amount of acetic and succinic acids being produced in IWBT Y121 under both anaerobic and semi-anaerobic conditions. Only the trends observed in ISA 1649 and AWRI 1499 under semi-anaerobic conditions

are therefore displayed on Figure 3.2 (IWBT Y121 data and anaerobic data are nevertheless displayed in Appendix A: Figure S1). As seen in Figure 3.2, the AWRI 1499 strain displayed overall a similar trend to strains ISA 1649 and IWBT Y121 however, its growth rate and metabolite consumption or production were marginally delayed in this strain when compared to the other two strains. The anaerobic and semi-anaerobic conditions showed similar trends in growth and metabolite flux, except that ethanol was not consumed under anaerobic conditions (Appendix A Figure S1). An initial utilisation of the sugars (glucose, fructose and mannose) was observed in all strains concurrently to a production of biomass and ethanol (Figures 3.2 A and B). In the AWRI 1499 strain, the process of sugar metabolism was prolonged when compared to the ISA 1649 and IWBT Y121 strains, which can be linked to a slower growth rate. For all 3 strains, upon complete consumption of the sugars, a concurrent and slow consumption of ethanol and malic acid was observed until the experiment was terminated on day 45. During this phase, no increase in biomass was observed but a constant culturable cell concentration was noted until the end point in all strains. Glycerol was neither significantly consumed nor utilised in all 3 strains over the course of the experiment. A slow and gradual yet significant increase of acetic acid and succinic acid was observed from the depletion of the sugars until the termination of the experiment in AWRI 1499 and ISA 1649. The same trend did not occur in IWBT Y121, under both conditions (Figure S1). No change was observed in citric and tartaric acids (data not shown).

In Figure 3.2 B, when no sugar was available for consumption, a similar trend in the consumption of malic acid was observed but this occurred from the onset of the experiment. Ethanol was also consumed to a lesser extent during the exponential phase. However, during stationary phase more ethanol was consumed than malic acid (17 g/L of ethanol was consumed). Indeed, the rate of consumption of ethanol remained unchanged while that of malic acid, already lower than that of ethanol, decreased slightly.

Glycerol was neither significantly consumed nor produced over the experimental period, regardless of the conditions, even when no sugars were initially present.

Since malic acid and ethanol did not allow growth, but only survival, it was surprising that the population managed to produce biomass in the absence of sugars. It was hypothesised that biomass could have been produced from stored sugars. The amount of sugars stored in the cells in the form of glucose, glycogen and trehalose was therefore assessed at the end of each preculture (Figure 3.3).

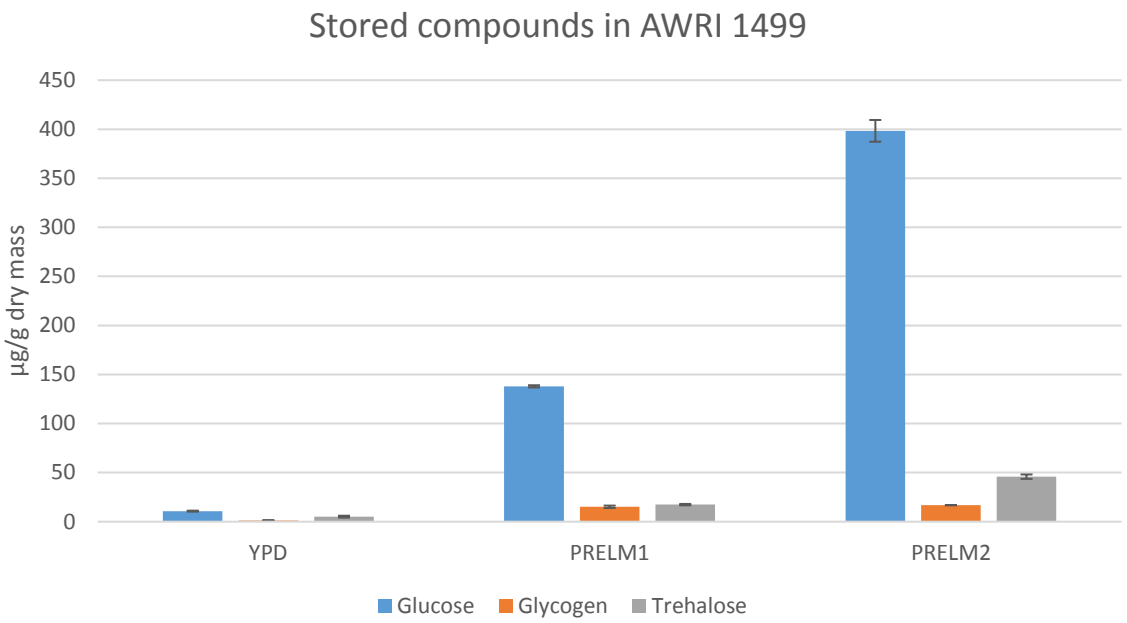

Figure 3.3: The stored compounds calculated as µg of compound/ g of dry mass, at the end of each preculture step before inoculation into the SWM.

Figure 3.3 shows that the AWRI 1499 strain did not seem to initially store much carbon sources from YPD. However, just before inoculation into the SWM at the end of the second preculture, 400 µg of glucose per g dry mass has been stored. This source was present along with 16 and 45 µg of glycogen and trehalose per g dry mass, respectively. These concentrations were calculated using an average of 13 glucose molecules per glycogen and 2 per trehalose. Indeed, glycogen is composed of 12-14 glucose molecules, as reported in *S. cerevisiae* for glycogen (François et al., 2011) and two α-glucose molecules are found per trehalose molecule.

In Table 3.6, the change in carbon is summarised as the molar concentration of carbon converted from the initial sugars into the various products quantified, during exponential phase. This was calculated using the theoretical molar concentration of carbon obtained from the sugars provided. This was compared to the molar concentration of carbon contained in the metabolites produced from the degradation of these sugars. Furthermore, the carbon recovery was calculated for each strain under both anaerobic and semi-anaerobic conditions. A good percentage recovery was observed in the AWRI 1499 strain, but the same cannot be said for the IWBT Y121 and ISA 1649 strains, pointing towards compounds being produced that were not outlined/quantified. From the table, ethanol appears to be the main carbon sink.

The stationary phase was not included in Table 3.6 as the resulting metabolites produced, from the assimilation of malic acid and ethanol, were not entirely known and only acetic acid and succinic acid were quantified.

Table 3.6: Moles of carbon per litre formed during the exponential phase of each strain under anaerobic (AN) and semi-anaerobic (SA) conditions. The number of moles of each compound formed from glucose, fructose and mannose were utilised in order to determine the moles of carbon formed from each metabolite. This was done utilising the molecular weight of each compound and the number of carbons per molecule. The molecular formula of a cell ($C_4H_{7.32}O_{2.24}N_{0.68}S_{0.006}$) was used to calculate the mol of carbon in each strain (van Dijken and Scheffers, 1986). From the initial sugars concentration (5.5 g/L), 183 mmol/L of carbon are provided, not taking into account any stored carbon compounds.

Strain	**AWRI 1499**		**ISA 1649**		**IWBT Y121**	
Condition	**AN**	**SA**	**AN**	**SA**	**AN**	**SA**
Metabolite						
CO_2	48.13	55.89	44.26	38.29	41.79	39.76
Ethanol	96.25	111.77	88.51	76.57	83.59	79.52
Biomass	2.62	2.86	8.90	22.10	11.87	2.29
Acetic acid	12.88	1.21	5.94	2.18	0.00	0.00
Succinic acid	6.09	10.09	0.00	0.00	0.00	0.00
Total mol C	165.97	181.83	147.61	139.14	137.25	121.58
% Carbon recovered	**90.69**	**99.36**	**80.66**	**76.03**	**75.00**	**66.44**

*CO_2 was extrapolated from the ethanol concentration produced.

Volatile phenol production in the B. bruxellensis strains

4-ethyl phenol production is displayed in Figure 3.4. 4-EP is the major volatile phenol produced in red wine, therefore its quantification as an indication of volatile phenols suited the purposes of this study.

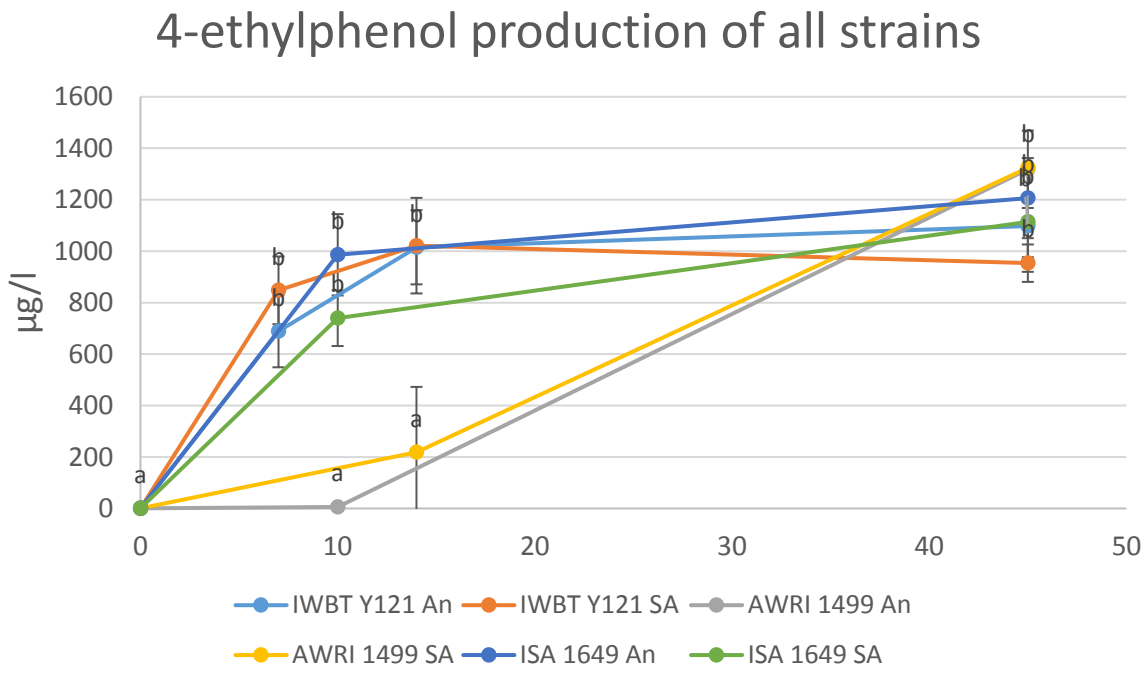

Figure 3.4: The 4-Ethylphenol production by three *B. bruxellensis* strains. Anaerobic conditions correlate to strains with "An" next to the name and Semi-anaerobic is indicated with "SA". Values with the same letter are statistically similar when compared with Tukey's HSD post-hoc test at 95 % confidence level.

The trend in 4-EP production in ISA 1649 and IWBT Y121 seemed to be linked to growth and is mostly produced during exponential phase, similar in the AWRI 1499 strain, as seen in Figure 3.4.

The initial lag in 4-EP production may be a result of its slower initial growth when compared to the other two strains. AWRI 1499 produced marginally more 4-EP than the other two strains however, all strains produced high levels of 4-EP (between 950 and 1300 µg/L). No significant difference is apparent when comparing anaerobic and semi-anaerobic conditions for all strains.

3.3.3 Nitrogen consumption in the *B. bruxellensis* strains

During the same experiment described above, the amount of ammonia and individual amino acids was determined between day 0 and day 10 (end of exponential phase for strains IWBT Y121 and ISA 1649) and between day 10 and the end point (i.e. day 45) the stationary phase. In Table 3.7, the nitrogen consumed is displayed, in mg N/L. Although similarities occurred between the anaerobic and semi-anaerobic conditions in each strain significant differences were observed between the strains.

Table 3.7: The nitrogen consumption in mg N/L by each *B. bruxellensis* strain under all conditions. A strong consumption (Green) characterises a consumption of above 1 mg/L N consumed, intermediate/weak (Orange/yellow) 0.2 to 0.9 and <0.2 mg N/L indicates a weak consumption and negative values indicate a production (Red). A stronger colour indicates a stronger consumption/production. The time periods listed in the table correspond to the exponential phase (Day 0-10), stationary phase (Day 10-45) and the overall experiment (Day 0-45).

Strain	**Initial amount**	**AWRI1499**			**AWRI1499**			**ISA 1649**			**ISA 1649**			**IWBT Y121**			**IWBT Y121**		
Condition	**(mg N/l)**	**Anaerobic**			**Semi-anaerobic**			**Anaerobic**			**Semi-anaerobic**			**Anaerobic**			**Semi-anaerobic**		
Day		**0-10**	**10-45**	**0-45**	**0-10**	**10-45**	**0-45**	**0-10**	**10-45**	**0-45**	**0-10**	**10-45**	**0-45**	**0-10**	**10-45**	**0-45**	**0-10**	**10-45**	**0-45**
Ammonia	88.04	-0.14	32.42	32.28	17.88	27.58	45.46	28.58	29.84	58.42	20.39	27.50	47.88	26.30	1.30	27.60	24.22	4.74	28.96
Asparagine	2.90	-0.19	-1.90	-2.09	-0.55	-0.49	-1.04	-0.58	-2.49	-3.07	-0.51	-1.69	-2.20	-0.73	-0.70	-1.43	-0.85	-0.79	-1.64
Glycine	1.35	-0.62	0.02	-0.61	-0.60	-1.20	-1.80	-0.74	-0.28	-1.02	-0.74	-0.46	-1.20	-0.47	-0.25	-0.72	-0.57	-0.31	-0.89
Threonine	1.46	-0.03	-0.06	-0.10	-0.09	0.01	-0.07	-0.28	0.26	-0.02	-0.30	-0.07	-0.37	-0.42	-0.16	-0.58	-0.46	-0.10	-0.55
Valine	1.66	-0.17	-0.01	-0.18	-0.20	-0.11	-0.31	-0.12	-0.02	-0.14	-0.13	-0.10	-0.23	-0.11	0.02	-0.09	-0.04	0.04	0.00
Hydroxyproline	0.60	-0.88	0.58	-0.30	-1.24	0.21	-1.03	-0.35	-0.04	-0.39	-0.55	-0.42	-0.97	0.08	0.00	0.08	-0.24	0.32	0.08
Alanine	6.15	-0.34	1.03	0.69	-0.78	1.08	0.31	-1.96	1.90	-0.06	-2.58	0.35	-2.23	-1.48	0.39	-1.09	-1.44	0.82	-0.63
Isoleucine	1.16	-0.05	0.08	0.03	-0.07	0.17	0.10	0.04	0.29	0.33	-0.04	0.02	-0.02	0.06	-0.08	-0.02	-0.02	-0.11	-0.13
γ-Aminobutyric acid	2.68	-0.33	1.45	1.12	0.14	1.59	1.73	-0.41	-0.62	-1.03	-0.18	-0.06	-0.24	-0.23	-0.06	-0.29	-0.46	-0.04	-0.50
Phenylalanine	1.39	-0.05	0.45	0.40	-0.02	0.49	0.47	0.27	0.49	0.76	0.10	0.28	0.38	0.24	-0.06	0.18	0.29	0.02	0.31
Aspartic acid	1.90	0.54	0.88	1.41	0.91	0.61	1.52	0.71	0.29	1.00	0.75	0.20	0.95	1.35	0.01	1.36	1.36	0.09	1.46
Glutamic acid	2.57	0.19	0.56	0.75	0.39	0.54	0.93	0.09	0.51	0.60	0.02	0.12	0.14	-0.11	-0.32	-0.43	-0.24	-0.64	-0.89
Serine	1.94	0.10	1.36	1.46	0.32	0.79	1.11	0.52	0.46	0.98	0.43	0.25	0.68	0.31	0.20	0.51	0.28	0.22	0.51
Histidine	0.94	-0.20	0.85	0.65	-0.03	0.58	0.55	0.46	0.18	0.64	0.29	0.11	0.40	0.16	-0.01	0.14	0.13	0.01	0.14
Arginine	4.56	0.49	3.93	4.41	2.29	1.84	4.14	4.27	-0.01	4.27	4.47	-0.17	4.30	3.67	-0.02	3.65	3.67	0.01	3.68
Tyrosine	0.54	-0.03	0.09	0.06	-0.02	0.14	0.13	0.02	-0.10	-0.08	-0.01	0.02	0.01	0.04	-0.01	0.03	0.05	0.02	0.06
Cysteine	1.56	-0.48	1.45	0.97	0.19	0.49	0.68	0.79	-0.01	0.79	0.69	-0.01	0.68	-0.08	-0.06	-0.14	-0.08	-0.08	-0.16
Methionine	0.38	0.12	0.02	0.14	0.20	0.01	0.21	0.28	-0.01	0.28	0.29	-0.01	0.27	0.18	-0.11	0.06	0.14	-0.07	0.07
Tryptophan	0.22	0.06	0.09	0.15	0.10	0.05	0.15	0.10	0.03	0.13	0.07	0.07	0.14	0.07	0.03	0.10	0.08	0.01	0.09
Ornithine	2.36	-0.09	1.89	1.81	0.14	-0.54	-0.40	-0.46	-0.60	-1.07	-0.39	-3.02	-3.41	0.53	0.31	0.83	0.54	0.32	0.86
Leucine	2.55	-0.03	2.28	2.24	-0.05	2.11	2.05	0.58	1.26	1.83	0.20	1.17	1.37	0.21	0.00	0.21	0.25	-0.06	0.19
Lysine	6.20	1.49	4.36	5.86	3.58	2.07	5.65	5.45	0.15	5.60	5.28	0.31	5.59	0.77	0.01	0.78	0.77	-0.28	0.48
Proline	54.08	-4.54	8.43	3.89	-1.98	13.82	11.84	-8.27	23.98	15.72	-8.23	19.74	11.51	-4.66	9.13	4.47	-1.00	9.80	8.79

The amino acids asparagine, glycine, threonine, valine, hydroxyproline, alanine, isoleucine and γ-aminobutyric acid were released in all strains under both conditions throughout the experiment, with the following few exceptions. Hydroxyproline was strongly consumed from day 10 until day 45 in AWRI 1499 under anaerobic conditions and only intermediately consumed under semi-anaerobic conditions. γ-Aminobutyric acid was strongly consumed (>1 mg N/L) between day 10 to day 45 only in strain AWRI 1499 but was released in the other two strains. Intriguingly, methionine and cysteine (the sulphur-containing amino acids) were intermediately to strongly consumed in the AWRI 1499 and ISA 1649 strains and were utilised to a lesser extent or released in the IWBT Y121 strain. AWRI 1499 strongly consumed the highest number of nitrogen sources, followed by ISA 1649 and finally IWBT Y121 with the least number of consumed nitrogen sources. The nitrogen compounds initially consumed in exponential phase (i.e. Phenylalanine, aspartic acid, glutamic acid, serine, histidine, arginine, tyrosine, cysteine, methionine, tryptophan, leucine and lysine) in strains ISA 1649 and IWBT Y121 (Table 3.7) are coupled to growth. As the same pattern is observed in AWRI 1499 however, it is only observed between days 10 and 45, as biomass is still being produced, probably due to a slower growth rate in this strain. Indeed, day 10 does not correspond to the end of exponential phase in the AWRI 1499 strain.

For the AWRI 1499 strain under anaerobic conditions from day 0 until day 10, few nitrogen sources were strongly consumed (lysine >1 mg N/L and to a lesser extent arginine and aspartic acid <1 mg N/L). During the same time period, under semi-anaerobic conditions, the following nitrogen sources were strongly consumed (>1 mg N/L): ammonia, aspartic acid, arginine, and lysine. Under semi-anaerobic conditions, from the beginning of the experiment until day 10, γ-aminobutyric acid, glutamic acid, serine, cysteine, methionine, tryptophan and ornithine were weakly consumed (<0.4 mg N/L) under semi-anaerobic conditions. Under anaerobic conditions for the same time period, the same amino acids were weakly consumed, with the exception of γ-aminobutyric acid, cysteine and ornithine being produced. Certain nitrogen sources (ammonia, hydroxyproline, alanine, isoleucine and γ-aminobutyric acid, phenylalanine, histidine, tyrosine, cysteine, ornithine, leucine and proline) were initially released in the initial growth period under anaerobic conditions from day 0 until day 10. Subsequently, the same amino acids were consumed between day 10 and day 45. The same trend in the aforementioned amino acids was observed under semi-anaerobic conditions for strain AWRI 1499.

For the ISA 1649 strain under both anaerobic and semi-anaerobic conditions from day 0 until day 10, ammonia, aspartic acid, arginine, cysteine and lysine were strongly consumed. Serine, histidine, methionine and leucine were moderately consumed (0.2-0.5 mg N/L) under both anaerobic and semi-anaerobic conditions for the same time period. Under both anaerobic and semi-anaerobic conditions for the initial time period, isoleucine, phenylalanine, glutamic acid, tyrosine and tryptophan were weakly consumed (<0.2 mg N/L). From day 10 until day 45 under both anaerobic and semi-anaerobic conditions ammonia, alanine, leucine and proline are strongly consumed (>1 mg/L), with

the exception of alanine under semi-anaerobic conditions which is only moderately consumed. With alanine, phenylalanine, aspartic acid, serine and lysine were moderately consumed (0.2-0.5 mg N/L). The same is observed under anaerobic conditions along with threonine, isoleucine and glutamic acid also being moderately consumed (0.2-0.5 mg N/L), while these sources are only weakly consumed (<0.2 mg N/L) under semi-anaerobic conditions. In both anaerobic and semi-anaerobic conditions, histidine, tryptophan and lysine were weakly consumed (<0.2 mg N/L).

Regarding the IWBT Y121 strain, ammonia, arginine, aspartic acid and to a slightly lesser extent lysine were strongly consumed (>1 mg N/L) under semi-anaerobic conditions, similarly to the anaerobic conditions. For both anaerobic and semi-anaerobic conditions from day 0 until day 10, phenylalanine, serine, methionine, ornithine and leucine were moderately consumed (0.2-0.5 mg N/L). For the same time period, hydroxyproline, isoleucine, histidine, tyrosine and tryptophan were weakly consumed under both anaerobic and semi-anaerobic conditions. From day 10 until the experiment was terminated, ammonia, proline and alanine to a lesser extent were strongly consumed (>1 mg N/L) with few sources being moderately consumed (i.e. alanine, serine and ornithine between 0.2 and 0.5 mg N/L) in this strain under both anaerobic and semi-anaerobic conditions. Valine, hydroxyproline, aspartic acid, histidine, arginine, tyrosine, tryptophan, leucine and lysine were weakly consumed (<0.2 mg N/L) under both anaerobic and semi-anaerobic conditions. The rest of the nitrogen compounds were released during this time period.

The two nitrogenous sources that add the highest concentration to the total potential nitrogen available are ammonia (±88 mg N/L) and the amino acid proline (±54 mg N/L), which can be utilised by *B. bruxellensis*. Indeed, proline, initially released in all strains and conditions, was greatly consumed between day 10 and 45, when compared to other amino acids in the medium (8.43-23.98 mg N/L of proline dependent on strain and similar for both conditions). Regarding ammonia, between 27-58 mg N/L was assimilated, depending on strain and condition tested. In comparison to the other nitrogen sources present, the next sources strongly consumed (arginine and lysine) only provided ±4.2 and ±5.6 mg N/L respectively. Therefore, of all the nitrogen sources provided ammonia and proline were most assimilated. It is interesting to note that ammonia, the most preferred and easily assimilable source of nitrogen in *S. cerevisiae*, was not fully utilised (globally ±35% consumed with the exception of AWRI 1499 and ISA 1649 under anaerobic conditions see Table S1) by any of the *B. bruxellensis* strains tested.

3.3.4 Investigating the metabolic role of volatile phenol production in *B. bruxellensis* strain AWRI 1499

In order to investigate the role of volatile phenol formation and their role in balancing the redox potential of the cell, strain AWRI 1499 was used as it was identified as the highest producer of 4-EP in the previous experiment. Figure 3.5 A, B, C and D depicts the different trends obtained in the experiment to determine whether the consumption of *p*-coumaric acid plays a role in balancing the redox potential of the *B. bruxellensis* cell.

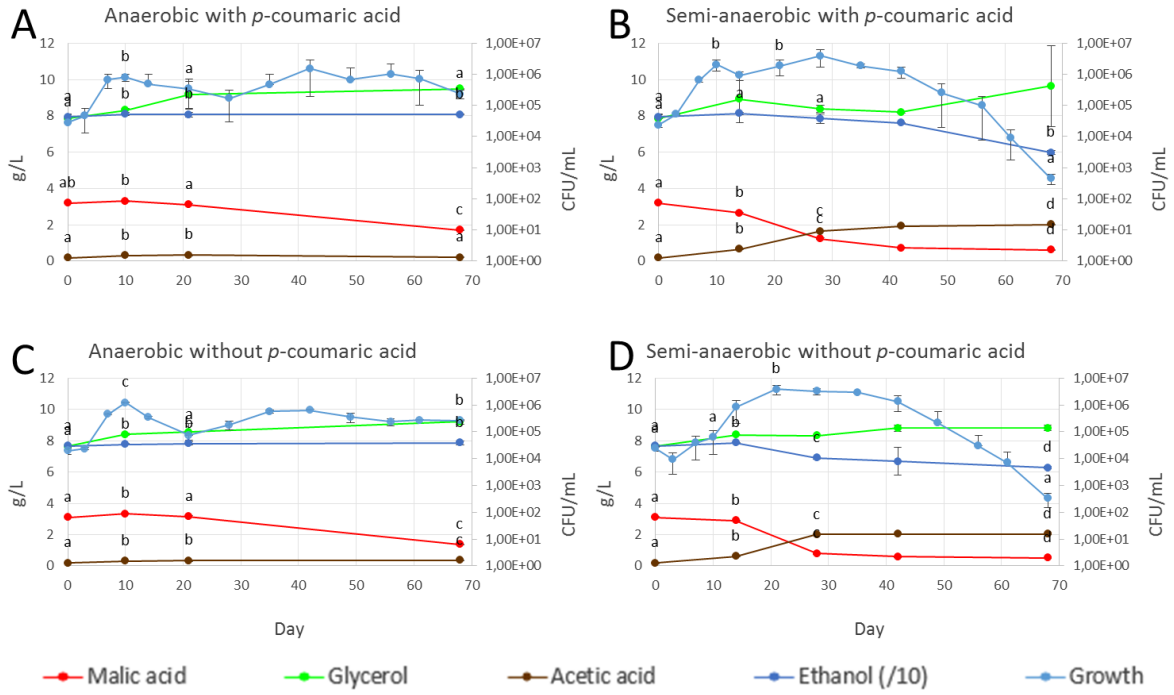

Figure 3.5: The growth kinetics of AWRI 1499 with *p*-coumaric acid added under anaerobic conditions (A) and semi anaerobic conditions (B), and without *p*-coumaric acid under anaerobic (C) and semi-anaerobic conditions (D) over the defined growth period. The various metabolites produced or consumed are displayed on the primary vertical axis in g/l, whereas growth in CFU/mL is displayed on the secondary vertical axis.

Under anaerobic conditions (Figure 3.5.A and C), a difference in growth was observed together with metabolite fluctuation, when compared to Figure 3.5 B and D which displays the effect of semi-anaerobic conditions. Under anaerobic conditions, the population grew up to day 10 and thereafter remained consistently high between 10^5 and 10^6 CFU/mL, with slight fluctuations, until the experiment was terminated on day 68. Contrary to this pattern, the population under semi-anaerobic conditions initially increased from day 0 until day 10, and then remained constant. Thereafter, the population declined steadily until the termination of the experiment. With regard to ethanol, a similar trend was observed when comparing the same strain in the previous experiment: an initial production was observed during the growth phase followed by ethanol utilisation under semi-anaerobic conditions. Under anaerobic conditions, the ethanol concentration increased concurrently with sugar consumption but no ethanol was subsequently consumed. Similar to the previous experiment, malic acid was consumed under both anaerobic and semi-anaerobic conditions. However, it was consumed faster under semi-anaerobic conditions at the beginning of the experiment (Day 0-28) and the consumption plateaued at day 42 (0.6 and 0.7 g/L without *p*-coumaric and with *p*-coumaric acid respectively). However, under anaerobic conditions, malic acid was linearly but very slowly consumed from day 10 and was not fully consumed at the termination of the experiment (1.69 and 1.37 g/L when *p*-coumaric acid was present in the medium and lacking, respectively). Similar to the previous experiment, acetic acid was produced substantially under semi-anaerobic conditions, and to a lesser extent under anaerobic conditions. Regarding the addition of *p*-coumaric acid (Figure 3.5

A and B) or the lack thereof in the medium (Figure 3.5 C and D), minor differences were observed in growth kinetics and metabolite consumption.

In Figure 3.6, the volatile phenol production under these different conditions is displayed as the concentration of 4-EP produced. As expected, no 4-EP was produced when no precursor (*p*-coumaric acid) was added. However, in the presence of *p*-coumaric acid, after an initial lag phase (until day 7) where no production was observed, 4-EP was produced under both anaerobic and semi-anaerobic conditions. Although more 4-EP was produced under semi-anaerobic conditions, the final concentrations were very similar (2.2 and 2.3 mg/L, under anaerobic and semi-anaerobic conditions, respectively), as the concentration of 4-EP decreased between day 42 and 68 under the latter conditions. Globally, the increase in 4-EP corresponded to the population's exponential phase. Under anaerobic conditions, the increase in 4-EP was slower when compared to the rate of 4-EP production under semi-anaerobic conditions, but relatively linear. Under semi-anaerobic conditions, the production occurred at a faster rate but 4-EP seems to have been assimilated as the population declined.

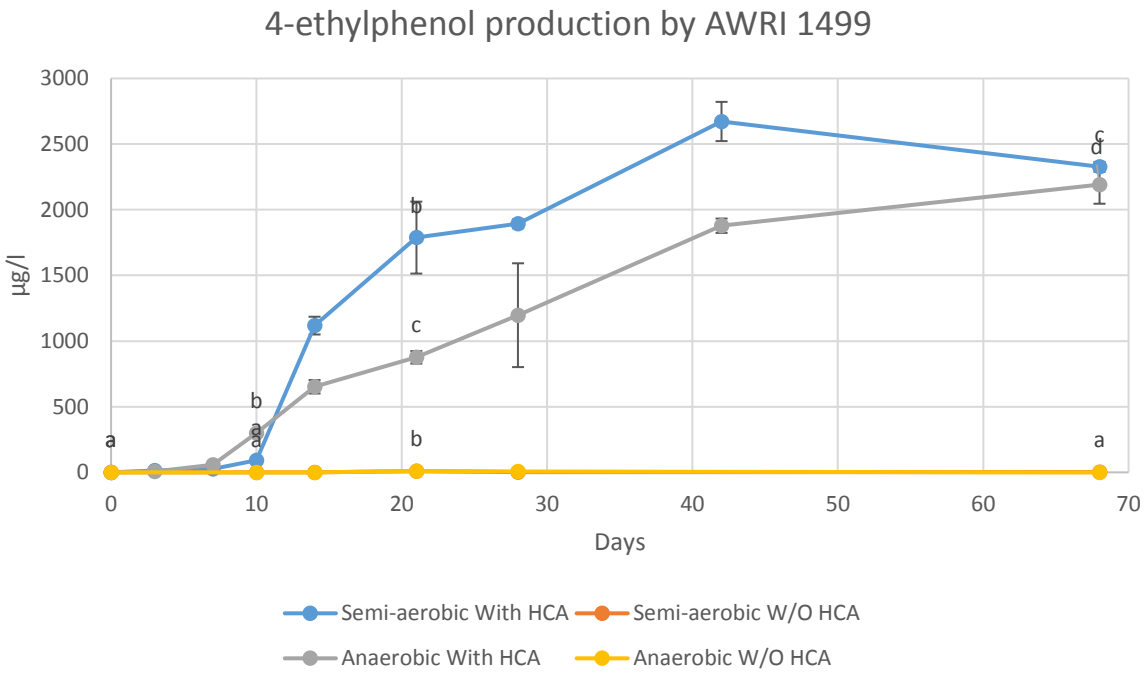

Figure 3.6: Concentration of 4-EP produced in the experiment to determine redox balancing potential of *p*-coumaric acid as a precursor for the strain AWRI 1499.

Table 3.8: Balance of NAD+/NADH for the AWRI 1499 strain in mol/L NAD+ or NADH produced. Green indicates a production of NAD+. Red and negative values indicate a production of NADH. Grey indicates neither a production of NAD+ nor a production of NADH, and is not included in the balance subtotal calculation. The higher alcohols and fatty acids were quantified at time 0 and 68. With regard to the balance subtotal "0" is considered at balance/equilibrium, therefore more negative depicts an unbalanced event with a total NADH present whereas more positive corresponds with more NAD+ present.

		Anaerobic			Semi-anaerobic		
	Time points	**0-10**	**10-30**	**30-68**	**0-10**	**10-30**	**30-68**
With p-coumaric acid	Carried over NAD+	0.00E+00	2.99E-02	2.90E-02	0.00E+00	2.90E-02	-6.06E-02
	Stored sugar		-8.33E-05			-8.33E-05	
	Malic acid	-5.83E-05	-5.82E-04	-1.06E-02	-4.16E-03	-1.05E-02	-4.60E-03
	Acetic acid	-1.91E-03	-3.52E-04	#N/A	-7.69E-03	-1.65E-02	-6.18E-03
	Ethanol	3.22E-02	0.00E+00	0.00E+00	4.18E-02	-6.26E-02	-4.04E-01
	Biomass	-3.06E-04	0.00E+00	0.00E+00	-8.13E-04	0.00E+00	0.00E+00
	Higher alcohols		1.02E-04			1.16E-04	
	Fatty Acids		-2.34E-05			-4.16E-05	
	4-EP	2.40E-06	7.34E-06	8.14E-06	6.44E-07	1.47E-05	3.55E-06
	Balance Subtotal	**0.030**	**0.029**	**0.018**	**0.029**	**-0.061**	**-0.475**
Without p-coumaric acid	Carried over NAD+	0.00E+00	1.74E-02	1.52E-02	0.00E+00	3.57E-02	-2.12E-01
	Stored sugar		-8.33E-05			-8.33E-05	
	Malic acid	-4.78E-05	-1.70E-03	-1.12E-02	-1.55E-03	-1.58E-02	-1.94E-03
	Acetic acid	-1.85E-03	-4.73E-04	-7.19E-04	-7.02E-03	-2.31E-02	-8.17E-04
	Ethanol	1.99E-02	0.00E+00	0.00E+00	4.44E-02	-2.09E-01	-1.34E-01
	Biomass	-4.80E-04	0.00E+00	0.00E+00	-1.57E-05	0.00E+00	0.00E+00
	Higher alcohols		7.48E-05			1.23E-04	
	Fatty Acids		-1.59E-05			-3.72E-05	
	4-EP	-	-	-	-	-	-
	Balance Subtotal	**0.017**	**0.015**	**0.003**	**0.036**	**-0.212**	**-0.349**

*The higher alcohols and fatty acids were not included in the NAD+ recovery calculation as they were only calculated at the end point.
*The stored sugars were included in the calculations between day 0-10.

The change in the redox potential of the population is outlined in Table 3.8. The time points are grouped to correspond with the exponential phase (0-10), stationary phase (10-30) and, when applicable, the decline phase (30-68 in semi-anaerobic conditions). Using the pathways shown in Figure 3.7, the NAD+/NADH balance was calculated. Initially in all instances biomass production sinks NAD+, whereas the consumption of ethanol leads to an increase in NAD+ as well as the production of 4-EP when *p*-coumaric acid is present. The consumption of malic acid as well as the formation of acetic acid produce NADH. The exponential phase (day 0-10) for all conditions is unbalanced favouring NAD+. However, the stationary phase (day 10-30) under semi-anaerobic conditions has become much more strongly unbalanced favouring NADH presence. In Table 3.8, the higher alcohols and fatty acids were measured at day zero and at day 68 for each variation of the experiment (Appendix A, Table S2). The fatty acids are comprised of propionic acid, iso-butyric acid, butyric acid and iso-valeric acid and fatty acid ethyl esters (Ethyl phenylacetate) and the higher alcohols are comprised of isobutanol, isoamyl alcohol and 2-phenylethanol), acetate esters (2-Phenylethyl Acetate). The formation of fatty acids and higher alcohols cause a production of NADH and NAD+ respectively. In each instance, the higher alcohols allow for more NAD+ production in comparison to the fatty acids. At the final time point the balance subtotal displayed in Table 3.8, is similar for both values under the anaerobic conditions with NAD+ being present (0.018 and 0.003 mol of NAD+ equivalents, respectively). These values are positive and close to "0" which indicates that more NAD+ is present but the cells are close to equilibrium. In contrary to this, the semi-

anaerobic conditions display a lack of NAD^+ at the final time point (-0.475 and -0.349 mol of NAD^+ equivalents, respectively) and indeed a net presence of NADH regardless of whether *p*-coumaric acid is present or not. This indicates a strong imbalance in the favour of NADH in the cells.

3.4 Discussion

3.4.1. Carbon and nitrogen screening

In this study, the abilities of three *B. bruxellensis* strains to grow on single sources of carbon and nitrogen present in wine were first investigated using plate assays. According to the results obtained in Tables 3.4 and 3.5, the strains of *B. bruxellensis* investigated can utilise many different carbon and nitrogen sources but clear variations between strains and growth conditions were observed.

Carbon

A recorded growth of between 2-5 AUs (Table 3.4) was attained in all strains under both anaerobic and aerobic growth conditions when utilising sugars (glucose, fructose and mannose). Under aerobic and anaerobic conditions, sugars generally allowed for stronger growth of *B. bruxellensis* strains when compared to the other carbon sources investigated. However, certain differences were observed in the strains, as anaerobic conditions recorded lower growth levels in most *B. bruxellensis* strains. Oxygen is not required for growth of *B. bruxellensis* but it has been previously noted that growth is depressed under oxygen limited conditions (Smith, 1998). Under these conditions, AF is inhibited and carbohydrates are catabolised to acetic acid rather than ethanol. This forms the basis of the so-called Custers effect (Wijsman et al. 1984; Ciani and Ferraro, 1997).

Under both aerobic and anaerobic conditions, a visibly lower growth occurred in certain strains (AWRI 1499) utilising certain compounds, when compared to the other strains (ISA 1649 and IWBT Y121), but it is likely attributable to an overall slower growth as was reported in Louw et al. (2016). It was noted that *B. bruxellensis* strains in general grew visibly slower when compared to the other two yeast species, *S. cerevisiae* Vin 13 and *S. polymorphus* var*. africanus.* Indeed, the latter yeasts attained 5 AU when grown on sugars (e.g. glucose and mannose) under aerobic conditions. However, under anaerobic conditions, *S. polymorphus* could not achieve any growth. *S. cerevisiae* managed to grow under strictly anaerobic conditions. The inclusion of sterols and unsaturated fatty acids in the media used, allowed *S. cerevisiae* and *B. bruxellensis* to grow (Andreasen and Stier, 1953; Verduyn et al., 1990). The weaker growth of the latter could be attributed to its overall slower growth than that of the former. The concentration and/or nature of these compounds were clearly not appropriate for *S. polymorphus* var. *africanus*.

As reported in Table 3.4, the *B. bruxellensis* strains were able to utilise the alternative carbon sources malic acid, ethanol and glycerol (between 1-3 AUs) under aerobic conditions. This was also observed in liquid medium for malic acid and ethanol, but not for glycerol (Figure 3.6). The growth however, was weaker when compared to that obtained on sugars. In liquid medium, it did not allow

for growth, but only survival. Acetic acid was also consumed but the growth observed on plate was even weaker (1-2 AUs). Numerous studies have outlined the growth of *B. bruxellensis* utilising ethanol and glycerol as sole carbon sources (Custers, 1940; Smith, 1998; Dias et al., 2003b; Conterno et al., 2006). Few sources of literature support the use of acetic acid as a source for growth in *B. bruxellensis*. Therefore, it is noteworthy that growth, utilising acetic acid as a sole source of carbon, is observed under aerobic conditions, albeit weak (Figure 3.7). Previously the consumption of acetic acid has only been reported upon complete consumption of glucose and ethanol (Wijsman et al., 1984). Under aerobic conditions, it is possible for *B. bruxellensis* to metabolise acetic acid via the TCA cycle as the electron transport chain is active (Figure 3.6).

Under anaerobic conditions, the *B. bruxellensis* strains attain weak growth when utilising malic acid, similar to the growth obtained by *S. cerevisiae*. Under these growth conditions, similarly to *S. cerevisiae*, the *B. bruxellensis* strains could not utilise ethanol, glycerol or acetic acid on plates. Indeed, *S. cerevisiae* is unable to metabolise these compounds in the absence of oxygen (Larsson et al., 1998; Piškur et al., 2006). In this regard, it seems that *B. bruxellensis* behaves similarly.

Nitrogen

Similar to the carbon source plate results, trends in nitrogen assimilation varied between strains and environmental conditions (Table 3.5). Many strains were not able to achieve strong growth utilising the provided nitrogen sources, under both aerobic and anaerobic conditions. This may be due to the limited sugar available, as only 0.2% w/v glucose was added to the nitrogen screening plates to simulate wine sugar levels. This minimal amount of sugar would only allow for a limited amount of growth on these plates when compared to the carbon screening plates. However, this glucose concentration in the nitrogen plate screening was standard for each nitrogen source. Furthermore, each nitrogen source was standardised at a set concentration of 200 mg N/L in order to compare the nitrogen compounds.

From Table 3.5, it is noted that growth between the strains was reduced under aerobic conditions, with stronger growth achieved in each strain under anaerobic conditions. Literature reveals that yeast extract and certain amino acids (i.e. arginine and leucine) aid in the growth of *B. bruxellensis* under anaerobic conditions, similar to the results of this study (Blomqvist et al., 2012). From this plate screening, it should be noted that under anaerobic conditions sodium nitrate allowed for high levels of growth (4-5 AU) in all strains of *B. bruxellensis* as well as *S. polymorphus*, but not *S. cerevisiae*. The assimilation of nitrate has previously been described in *B. bruxellensis*. Furthermore, the corresponding genes, allowing for the uptake of nitrate, and assimilation has been proposed to confer an advantage over *S. cerevisiae* by *B. bruxellensis* (Woolfit et al., 2007; de Barros et al., 2011; Galafassi et al., 2013). The utilisation of proline and arginine by *S. cerevisiae* is unsurprising as this yeast is able to utilise alternative nitrogen sources such as arginine, urea, or proline when preferred nitrogen sources (glutamine, asparagine, or ammonium ions) are unavailable (Xu et al., 1995). Interestingly, the *B. bruxellensis* strains all attained stronger growth on proline, albeit varied, than

S. cerevisiae. S. cerevisiae requires oxygen in order to assimilate proline but *B. bruxellensis* may have other pathways that do not require oxygen for the assimilation of proline.

It has also been stated in other studies, that *B. bruxellensis* has higher nitrogen requirements when compared to *S. cerevisiae* regarding anaerobic growth (Blomqvist et al., 2012). Indeed, this can be observed in Table 3.5 as *S. cerevisiae* obtains stronger growth as opposed to the *B. bruxellensis* strains. This could potentially be misleading as the growth rate of *S. cerevisiae* is known to be faster than that of *B. bruxellensis*. The growth trends under anaerobic conditions, when comparing strains (Table 3.5), seem to be strain dependent as opposed to being dependent on the source in question, as certain strains attain stronger growth compared to other strains on all the nitrogen sources. This may reveal that certain strains are better adapted to growth utilising these nitrogenous sources or assimilating nitrogen (CB63, LO2E2 and ISA1649) when compared to others that do not assimilate as much or attain weak growth (AWRI 1499 and IWBT Y121). This displays strain variation that can be attributed to varying growth rates between strains.

3.4.2. Growth kinetics and carbon metabolism

B. bruxellensis and other budding yeasts are able to produce ethanol in the presence of oxygen and excess of glucose, via the glycolysis pathway as “Crabtree-positive” yeasts (Rozpędowska et al., 2011; Rhind et al., 2011). The three strains of *B. bruxellensis* used in this study (AWRI 1499, ISA 1649 and IWBT Y121) utilised the same carbon sources in the synthetic wine medium as in the plate screening, with the exception of glycerol, as mentioned above (Figure 3.2 A and B). The consumption of sugars corresponded to the concurrent increase in biomass and ethanol production (Figure 3.7). Therefore, the longer sugar consumption time observed in the AWRI 1499 strain, is most certainly due to its slower growth rate (Figure 3.2 B). The initial sugar concentration in the SWM (5.5 g/L) was therefore mostly utilised by each strain in order to concurrently produce ethanol (1.2-3 g/L) and biomass (10^3-10^4 CFU/mL), which does not seem plausible from the amount of sugar supplied. It was then hypothesised that certain stored carbon sources, from the preculture media, could be utilised during this exponential growth phase (Figure 3.3). The data confirms that stored carbon sources were indeed available to *B. bruxellensis* in the forms of glucose, glycogen and trehalose. Of the sources mentioned, the storage of the latter contradicts a study by Leite et al, (2016) which states that the high trehalase activity, prevents the accumulation of trehalose in *B. bruxellensis* strain GDB 248. This could be due to strain variation as different strains were utilised in this study. These stored sources could certainly contribute to the accumulation of both ethanol and biomass yields. Indeed, in other yeast species, it has been shown that glycogen and trehalose are accumulated by cells experiencing carbon and energy limitation (Lillie and Pringle, 1980). In *S. cerevisiae*, these sources can represent 41-87 or 205-235 µg of carbohydrate per g of the dry weight of the cells, of trehalose and glycogen respectively, depending on the strain in question and growth phase under carbon limited conditions (Gunja-Smith et al., 1977; Küenzi and Fiechter, 1972). In this study, *B. bruxellensis* strain AWRI 1499 was able to store 45 and 16 µg, of trehalose and glycogen, respectively, per g of

dry cell mass. Although lower than the amounts stored in *S. cerevisiae*, these carbon reserves help to explain how the AWRI 1499 strain was able to attain 0.18 g of dry mass (4 logs CFU/mL) when no sugar was supplied in the medium. However, certain other sources may be utilised. It is interesting to note that *B. bruxellensis* was able to attain growth 3-4 log CFU/mL utilising scarce amounts of these carbon sources and maintain its population for 45 days. *B. bruxellensis* was indeed able to make efficient use of these stored and scarce initial sugar sources when compared to *S. cerevisiae*. When *S. cerevisiae* was placed under the same conditions, it was not able to grow or survive in the initial preculture medium and could not be inoculated into the SWM (data not shown). This highlights the adaptation of this yeast to utilising limited nutrients in an efficient manner in a stressful environment.

The ethanol flux in this study differed under anaerobic or semi-anaerobic conditions. Under anaerobic conditions, ethanol was produced but could not be consumed. This is due to the fact that no oxygen was present to activate the electron transport chain (See pathways 3, 4 and 8 of Figure 3.7). Once the sugars were fully consumed by the *B. bruxellensis* strains into ethanol and biomass, it was observed that the strains subsequently utilised alternative sources of carbon, such as malic acid and ethanol under semi-anaerobic conditions and only malic acid under anaerobic conditions (Figure 3.2 A and B). These results correspond to those obtained in the plate trials, where ethanol and malic acid are both similarly utilised under semi-anaerobic conditions, and only malic acid was assimilated under anaerobic conditions. This pattern was similar in all strains (Figure S1 supplementary material). These sources seem to be concurrently but incompletely utilised from day 10 until day 45. Malic acid was assimilated over this period under both anaerobic and semi-anaerobic conditions. Ethanol was catabolised under semi-anaerobic conditions. The assimilation of malic acid and ethanol was slow and not complete in any of the strains but seemed to have been sufficient to allow cell maintenance and survival, but not multiplication as no population growth was observed during this period. The complete consumption of these sources may have occurred had the experiment not been terminated at day 45. The Crabtree-positive yeasts *S. cerevisiae* and *B. bruxellensis* can efficiently catabolise ethanol; this mechanism is well documented as a “make, accumulate and consume” strategy (Thomson et al., 2005; Piškur et al., 2006; Rozpędowska et al., 2011). It is interesting to note that with so little oxygen present, 7 mg/L, the assimilation of ethanol was still possible.

In the experiment without initial sugar sources the strain AWRI 1499 made use of malic acid and ethanol concurrently from the onset of the experiment. Initially, between day 0 and day 10, the consumption was low, possibly due to consumption of the stored carbon compounds (i.e. glucose, glycogen and trehalose). Furthermore, from day 10, the consumption rate increased but, similarly to the experiment in which sugar was provided, neither malic acid nor ethanol was fully consumed by the *B. bruxellensis* strains. Furthermore, as stated above, these substrates may have been completely consumed had the experiment time been extended. It is interesting to note that 17 g/L of

ethanol was assimilated during the stationary phase in this experiment. This is exceptionally higher than the concentration of malic acid consumed. Furthermore, the ability of this yeast to assimilate so much ethanol with so little oxygen present (7 mg/L) is fascinating. Additionally, assimilation of ethanol could correspond with the increase in acetic acid as found in a study by Aguilar-Uscanga et al, (2007). Furthermore, the consumption of acetic acid occurred after both glucose and ethanol had been depleted in a study by Wijsman et al. (1984). This was not observed in any of the experiments as ethanol was never fully consumed.

There seems to be neither a significant production nor a significant consumption of glycerol by any of the *B. bruxellensis* strains. These results are contrary to those obtained in the plate assays as when supplied as a single carbon source under aerobic conditions glycerol was assimilated by the strains. This demonstrates that *B. bruxellensis* has the enzymatic machinery to utilise glycerol as a source of carbon but this process is possibly repressed when other carbon sources are present. Literature nevertheless confirms that *B. bruxellensis* has a low capacity for glycerol production (Aguilar-Uscanga et al., 2003; de Souza-Liberal et al., 2007; Blomqvist et al., 2010). This absence of glycerol production during sugar consumption may lead to an eventual redox imbalance in the yeast cell. This is compounded by its inability to reoxidise NADH to NAD^+, especially under anaerobic conditions, which occurs during the formation of glycerol in *S. cerevisiae*. When oxygen is present, certain other mechanisms could be utilised in order to reoxidise NADH, such as converting ethanol to acetate and eventually the citric acid cycle and the electron transport chain (Figure 3.6).

A release of acetic and succinic acids was also observed in AWRI 1499 but not IWBT Y121 (Figure 3.2 A and B). However, this only occurred under semi-anaerobic conditions, when oxygen was present. In a previous study by Freer. (2002) reporting a screening of *B. bruxellensis* strains for acetic acid production, the majority of the *Brettanomyces* spp. were found capable of producing acetic acid when either glucose or ethanol was used as a carbon/energy source. The formation of acetic acid in *B. bruxellensis* is strain dependent (Castro-Martinez et al., 2005; Rozpędowska et al., 2011), thereby making IWBT Y121 a low producing strain. Acetic acid was not consumed by any of the strains under both conditions (i.e. anaerobic and semi-anaerobic). This is contrary to the results on the plate trials under aerobic conditions as most strains could attain weak growth utilising acetic acid as a single source. It is possible that due to various other substrates being present that *B. bruxellensis* would initially assimilate these preferred compounds. It has been reported that once ethanol is completely metabolised acetic acid is then metabolised (Wijsman et al., 1984). Therefore, *B. bruxellensis* has the enzymatic machinery to assimilate acetic acid, but due to the presence of other preferential sources acetic acid like glycerol was not assimilated. Regarding the production of succinic acid, most is excreted by fermenting yeasts during the early stages of alcoholic fermentation (Heerde and Radler, 1978; Arikawa et al. 1999). Indeed, fermenting yeasts can produce succinic acid both during the exponential phase as well as during stationary phase (Heerde and Radler, 1978; Arikawa et al., 1999). Regarding the *B. bruxellensis* strains investigated in this study, AWRI 1499

produced more succinic acid than ISA 1649 and IWBT Y121 did not produce any. The production mainly occurred during stationary phase (Figure 3.2 A and B), in order to potentially produce ATP (Figure 3.7). Therefore, this could suggest that AWRI 1499 and to a lesser extent ISA 1649 require more energy during this growth phase when compared to IWBT Y121.

For the entire experiment period, the trend in conservation of carbon compounds is evident (Table 3.6). It should be noted that the table displays the balance of the carbon in the compounds, some of which are consumed while others are produced, in mol/L over the experimental period. In addition, the table shows that the compounds whose concentrations were determined in the study explained 66 - 99% carbon recovery, which is acceptable (Table 3.6). Furthermore, there are instances where the carbon recovery is not optimal; this may be due to the production of certain compounds that have not been taken into consideration. For example, certain stored compounds could be contained in the cell (e.g. stored sugar, pyruvate or acetaldehyde to name a few) as well as other polyols (e.g. ribitol and arabitol) necessary to protect the cell from stress (Vigentini et al., 2013). Indeed, the production of polyols could be a possible mechanism that replaces the production of glycerol in *B. bruxellensis*.

Volatile phenol production is linked to growth (Figure 3.4) and occurred mostly towards the end of exponential phase and into stationary phase, as previously observed (Barata et al., 2008). At this physiological stage, the sugars have been metabolised, leading to a redox imbalance as the sugar is metabolised into ethanol and biomass. Therefore, NAD^+ should be regenerated in order to allow for the maintenance of cell functioning until day 45 which occurs in all strains. Potentially, this could be achieved by production of these volatile phenols which is characteristic of *B. bruxellensis*. The formation of 4-EP in the three strains is displayed in Figure 3.4. The strains produced 4-EP that follows the trend of the population's growth. There is an initial exponential production of 4-EP followed by a slower rate of production during the stationary phase. ISA 1649 and IWBT Y121 produce 4-EP, synchronously with exponential growth, from the onset of the experiment. AWRI 1499 produced a lower concentration at the beginning of exponential phase (Day 0-10) and only produced higher concentrations from day 10 to the end of the experiment period as the end of its exponential phase is after day 10 (Figure 3.4), due to its slower growth kinetics. All strains fall under the category of medium 4-EP producers falling between 1000 and 2000 µg/L (Conterno et al., 2006). This is a significant amount of 4-EP which could lead to a balance in the redox potential of the cell by regenerating NAD^+. In order to test this hypothesis, the experiment to test the redox balancing ability of the *B. bruxellensis* strain AWRI 1499 was implemented (see paragraph 3.4.4, below).

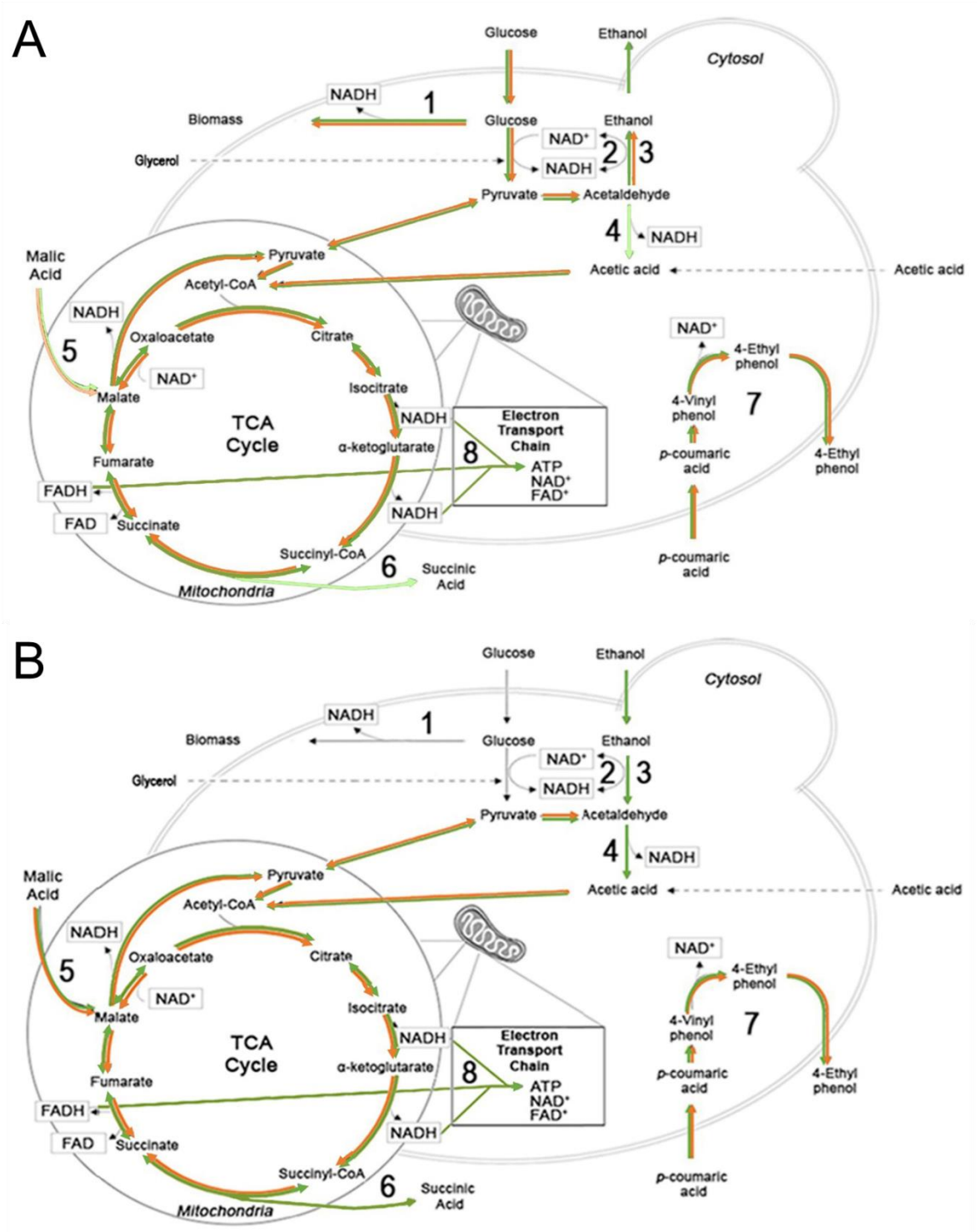

Figure 3.7: The various metabolic pathways occurring in both liquid and plate experiments during A) exponential and B) stationary phase, in *B. bruxellensis.* 1) The production of biomass from glucose simplistically represented as several pathways are involved. 2) Glucose is converted into ethanol during alcoholic fermentation, a redox neutral reaction. 3) Ethanol can be assimilated into acetic acid and conducted through the TCA cycle and eventually the electron transport chain when oxygen is present. 4) Formation of acetic acid which can also originate from Acetyl-CoA. 5) The utilisation of malic acid can occur either under aerobic or anaerobic conditions. 6) Formation of the metabolite succinic acid. 7) The formation of 4-EP, a volatile phenol, as a proposed means to regenerate NAD^+. 8) The final products of the electron transport chain namely ATP, NAD^+ and FAD^+. The green arrows represent the metabolic pathways that are active in the presence of oxygen (semi-anaerobic and aerobic conditions). Orange arrows represent the metabolic pathways that occur under anaerobic conditions. Lighter shades of green or orange represent poorly produced/consumed compounds. Dashed lines represent the carbon metabolism by *B. bruxellensis* when supplied as a single source. Adapted from Smith and Divol et al., 2016.

3.4.3 Nitrogen consumption in the *B. bruxellensis* strains

The formation of biomass is related to initial YAN but the quantitative relationship depends on many factors, including yeast demand for nitrogen, type of nitrogen source and other factors (e.g. temperature of fermentation). In this study, the initial YAN was calculated at 120 mg N/L, at the onset of the experiment. This is well above the needs of *B. bruxellensis* as previously reported in literature (Childs et al., 2015). However, as the medium was proposed from concentrations reviewed in literature, it is representative of what is present in wine.

It has been proposed that organic nitrogen compounds that are metabolized by yeasts may ultimately yield three metabolic outcomes. Firstly, they may be incorporated without alteration as amino acids into proteins. Secondly, the carbon skeleton of the organic nitrogen compound may be used by the cell for synthetic purposes and may act as a carbon source. Finally, they may be deaminated to produce ammonia, transaminated or assimilated by the Ehrlich pathway to form fusel alcohols (Hazelwood et al., 2008). For the purpose of this discussion a focus will be placed on the latter of these eventualities. The uptake of nitrogen sources, in *S. cerevisiae*, is facilitated by a large number of permeases which are controlled by two major regulatory mechanisms either SPS-mediated control of specific permeases or Nitrogen catabolic repression (NCR) mediated. The SPS-mediated permeases are controlled by a plasma membrane sensor, which is made up of three core proteins (Ssy1p, Ptr3d and Ssy5p). Furthermore, these proteins are responsible for externally sensing amino acids and regulate the transport of certain amino acids (Forsberg et al., 2001). In a study by Crépin et al. (2012) on *S. cerevisiae*, most nitrogenous sources were assimilated sequentially according to preference: the initial sources assimilated were SPS-mediated (i.e. lysine, aspartic acid, threonine, leucine, methionine and serine). In this study, *B. bruxellensis* strains acted differently in this regard and initially utilised (Day 0-10) sources that are both the SPS and NCR-mediated (i.e. aspartic acid, serine, arginine, leucine, lysine, ammonia and proline). Therefore, it is possible that *B. bruxellensis* has a different regulatory mechanism with regard to nitrogen source assimilation.

In wine, nitrogenous sources are limited when compared to those present in grape juice. Indeed, two major nitrogenous sources generally make up the majority of the total nitrogen available in wine, namely ammonia and the amino acid proline. Next in abundance is arginine but its concentration is low (0.04-0.1 g/L), when compared to ammonia and proline (Lehtonen, 1996). Ammonia is generally present from diammonium phosphate (DAP) additions at the beginning of the AF, and if unutilised by the yeasts during AF could be available. It has been reported in literature that proline is only utilised when it is a sole source of nitrogen by *S. cerevisiae* and is non-preferred, therefore it is available as another source for *B. bruxellensis* (Perpete et al., 2005; Crépin et al., 2012). In this study, the consumption of arginine was between 0.49-4.47 mg N/L between day 0-10 in all *B. bruxellensis* strains (Table 3.7). This is consistent with the results obtained under anaerobic conditions in the plate trials (Table 3.5). Ammonia was greatly consumed in liquid trials, however the use of ammonia as a sole nitrogen source resulted in weak growth in the plate trials under both

aerobic and anaerobic conditions. This could possibly be due to the fact that the plates were only incubated for seven days. However, regarding the exponential stage the consumption of ammonia provided the highest concentration of nitrogen for growth and biomass production. Proline was released in the liquid trials between day 0 and day 10, however in the plate trials it was assimilated moderately to strongly for growth. Therefore, proline may be used as a sole nitrogen source but may also be utilised in other capacities. Ammonia and proline are more abundant than the other amino acids in the SWM, which make these sources of prime importance to *B. bruxellensis*. In a study by Conterno et al. (2006), all isolates grew utilising ammonium, proline, and arginine as a nitrogen source similar to the results obtained in this study. During biomass production, the strains released proline (2-15% production); this phenomenon is often initially observed following arginine metabolism in *Saccharomyces* spp. and other yeast species (Large, 1986, Ingledew et al., 1987, Ough et al., 1991). During stationary phase, proline was assimilated regardless of the presence of oxygen which is necessary for the catabolism of proline. Therefore, proline may not be involved strictly as a nitrogen source used for growth. It may act to counteract stressors acting on the cell, for example ethanol, acetic acid and osmotic stress. As it has been found that proline counteracts ethanol and acetic acid stress, desiccation and the effect of peroxide in *S. cerevisiae* (Takagi et al., 2005; Takagi, 2008; Greetham et al., 2014). Furthermore, when considering the acetic acid (±2 g/L) and ethanol (±80 g/L) concentrations during stationary phase, which are incredibly elevated. It is plausible that these stressors could averted by the assimilation of proline during the stationary phase.

Arginine, aspartic acid, glutamic acid, threonine and lysine represent the amino acids assimilated first in *S. cerevisiae* (Perpete et al., 2005) and also supported the anaerobic growth of a *B. bruxellensis* strain in a study by Blomqvist, (2012). Similar results were obtained in the strains and conditions utilised in this study, with the exception of threonine. Furthermore, in the same study by Blomqvist, (2012), arginine, lysine and, to some extent, methionine and glutamic acid, were found to support growth. Leucine (80-88% in AWRI 1499 and 50-70% in ISA 1649 under both oxygen conditions) and lysine (91-94% in AWRI 1499 and 90% in ISA 1649 under both oxygen conditions) were strongly assimilated, with the exception of IWBT Y121 (leucine, 8% and lysine, 11-17%) (see Table SD1 of Appendix A). Indeed, it has previously been proposed that these sources along with isoleucine and valine are utilised along with volatile phenol production (Oelofse, 2008; Grbin, 1998). It is noteworthy that leucine and lysine were only consumed (day 0-10 in ISA 1649 and IWBT Y121 and day 10-45 in AWRI 1499) when 4-EP was produced, thereby confirming this link (Figure 3.4).

The aromatic amino acids, phenylalanine (<0.5 mg N/L) and tyrosine (<0.2 mg N/L) were moderately to weakly assimilated, respectively. Furthermore, these amino acids also form part of the hydrophobic amino acids which additionally contain glycine, alanine, valine, isoleucine, proline, phenylalanine and methionine which were poorly assimilated and even sometimes released, apart from methionine which varies between strains and conditions in question. *B. bruxellensis* could be unable to assimilate these hydrophobic amino acids easily. The polar amino acids resulted in diverse

uptake percentages: asparagine and threonine were not utilised (0.1-2 mg N/L produced), tyrosine was consumed weakly under most conditions and trends regarding histidine, serine, methionine and tryptophan varied between strains and condition (i.e. anaerobic/semi-anaerobic).

It has been reported that γ-aminobutyric acid (GABA) is transported into yeast cells is assimilated through the GABA pathway. In the first step of this pathway, the GABA transaminase catalyses the transfer of the amine group from GABA to α-ketoglutarate (α-KG), producing glutamate. This reaction generates succinate semi-aldehyde, which is further oxidized into succinate leading to a regeneration of energy in the form of ATP or NAD^+ (Bach et al., 2009). It is intriguing as with regard to the strains tested, AWRI 1499 assimilated a net (41-64%, equating to 1.1-1.7 mg N/L) moderate to a high percentage of the GABA that was initially supplied. In comparison, ISA 1649 (9-38% net production) and IWBT Y121 (15-26% net production) did not utilise GABA, and in fact even produced this amino acid (Table 3.7 and Table SD1, Appendix A). This would suggest a stronger need of ATP and/or NAD^+ in the AWRI 1499 strain.

Aspartic acid was assimilated under most conditions (Table 3.7) and is most likely synthesised to form glutamic acid, via the TCA cycle, which is itself taken up in order to facilitate anaerobic growth. Cystine is involved in cysteine and glutathione metabolism. In the AWRI 1499 strain and the ISA 1649 strain, it is moderately assimilated (43-62% net consumption). However, contrary to this, the IWBT Y121 strain produced between 9 and 11% (Table SD1, Appendix A). Furthermore, more methionine, which also contains sulphur, was also assimilated by both AWRI 1499 and ISA 1649. This could suggest a higher need for the sulphur, contained in cysteine, in AWRI 1499 and ISA 1649. Hydroxyproline is an intermediate formed when proline is assimilated to glutamic acid in *S. cerevisiae*. Therefore, the initial production and subsequent assimilation of this compound may be due to the conversion of proline into glutamic acid. This can further be utilised to biochemically produce an α-ketoacid and ammonium an easily assimilated nitrogen source. Nitrate was a preferred as a single nitrogen source in the plate screening however, could not be measured in the liquid media due to other compounds interfering with the nitrate quantification methods attempted.

It is interesting to note that the initial total available nitrogen of 120 mg N/L was not fully assimilated by the *B. bruxellensis* strains unlike what is typically observed in *S. cerevisiae* (Crépin et al., 2012). It is possible that due to the slow growth of *B. bruxellensis* the total consumption of these compounds would not be observed in this short time frame. In addition, AWRI 1499 and ISA 1649 consumed more nitrogen sources strongly when compared to IWBT Y121. This suggests a lesser need for nitrogen sources by the IWBT Y121 strain in comparison to AWRI 1499 and ISA 1649. Furthermore, globally *B. bruxellensis* did not require as much nitrogen and seems to efficiently utilise whatever is available.

3.4.4 Redox balancing in *B. bruxellensis* strain AWRI 1499

In order to determine the efficiency of the *B. bruxellensis* strain AWRI 1499 and its ability to correct any potential redox imbalance that could occur, a further experiment was conducted. The results are outlined in Figures 3.5 and 3.6. The medium was devoid of sugars. However, certain stored carbon sources as discussed above were likely present in the cells that were inoculated as the same pre-culture procedure was followed. This experiment was conducted for a longer period. In addition to the trends observed in Figure 3.2 B, under semi-anaerobic conditions, the consumption of malic acid by this strain seemed to taper off after day 45 and was not fully consumed (0.6 g/L is recorded from day 42 until the experiment was terminated). Furthermore, ethanol was further consumed, under the same conditions, possibly to regenerate NAD^+ through the electron transport chain (Figure 3.6). The production of acetic acid seems to plateau as malic acid is no longer utilised. Under semi-anaerobic conditions, a decline in culturable cells occurred after day 45, regardless of the presence of *p*-coumaric acid, possibly due to the strong NADH imbalance (Table 3.8). This phenomenon was not observed in the initial experiment, since day 45 was the endpoint of the initial experiment. Again the presence of minimal amounts of O_2 (semi-anaerobic conditions), allowed for the metabolism of ethanol in *B. bruxellensis*. The result of this metabolism could have led to an increased NADH concentration that could not be reoxidised as not enough oxygen was available to activate the electron transport chain after a longer period of time. This could potentially explain the decline in the population observed as no NAD^+ is present to maintain the population. Furthermore, neither the consumption of *p*-coumaric acid nor ethanol under semi-anaerobic conditions allowed for the maintenance of cell viability (Table 3.8). Conversely, prolonged cell viability was observed under anaerobic conditions with and without *p*-coumaric acid (Figure 3.5 A and C). Under these conditions, the absence of oxygen did not allow for ethanol to be utilised. Interestingly, the absence of ethanol degradation seems to have maintained the redox balance (Table 3.8) and therefore favour *B. bruxellensis*'s survival.

AWRI 1499 produced a similar amount of 4-EP under both anaerobic conditions and semi-anaerobic conditions (2.2-2.4 mg/L). Over the additional time of this experiment more 4-EP was produced between day 45 and day 68, when compared to the initial experiment. As expected, no 4-EP was produced in the medium lacking *p*-coumaric acid. 4-EP production seems to be independent with regard to the presence/absence of oxygen. The production of 4-EP did not seem to have a significant effect on correcting the redox balance (Table 3.8). However, a certain amount of NAD^+ was regenerated by the production of 4-EP which could be utilised in conjunction with other pathways to further regenerate NAD^+. The NAD^+/NADH equivalent production was calculated in order to determine the redox balance between certain time points during the experiment and outline the NAD^+ recovery in each situation (Table 3.8). In the table regarding the anaerobic conditions, NAD^+ is present after each metabolic pathway is taken into account. Furthermore, the maintenance of cell culturability is observed until the end of the experiment under anaerobic conditions (Figure 3.5 A and

C). Under semi-anaerobic conditions however, no NAD^+ is recovered, as it is mostly in its reduced form NADH (Table 3.8). The net NADH concentration available under the semi-anaerobic conditions without *p*-coumaric acid is more than the concentration present when *p*-coumaric acid is present. Therefore, some NAD^+ can be regenerated when *p*-coumaric acid is present but this does not maintain the cell viability. It is interesting to note that the semi-anaerobic conditions allowed for a vast imbalance of the redox potential as the availability of oxygen lead to the consumption of more malic acid and ethanol when compared to the anaerobic conditions. Therefore, the imbalance of the redox potential of the cell can be observed in decline of the population under semi-anaerobic conditions. With regard to the higher alcohols and fatty acids tested under both anaerobic and semi anaerobic conditions, the higher alcohol concentration is higher than the fatty acid concentration. However, this production of both higher alcohols and fatty acids was similar in each variation of this experiment. Seemingly, this reflects a need for more NAD^+ as the higher alcohols concentration was higher than the fatty acid concentration leading to more NAD^+ availability. Ultimately, the total production of higher alcohols therefore could have marginally aided in the correction of the redox balance and maintenance of the NAD^+ levels. Therefore, this is not a sole mechanism, but could be one mechanism utilised synchronously with many others, in order for *B. bruxellensis* to restore the redox potential. Of the higher alcohols tested, isoamyl alcohol had an average concentration of 6 mg/L (Appendix A, Table S2) which suggests that most of the leucine initially added (9 mg/L) to the medium was utilised to form Isoamyl alcohol. Indeed, AWRI 1499 was observed to strongly consume leucine (88-80%).

3.5 Conclusion

B. bruxellensis is a common contaminant found in wine, its survival in this medium is intriguing and somewhat perplexing. From this study, it has been found that *B. bruxellensis* strains survive efficiently utilising numerous sources in order to maintain cell viability and grow in a synthetic wine medium. Regarding the carbon sources tested in a synthetic wine medium it is observed that the strains fully utilise sugars (glucose, fructose and mannose) and then subsequently utilise alternative carbon sources concurrently but very slowly, for cell maintenance. No significant differences were observed when anaerobic conditions were compared to semi-anaerobic conditions, with the exception of ethanol that cannot be assimilated under the former conditions. Interestingly, ethanol assimilation in the presence of small amounts of dissolved oxygen leads to a strong redox imbalance that ultimately leads to slow cell death. Carbon source utilisation was remarkably conserved between strains. The nitrogen preferences were found to be a lot more variable with noticeable differences between strains, with the exception of ammonium and arginine. Various nitrogen sources were utilised but never completely assimilated or consumed. The strain AWRI 1499 seemed to assimilate more nitrogen sources, whereas the strains ISA 1649 and IWBT Y121 only utilised the nitrogen sources weakly. Proline assimilation in the *B. bruxellensis* strains during stationary phase was possibly not utilised as a nitrogen source but as a stress alleviator, as similar observations have

been reported in *S. cerevisiae* and certain bacteria. It was initially proposed that the production of 4-EP could potentially be a mechanism which allows *B. bruxellensis* to survive and maintain its population by balancing the redox potential of the cell. The data confirms that *p*-coumaric acid, contributes to the balance of the redox potential of the cell, but that it is not the only mechanism. Indeed, the regeneration of NAD^+ from *p*-coumaric acid did not allow for continued cell viability. Future studies could utilise a metabolomic approach to determine the compounds through which *B. bruxellensis* maintains its redox potential. Furthermore, the addition of small amounts of oxygen seemed to accelerate the decline of the population and could possibly be detrimental to *B. bruxellensis*. Therefore, the effect of oxygen on survival and cell viability should be investigated.

3.6 Acknowledgements

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3.7 References

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Appendix A: Supplementary material

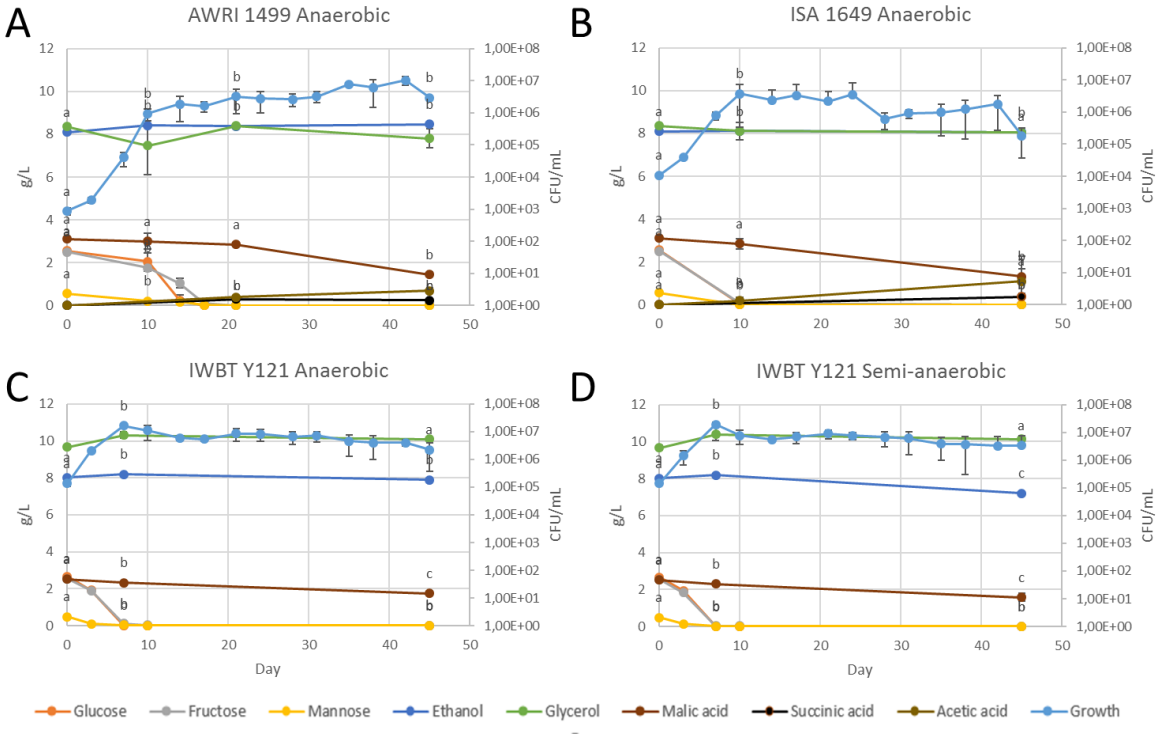

Figure S1: The growth kinetics of AWRI 1499 under anaerobic conditions (A), ISA 1649 under anaerobic conditions (B), and IWBT Y121 under anaerobic (C) and semi anaerobic conditions (D) over the defined growth period. The various metabolites produced or consumed are displayed on the primary vertical axis in g/l, whereas CFU/mL are displayed on the secondary vertical axis. Values with the same letter for the same compound are statistically similar when compared with Tukey's HSD post-hoc test at 95 % confidence level.

Table S1: The percentage nitrogen consumption by each *B. bruxellensis* strain under all conditions. A strong consumption (Green) characterises a consumption of between 60-100% nitrogen consumed, intermediate (Orange) 30-60% and weak (Red) is between 0-30% nitrogen consumed. The non-utilised indication (Grey) outlines sources either not utilised or released/produced between the different time points.

Strain	Initial amount	AWRI1499			AWRI1499			ISA 1649			ISA 1649			IWBT Y121			IWBT Y121		
Condition	(mg N/l)	Anaerobic			Semi-anaerobic			Anaerobic			Semi-anaerobic			Anaerobic			Semi-anaerobic		
Day		0-10	10-45	0-45	0-10	10-45	0-45	0-10	10-45	0-45	0-10	10-45	0-45	0-10	10-45	0-45	0-10	10-45	0-45
Ammonia	88.04	-0.16	36.82	36.66	20.31	31.33	51.63	32.46	33.89	66.36	23.16	31.23	54.39	30.35	1.50	31.85	27.96	5.47	33.42
Asparagine	2.90	-6.66	-65.59	-72.25	-20.13	-85.77	-105.91	-18.92	-17.03	-35.94	-17.50	-58.28	-75.77	-25.70	-24.72	-50.42	-29.77	-27.93	-57.70
Glycine	1.35	-46.03	1.12	-44.90	-44.57	-88.89	-133.46	-54.60	-20.86	-75.45	-54.82	-34.43	-89.25	-38.81	-20.40	-59.20	-47.01	-25.70	-72.71
Threonine	1.46	-2.29	-4.30	-6.59	-5.87	0.78	-5.09	-18.96	17.57	-1.39	-20.82	-4.84	-25.66	-26.73	-10.11	-36.84	-29.22	-6.07	-35.28
Valine	1.66	-10.44	-0.51	-10.95	-12.36	-6.45	-18.81	-7.31	-1.34	-8.64	-7.91	-5.88	-13.79	-5.89	1.11	-4.77	-1.91	2.02	0.10
Hydroxyproline	0.60	-146.52	96.63	-49.89	-207.90	34.75	-173.15	-58.04	-6.48	-64.52	-91.73	-70.37	-162.10	14.51	0.44	14.96	-43.15	58.04	14.90
Alanine	6.15	-5.58	16.73	11.14	-12.66	17.63	4.97	-31.88	30.92	-0.97	-41.88	5.64	-36.24	-24.33	6.46	-17.87	-23.68	13.41	-10.27
Isoleucine	1.16	-4.18	6.48	2.30	-5.84	14.64	8.80	3.61	24.65	28.25	-3.35	1.79	-1.56	5.24	-7.14	-1.90	-1.73	-9.56	-11.29
γ-Aminobutyric acid	2.68	-12.15	54.10	41.96	5.15	59.55	64.70	-15.25	-23.21	-38.47	-6.91	-2.17	-9.09	-11.97	-3.24	-15.21	-24.42	-2.30	-26.73
Phenylalanine	1.39	-3.70	32.29	28.59	-1.71	35.64	33.94	19.50	35.15	54.64	7.17	20.34	27.51	17.42	-4.37	13.05	21.45	1.42	22.86
Aspartic acid	1.90	28.28	46.04	74.32	47.81	31.91	79.71	37.48	15.18	52.65	39.21	10.71	49.92	59.46	0.63	60.09	60.22	4.14	64.35
Glutamic acid	2.57	7.53	21.62	29.15	15.04	21.17	36.20	3.46	19.83	23.29	0.96	4.47	5.43	-4.55	-12.58	-17.12	-9.52	-25.51	-35.03
Serine	1.94	5.39	69.73	75.12	16.45	40.65	57.10	26.57	23.87	50.44	22.20	12.69	34.88	17.52	11.21	28.73	16.04	12.57	28.61
Histidine	0.94	-21.43	90.11	68.68	-2.87	61.82	58.95	48.55	19.25	67.80	30.56	12.05	42.61	20.56	-1.86	18.71	17.60	0.68	18.27
Arginine	4.56	10.66	86.09	96.75	50.24	40.41	90.65	93.66	-0.17	93.49	97.89	-3.73	94.16	96.61	-0.43	96.18	96.78	0.21	96.98
Tyrosine	0.54	-5.74	16.49	10.75	-2.91	26.28	23.37	2.91	-18.38	-15.47	-1.20	3.59	2.39	6.67	-2.34	4.33	7.21	2.68	9.89
Cysteine	1.56	-30.74	92.81	62.06	12.29	31.41	43.70	50.70	-0.36	50.34	44.15	-0.57	43.57	-5.63	-4.32	-9.95	-6.07	-5.84	-11.90
Methionine	0.38	31.19	4.27	35.47	51.79	3.86	55.65	74.05	-1.75	72.30	74.91	-3.28	71.63	47.24	-29.94	17.30	37.96	-19.31	18.65
Tryptophan	0.22	27.67	37.91	65.58	43.89	20.86	64.74	42.38	14.26	56.63	32.23	29.16	61.39	33.53	15.89	49.42	39.28	3.60	42.88
Ornithine	2.36	-3.70	80.26	76.56	6.12	-23.07	-16.95	-19.69	-25.44	-45.13	-16.44	-128.11	-144.55	19.96	11.60	31.55	20.44	12.03	32.47
Leucine	2.55	-1.33	89.42	88.09	-2.15	82.64	80.49	22.60	49.33	71.93	7.94	45.91	53.85	8.16	-0.05	8.12	9.93	-2.23	7.69
Lysine	6.20	24.09	70.40	94.49	57.79	33.38	91.17	87.88	2.45	90.33	85.20	5.08	90.28	17.55	0.21	17.76	17.56	-6.46	11.10
Proline	54.08	-8.40	15.58	7.19	-3.67	25.57	21.90	-15.29	44.35	29.07	-15.21	36.50	21.29	-7.47	14.64	7.17	-1.61	15.72	14.11

Table S2: Concentrations of major volatile compounds (mg/L) for the redox balancing experiment in *B. bruxellensis* strain AWRI 1499. Anaerobic and semi-anaerobic conditions are displayed in the table. The values represented were quantified when the experiment was terminated at day 68.

	Compound	Anaerobic with p-coumaric acid	Anaerobic without p-coumaric acid	Semi-anaerobic with p-coumaric acid	Semi-anaerobic without p-coumaric acid
Higher alcohols	Isobutanol	1.36	0.00	1.35	1.41
	Isoamyl alcohol	6.13	5.65	7.02	6.96
	2-Phenyl Ethanol	1.45	1.04	1.99	2.80
	2-Phenylethyl Acetate	0.36	0.36	0.37	0.37
	Total Higher alcohols	**9.29**	**7.06**	**10.73**	**11.55**
Fatty Acid	Propionic Acid	0.54	0.31	1.20	0.99
	Isobutyric acid	0.48	0.42	0.75	0.83
	Butyric Acid	0.36	0.21	0.52	0.26
	Iso-Valeric Acid	0.62	0.48	1.04	1.09
	ethyl phenylacetate	0.10	0.00	0.12	0.11
	Total Fatty acids	**2.09**	**1.41**	**3.64**	**3.29**

Chapter 4

General discussion and conclusions

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4.1 General discussion and conclusion

The spoilage wine yeast *Brettanomyces bruxellensis* is one of very few yeasts able to survive in wine (Fugelsang, 1998). Indeed, *B. bruxellensis* has been isolated/cultured from wines that are decades old (Arvik and Henick-Kling, 2002). The ability of this yeast to survive in such a nutrient-deficient medium is intriguing, especially since its biological characteristics are similar to those of its distant relative *Saccharomyces cerevisiae* which cannot survive in wine. Unlike *S. cerevisiae,* which dominates the initial alcoholic fermentation of grape juice, *B. bruxellensis* is well adapted to survival in wine (Oelofse et al., 2008). This is most probably due to its ability to utilise a vast array of substrates (i.e. ethanol and malic acid) (Conterno et al., 2006; Smith and Divol, 2016). It is also an exceptionally slow growing yeast, even in rich culture media, (compared to most yeast species) and utilises nutrients sparingly over a long period of time (Fugelsang et al., 1993; Steensels et al., 2015), an adaptation that seems to allow it to prevail in such a medium and endure unfavourable conditions such as the high SO_2 concentrations added to wine. Certain strains have also been reported as resistant to the antimicrobial agent SO_2 (Van der Walt and Van Kerken, 1961; Froudière and Larue, 1988).

In this study, it was shown that *B. bruxellensis* is able to utilise various substrates for different purposes. Interestingly, *B. bruxellensis* is able to store various carbon sources (i.e. in the forms of glucose, glycogen and trehalose) that can be utilised when preferential compounds are scarce in the medium. The initial use of sugars provided a means for growth and the production of ethanol concurrently. Subsequently, the slow utilisation of ethanol (in the presence of oxygen, as minimal as it may be) and malic acid, concurrently allowed for the maintenance of the cell numbers over a long stationary phase. Therefore, it would be advantageous for winemakers to ensure that the alcoholic fermentation by *S. cerevisiae* exhausts all possible sugar sources, in order to suppress the formation of biomass in *B. bruxellensis*. Subsequently, the dead *S. cerevisiae* cells should be removed timeously by racking, to reduce the compounds released during autolysis (i.e. stored sugars, mannoproteins and certain amino acids). Secondly, the malolactic fermentation should run to completion and exhaust all malic acid present, to ensure no residues occur for cell maintenance in *B. bruxellensis*. Furthermore, *B. bruxellensis* was able to utilise other compounds such as glycerol and acetic acid when provided as a sole carbon source on plates. Therefore, it could potentially utilise these sources upon complete consumption of the initially assimilated sources. However, this could take a considerable amount of time, potentially decades, to fully utilise all ethanol present.

Of the nitrogen sources available in wine, the dominant and strongly assimilated sources include, ammonia, proline and to a lesser extent arginine and other amino acids. These nitrogen sources aided in growth (ammonia and arginine) and possibly provided protection from stressors (proline)

that occur in wine i.e. ethanol and acetic acid stress. The presence of ammonia in wine is dependent on many factors but most importantly would be subjected to the amount of Diammonium Phosphate (DAP) added during the fermentation and the amount consumed by *S. cerevisiae*. Therefore, in order to discourage the growth and survival of *B. bruxellensis* nitrogen enhancing products such as DAP and complex yeast-derived nitrogen sources should be utilised sparingly, especially towards the end of alcoholic fermentation, to avoid excess residual nitrogen. Furthermore, products such a yeast hulls should also be added to fermenting wine with care, as these products contain various amino acids in varying quantities. An overdose of such products could lead to excess amounts of amino acids being present which can allow for growth and the protection of *B. bruxellensis* cells from possible stressors.

An attempt was initiated to determine the metabolic mechanisms utilised by *B. bruxellensis* to maintain the redox potential in the cell. The formation of higher alcohols and the volatile phenol 4-Ethylphenol (4-EP) aided in partially correcting any imbalance in the cell, but it cannot be considered sufficient. Therefore, more mechanisms are possibly utilised in order to allow for the maintenance of the population over decades. The presence of oxygen in this study did not significantly affect the growth kinetics of the strains tested. Interestingly, the provided oxygen, equating to a minimal amount, taken up by the medium during bottling, racking or ageing in a barrel was sufficient to allow for ethanol assimilation by *B. bruxellensis*. However, this consumption of ethanol induced an increase in the redox imbalance in the cell, which ultimately caused a slow but irrevocable decline in the population. Furthermore, 4-EP was still produced therefore the oxygenation of wine is not encouraged to cause a decline in the *B. bruxellensis* population. Therefore, in order to combat the maintenance of a *B. bruxellensis* population, through the assimilation of ethanol and subsequent production of acetic acid, winemaking practices should avoid the uptake of oxygen in the wine. This can be facilitated by fewer rackings and the use of CO_2 when bottling, racking or filling barrels and avoiding the introduction of oxygen when pumping wine.

In summary, this study shows that the spoilage caused by *B. bruxellensis* is inevitable if conditions favouring their proliferation are optimal. However, there are a few preventive measures that can be considered, as mentioned above. Furthermore, the control of this unwanted and potentially harmful microorganism requires an integrated approach. Indeed, the use of SO_2 is a common practice by winemakers. However, this yeast has adapted to survival in the harsh medium that is wine and is able to prevail under these conditions and enumerate when conditions are favourable. Therefore, the use of SO_2 alone is not sufficient. Furthermore, certain strains have adapted a resistance to SO_2. Therefore, an unfavourable environment should be established, using winemaking practices. This environment should be devoid of potentially assimilable carbon and nitrogen sources. The addition of oxygen to the medium should be minimal and if possible avoided during winemaking practices.

4.2 Limitations of the study

Certain limitations were outlined during the progress of this study. Firstly, the synthetic wine medium (SWM) utilised, for both anaerobic and semi-anaerobic experiments, contained anaerobic factors (i.e. Tween 80 and ergosterol). The presence of these anaerobic factors seem to have caused similar results in certain experiments. Furthermore, the SWM was very complex and caused interference when testing certain compounds e.g. nitrates and nitrites whose concentrations could therefore not be determined. In addition, certain compounds such as CO_2 release and the quantification of certain yet-to-identify metabolites were not quantified which could have aided in recovering the carbon flux in the yeast (e.g. pyruvate and acetaldehyde).

4.3 Potential future studies

Future research should focus on identifying a greater array of compounds produced and potentially stored in *B. bruxellensis*. Furthermore, a metabolomic approach to determine the metabolic pathways through which *B. bruxellensis* maintains its redox potential should be further investigated, testing as many compounds as possible (or even maybe using an untargeted approach). Indeed, certain mechanisms were outlined in this study, however certain compounds were missed and flux of compounds were not fully recovered. It is proposed that polyol formation could be a replacement of glycerol in *B. bruxellensis* to counteract stressors and possibly regenerate NAD^+ in the cell. Therefore, a future study could test the production of polyols in *B. bruxellensis* strains as well as their effect on the cell. One could investigate the role of proline to confirm its potential as a stress-alleviator. Finally, the efficiency of nutrient utilisation in terms of growth kinetics and cellular maintenance of *B. bruxellensis* should be compared to that of *S. cerevisiae*, in minimal media, as the latter could not utilise minimal amounts of nutrients in order to survive in the same environment.

4.4 References

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