Metabolic, genetic and physiological responses to SO_2 exposure and nutrient-limiting conditions in *Brettanomyces bruxellensis*

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Declaration

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Summary

Brettanomyces bruxellensis has become of increasing interest over the past few decades yet this complex red wine spoilage yeast is still poorly understood and strain variance also leads to the contradictory results reported in literature. This yeast is responsible for the production of phenolic compounds, associated with off-flavours that render wine unpalatable. Sulphur dioxide (SO_2) is the most commonly used antioxidant and antimicrobial preservative instrumental in the control of spoilage yeasts such as *B. bruxellensis*. However, its diploid/triploid genome is enriched for genes that provide the yeast a fortuitous advantage, under conditions permissive for growth, with genotype-dependent SO_2 tolerance phenotypes observed among numerous strains.

This study investigates the metabolic, physiological and genetic responses associated with SO_2 exposure. It also explores the environmental cues responsible for the onset of non-SO_2 induced morphological characteristics. These morphological characteristics were investigated using fluorescent probes and microscopy in the presence of SO_2 and in the absence thereof, in YPD media. Pseudohyphae formation was observed to be a highly strain dependent feature and less pronounced in the presence of 0.6 mg/L molecular SO_2. This study also reports on the metabolic response observed over a 3-week period, following exposure to SO_2, in a synthetic wine medium. The following metabolites were consistently monitored during the course of the experiment: acetic acid, acetaldehyde, D-glucose and D-fructose. Utilization of sugars was retarded in the presence of SO_2 for up to 10 days in the presence of 1.2 mg/L molecular SO_2 and overproduction of acetaldehyde was prominent, with a peak at day 10. The study further highlights the expression profiles observed for the *SSU1* gene (referring to SO_2 tolerance) and the *PAD* gene (referring to production of volatile compounds) under SO_2 induced conditions in SWM, using qRT-PCR. The co-involvement of increased acetaldehyde production and elevated gene expression were indicative of *B. bruxellensis* yeast adapting to the presence of molecular SO_2, allowing survival of this fascinating yeast. Sequencing of the *SSU1* and *PAD* genes suggests the probable existence of different alleles of these genes that could explicate SO_2 tolerance and phenolic compound production associated differences among strains of this species.

Opsomming

Hoewel *Brettanomyces bruxellensis* oor die afgelope paar dekades toenemende belangstelling gewek het, word hierdie komplekse rooiwynbederfgis steeds swak verstaan en lei rasvariasie ook tot teenstrydige resultate in die literatuur. Hierdie gis is verantwoordelik vir die produksie van fenoliese verbindings, wat geassosieer word met afgeure, wat die wyn onsmaaklik laat. Swaweldioksied (SO_2) is die algemeenste preserveermiddel wat, weens antioksidant- en antimikrobiese eienskappe, instrumenteel in die beheer van bederforganismes, soos *B. bruxellensis,* gebruik word. Nogtans is die diploïede/triploïede genoom vir gene verryk, wat die gis 'n toevallige voordeel bied tydens ongunstige toestande, met genotipe-afhanklike SO_2 weerstandbiedende fenotipes wat onder verskeie rasse waargeneem word.

Hierdie studie ondersoek die metaboliese, fisologiese en genetiese reaksies tydens SO_2-blootstelling. Dit bestudeer verder die omgewingsleidrade wat vir die aanvang van die nie-SO_2 geassosiseerde morfologiese eienskappe verantwoordelik is. Hierdie morfologiese eienskappe is ondersoek met behulp van fluoresserende bakens en mikroskopie in die teenwoordigheid van molekulêre SO_2 en, in die afwesigheid daarvan, in YPD-medium. Pseudohyphae-vorming is as 'n baie rasspesifieke eienskap waargeneem en is minder prominent in die teenwoordigheid van molekulêre SO_2. Hierdie studie rappoteer ook oor die metaboliese reaksies waargeneem oor 'n 3-weke tydperk, na blootstelling aan SO_2, in 'n sintetiese wynmedium. Die volgende metaboliete was voordurend gemonitor tydens die verloop van die eksperiment: asynsuur, asetaldehied, D-glukose en D-fruktose. Benutting van die suikers is in die teenwoordigheid van SO_2 vertraag en oorproduksie van asetaldehied is prominent waargeneem. Hierdie studie beklemtoon verder die uitdrukkingsprofiele vir die *SSU1*-geen (verwys na SO_2-weerstandbiedendheid) en die *PAD*-geen (verwys na die produksie van vlugtige verbindings) in SO_2-geïnduseerde toestande in SWM, met behulp van qRT-PCR. Die gesamentlike invloed van beide verhoogde asetaldehied produksie en verhoogde uitdrukking van gene, was beduidend van *B. bruxellensis*-gis wat aanpas in die teenwoordigheid van molekulêre SO_2, wat die oorlewing van hierdie fassinerende gis verseker. Volgordebepaling van die *SSU1*- en *PAD*-geen dui daarop dat daar waarskynlik meer as een verskillende alleel vir dié gene bestaan, wat die SO_2-verdraagsaamheid en produksie van fenoliese verbindings, wat tans tussen verskeie spesies teenwoordig is, kan verduidelik.

This thesis is dedicated to

My loving parents and my deceased mother, for I know she will be proud

Biographical sketch

Marli Louw was born in Hartswater, NorthernCape of South Africa on 16 of April 1984 and completed her matric in 2002 at Vaalharts High School. She worked for 5 years prior to enrolment at University. She enrolled at Stellenbosch University, South Africa in 2007 and obtained a BSc-degree in Human Life Sciences, majoring in Microbiology and Biochemistry, in 2010. She then pursued her HonsBSc in Microbiology at Stellenbosch University, which she received in 2011.

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Preface

This thesis is presented as a compilation of 5 chapters. Each chapter is introduced separately.

Chapter 1	**General Introduction and project aims**
Chapter 2	**Literature review** Metabolic, genetic and physiological responses to SO_2 exposure and nutrient-limiting conditions in *Brettanomyces bruxellensis*
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Chapter 1

General introduction and project aims

1. General introduction and project aims

1.1 Introduction

Brettanomyces bruxellensis has been described as one of the major yeast contaminants associated with red wine spoilage worldwide (Loureiro and Malfeito-Ferreira 2003; Jensen et al. 2009; Leite et al. 2013). It has been detected on the surfaces of grapes, but is more commonly found in the barrels during ageing of wine and in bottled wine (Boulton et al. 1996; Suárez et al. 2007). Spoilage caused by *B. bruxellensis* yeasts can include colour loss of the wine (Mansfield et al. 2002; Dias et al. 2003; Tchobanov et al. 2008), gas production (Chatonnet et al. 1992, 1995, 1997; Echeverrigaray et al. 2013) and the formation of biofilms and cloudiness (Fugelsang et al. 1993; Fugelsang 1997). However, this yeast is more often renowned for the production of volatile off-flavour compounds that negatively affects the organoleptic properties of the wine (Dias et al. 2003; Loureiro and Malfeito-Ferreira 2003; Joseph and Bisson 2004). *B. bruxellensis* yeasts are exceptionally well suited for wine conditions. They are able to survive in nutrient-limiting conditions and also enter a viable but not culturable (VBNC) state, where the cells remain metabolically active, yet are undetectable using conventional detection methods (Divol and Lonvaud Funel 2005; Agnolucci et al. 2010; Coulon et al. 2011; Serpaggi et al. 2011). This yeast can utilise a vast number of carbon and nitrogen sources (Conterno et al. 2006) and has been reported to exhibit tolerance to ethanol (Woolfit et al. 2007; Hellborg and Piškur 2009; Galafassi et al. 2011) and SO_2 (Conterno et al. 2006; Curtin et al. 2012b). Adaptations to the presence of molecular SO_2 are indeed crucial for the survival of this yeast in wine conditions. Various cellular responses to the presence of SO_2 have been identified and include sulphur reduction (Yoshimoto and Sato 1968; Kobayashi and Yoshimoto 1982), sulphur oxidation (Heimberg et al. 1953; Beck-Speier et al. 1985), acetaldehyde production (Stratford et al. 1987), glutathione sulphitolysis (Mannervik et al. 1974; Kåtgedal et al. 1986) and sulphite efflux (Park and Bakalinsky 2000). The significance of the *SSU1* gene (encoding a sulphite pump), with regards to SO_2 tolerance, has been extensively studied in *S. cerevisiae,* and *B. bruxellensis* is assumed to possess an ortholog of this gene (Curtin et al. 2012a). *B. bruxellensis* also contained a homolog to the *PAD* (phenolic acid decarboxylase) gene that refers to phenolic compound production. These two genes play important roles in spoilage and tolerance to SO_2. *B. bruxellensis* has also been characterised to exhibit a high degree of intraspecific polymorphism that consequently results in the increase in variance associated within this yeast (Curtin et al. 2007; Agnolucci et al. 2010; Hellborg & Piškur 2009; Vigentini

et al. 2013). This may explain strain dependent characteristics and variations noted among numerous studies. This does however present increasing challenges to comprehend aspects of interest among strains, due to the high level of associated variance. In order to broaden our current knowledge on the effect of molecular SO_2 on *B. bruxellensis* yeast cells, the intracellular as well extracellular responses of the cell need to be examined, to allow for a holistic view of various mechanisms involve in spoilage and SO_2 tolerance. Insight into these complex mechanisms could potentially lead to better management of SO_2 during wine making.

1.2 Rationale and scope of study

The primary focus of this study is aimed at elucidating the impact of SO_2 on *B. bruxellensis* on metabolic, genetic and physiological levels.

The specific objectives of this study were the following:

a) To investigate the growth rate and morphological characteristics of *B. bruxellensis* strains from geographically different areas under normal and SO_2 induced conditions.

b) To ascertain the energy metabolism of *B. bruxellensis* strains in the presence of different concentrations of molecular SO_2 by evaluating the levels of primary and secondary metabolites (acetaldehyde, acetic acid and D-glucose/D-fructose) in a synthetic wine medium.

c) To sequence the *PAD* and *SSU1* genes in order to identify potential alleles for these spoilage genes.

d) To establish the gene expression profiles of the *PAD* and *SSU1* genes of *B. bruxellensis* during SO_2 exposure and correlate gene expression with metabolic responses.

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Chapter 2

Literature review

Brettanomyces bruxellensis, ruler among wine spoilage yeasts: Complex, unique and possibly, one of the most fascinating wine spoilage microorganisms

2. Literature Review

Brettanomyces bruxellensis, ruler among wine spoilage yeasts: Complex, unique and possibly, one of the most fascinating wine spoilage microorganisms.

2.1. Introduction

Wine is the result of alcoholic fermentation of grape must. It obtains its character and aromas from a large number of metabolites produced as a result of complex interactions between microorganisms and the grape must. *Saccharomyces cerevisiae* is the prevalent yeast associated with winemaking, but various other yeast and bacterial species also occur in grape must and wine. Some of them are undesirable for the production and quality of wine. One such undesirable microorganism is *Dekkera*/*Brettanomyces bruxellensis*, a well-known red wine spoilage yeast (Boulton et al. 1996; Fugelsang 1997; Delfini and Formica 2001; Loureiro and Malfeito- Ferreira 2003; Suárez et al. 2007; Duckitt 2012).

2.1.1. *Brettanomyces/ Dekkera* spp: discovery and taxonomy

At present, five species belonging to the genus *Brettanomyces* are described (Table 1): *Brettanomyces custersianus, Brettanomyces naardenensis, Brettanomyces nanus, Brettanomyces anomalus* and *Brettanomyces bruxellensis*. Teleomorphs (sporulating forms) are known for the latter two species, *Dekkera anomala* and *Dekkera bruxellensis* respectively (Du Toit et al. 2005; Loureiro and Malfeito-Ferreira 2006; Barata et al. 2008).

Various fermented food and beverage products have been shown in literature to be affected by the growth of the different *Brettanomyces* species. Some of these include dairy products (Ibeas et al. 1996; Cosentino et al. 2001), olive products (Ibeas et al. 1996; Coton et al. 2006), numerous soft drinks (Kolfschoten and Yarrow 1970) and fermented beverages (Van der Walt 1964; Lachance 1995; Teoh et al. 2004; Harris et al. 2008) (Table 1).

B. bruxellensis is involved in the production of old English stock beers (Andrews and Gilliland 1952), lambic and speciality sour ales (Van Oevelen et al. 1976; Vanderhaegen et al. 2003), where they seem to facilitate a second fermentation step. These beers owe their unique flavour profile to *B. bruxellensis* (Wedral et al. 2010). However, this yeast is also responsible for spoilage in red wines, which are more susceptible, than white wines, due to their lower acidity, higher polyphenol content and lengthy barrel aging (Wedral et al. 2010). *B. bruxellensis* has

detrimental effects on the visual and organoleptic quality of wines by not only producing off-flavours (Chatonnet et al. 1992, 1995, 1997; Dias et al. 2003; Loureiro and Malfeito-Ferreira 2003; Joseph and Bisson 2004), but also resulting in cloudiness (Fugelsang et al. 1993; Fugelsang 1997) and colour loss of the wine (Chatonnet et al. 1992; Mansfield et al. 2002; Dias et al. 2003; Tchobanov et al. 2008).

2.1.2. Identification and enumeration of *Brettanomyces/Dekkera* spp. in wine

Traditionally, the isolation and enumeration of *B. bruxellensis* are carried out by using conventional microbiological methods such as selective/ microbiological media (Yarrow 1998). The use of these techniques is, although imperative and extensively used as an initial identification step, limited by the degree of accuracy obtained using these methods. The slow growth rate of these yeasts and the low cell density that occurs in wine, further limit these conventional methods. It has also been noted in literature that these yeasts are able to enter into a Viable But Not Culturable state (VBNC), where the cells are metabolically active but no longer detectable on culture media (Millet and Lonvaud-Funel 2000; Du Toit et al. 2005; Serpaggi et al. 2011). This physiological state, that prevents the yeast from being detected by plating, allows the yeast to survive under severe environmental conditions without sporulating. The need for alternative methods for isolation and enumeration was therefore evident.

Various studies reported alternative techniques for the immediate detection and classification of *B. bruxellensis* yeasts (Hayashi et al. 2007; Röder et al. 2007), directly from wine (Cocolin et al. 2004; Delaherche et al. 2004) and even from different grape varieties (Agnolucci et al. 2007; Renouf et al. 2007). Numerous molecular methods such as random amplified polymorphism DNA (RAPD) PCR (Mitrakul et al. 1990; Martorell et al. 2006; Miot-Sertier and Lonvaud-Funel 2007), amplified fragment length polymorphism (AFLP) analysis (Curtin et al. 2007), mitochondrial DNA (mtDNA) restriction analysis (Martorell et al. 2006), intron splice site PCR amplification (ISS-PCR) (Vigentini et al. 2010), loop-mediated isothermal amplification (Hayashi et al. 2007), fluorescent-based detection (Röder et al. 2007) and restriction enzyme analysis coupled to pulse field gel electrophoresis (REA–PFGE) (Miot-Sertier and Lonvaud-Funel 2007) are just some of the current techniques that have been applied to characterize *Brettanomyces* isolates to the strain level. For a full review, refer to Duckitt (2012).

Table 1. Teleomorphic and anamorphic species of Genus *Brettanomyces* and typical sources of isolation/ habits.

Teleomorph (sporulating)	Anamorph (non-sporulating)	Typical source of isolation	References
Dekkera anomala	*Brettanomyces anomalus*	Beer (first isolation 1904)	Heresztyn (1986)
Dekkera bruxellensis	*Brettanomyces bruxellensis*	Beer, Ginger ale, Wine	Andrews and Gilliland (1952); Yarrow and Ahearn (1971); Barret et al. (1955); Peynaud and Domercq (1956); Van der Walt and Van Kerken (1958); Van der Walt (1961,1964)
n/a	*Brettanomyces naardenensis*	Soft drinks (first isolation 1970)	Kolfschoten and Yarrow (1970)
n/a	*Brettanomyces nanus*	Soft drinks and fermented food	Kolfschoten and Yarrow (1970); Cosentino et al. 2001; Coton et al. 2006
n/a	*Brettanomyces custersianus*	Fermented drinks	Yarrow and Ahearn (1971)

2.1.3. Physiological and morphological observations with regards to growth and nutrients

2.1.3.1. Physiological characteristics and growth requirements

B. bruxellensis is a very complex microorganism (on genetic and physiological levels) that has independently evolved into an organism possessing a number of adaptations, which allows it to survive and remain viable in physiologically challenging environments (Woolfit et al. 2007; Hellborg and Piškur 2009). The various adaptations and characteristics associated with this yeast is highly strain dependent. One such an adaptation is the ability to utilise various carbon sources (Table 2) (Conterno et al. 2006), including ethanol (Dias et al. 2003), even under nutrient-limiting conditions. *B. bruxellensis* is tolerant to ethanol, facultatively anaerobic, and can survive without the presence of mitochondrial DNA (petite positive), very similar to what is observed for *S. cerevisiae* (Woolfit et al. 2007; Hellborg and Piškur 2009; Galafassi et al. 2011). *B. bruxellensis* is Crabtree-positive since it preferentially ferment in high glucose medium under aerobic conditions (Kurtzman and Fell 1998; Piškur et al. 2006; Woolfit et al. 2007), with the substantial production of ethanol and acetic acid (Wijsman et al. 1984), however cultures previously cultivated under glucose limiting conditions, transferred to a high glucose medium, exhibited a weak fermentative response that corresponds with a high-affinity uptake system, associated with Crabtree-negative yeast (van Urk et al. 1989). Silva et al. (2004) reported that *B. bruxellensis* utilizes glucose less efficiently, than *S. cerevisiae*, which correlates with the study by Blomqvist et al. (2010) and Nardi et al. (2010a), where it was shown that *B.*

bruxellensis produces less ethanol, than *S. cerevisiae*. However Silva et al. (2004) and Nardi et al. (2010a) reported that *B. bruxellensis* proliferates considerably slower than *S. cerevisiae,* contradictory to the higher biomass production noted for *B. bruxellensis* compared to *S. cerevisiae* (Blomqvist et al. 2010). According to latter authors their results are suggestive of a less pronounced Crabtree effect, during aerobic growth. Under oxygen limiting conditions, the ethanol yield of *B. bruxellensis* is almost the same as in *S. cerevisiae* (Galafassi et al. 2011; Piškur et al. 2012), which could exemplify the ability of this spoilage yeast to ferment just as well as *S. cerevisiae*.

It is interesting to note that the proliferation of *B. bruxellensis* has been shown to improve in a complex culture medium, for example grape juice and molasses, as compared to that in media containing only glucose or refined cane sugar (Aguilar Uscanga et al. 2007). It can be speculated that this might be due to the presence of several other components, such as cofactors, vitamins or enzymes (e.g. pyridoxine) (Rose and Harrison 1971) and that these components could influence yeast cell growth. *B. bruxellensis* can assimilate a wider range of alternative carbon sources (Conterno et al. 2006; Woolfit et al. 2007). These minimal nutritional requirements for *B. bruxellensis* allow it to survive in unfavourable environments. These characteristics might explain the typical ecological progression observed during the course of alcoholic fermentation, with *S. cerevisiae* dominating during the major phase of the fermentation, after which it is replaced by *B. bruxellensis* in the maturation phase, when elevated ethanol concentrations and insignificant amounts of residual sugars are present (Renouf et al. 2006; Woolfit et al. 2007).

During aerobic growth of *B. bruxellensis,* in a medium containing glucose and yeast extract, a fascinating trend was noted by Wijsman et al. (1984). Glucose was fermented to almost equivalent amounts of ethanol and acetic acid. According to the authors, growth continued, following glucose depletion, by utilization of the ethanol initially produced, which was converted to acetic acid. The ability to use ethanol as sole carbon source however seems to be strain dependent as Conterno et al. (2006) found that 25% of the 35 strains they tested could utilize ethanol. Interestingly it also seems that *B. bruxellensis* not only has the ability to utilize ethanol, but also after a long lag phase, to resume growth with the concurrent utilization of acetic acid once all the ethanol is consumed (Wijsman et al. 1984).

Cultures incubated in an aerobic medium suddenly made anaerobic come to a sudden halt in growth, glucose consumption and metabolite production. This inhibition of alcoholic fermentation as a result of anaerobic conditions is known as the Custers effect (Custers 1940; Scheffers and Wiken 1969). Yeast belonging to *Brettanomyces* species show an amplified Custers effect (Scheffers 1979), which is indicated by a long lag phase, as the cells adapt to anaerobiosis, before growth and ethanol production can resume. According to Wijsman et al

(1984) glycerol production was not observed during any phase of growth, while Aguilar Uscanga et al (2003) found that low amounts of glycerol were produced under anaerobic conditions and trace amounts under aerobic conditions. These contradictory results in glycerol do not affect the consensus among these authors that the Custers effect in this yeast, is due to a disturbance of the redox balance, resulting from the tendency of the organism to produce acetic acid, and its inability to restore the balance by the production of reduced metabolites, like glycerol (Wijsman et al. 1984; Aguilar Uscanga et al. 2003) The addition of suitable hydrogen acceptors (e.g. oxygen, acetone, acetaldehyde and 3-hydroxy-butan-2-one), have been shown by Gaunt et al (1988) to alleviate the Custers effect, by restoring the redox balance (Aguilar Uscanga et al. 2003).

A study in 2000 showed that, potassium phosphate and magnesium sulphate did not affect the culture kinetics of *B. bruxellensis* strains. However, the absence of ammonium sulphate and yeast extract had detrimental effects on the yeast and the study clearly indicates that the lack of these two components greatly inhibited the growth and led to changes in the metabolic behaviour of the yeast (Aguilar Uscanga et al. 2000). The absence of ammonium sulphate resulted in a 30% decrease in biomass formation, an elevated rate of glucose consumption, which started to decrease after 50 h and halted at around 180 h, with 50% residual sugar left. A significant effect on the ethanol production, without ammonium sulphate was noted, resulting in a 0.17 g/g ethanol produced, compared to 0.46 g/g for the control (with ammonium sulphate) (Aguilar Uscanga et al. 2000). Elevated levels of acetic acid (7.6 g/L) were also observed without the presence of ammonium sulphate. High levels of acetic acid production (beyond 4 g/L) might act as an inhibitor of glucose metabolism of *B. bruxellensis*, as described by Phowchinda et al (1997). The effect of an excess of ammonium sulphate, poorly stimulated biomass formation by 12%, with the addition of 1 g/L. When 2 g/L ammonium sulphate was added, it induced a negative response in *B. bruxellensis*, resulting in a 6.5% decrease in growth (Aguilar Uscanga et al. 2000).

B. bruxellensis yeasts are also able to assimilate nitrate as nitrogen source, unlike *S. cerevisiae* which is unable to assimilate nitrate (Kurtzman et al. 2011; de Barros Pita et al. 2011; Blomqvist et al. 2012). According to Conterno et al. (2006), 71% of *B. bruxellensis* strains tested, were able to utilize nitrate (Table 2) (Conterno et al. 2006). When nitrate was the sole nitrogen source, an 45% lower growth rate was observed, compared to what is observed for medium containing ammonium (de Barros Pita et al. 2013). According to the latter authors the lower growth rate was in accordance with the low sugar consumption observed for cells growing on nitrate (de Barros Pita et al. 2013). Ethanol production was reduced, which corresponded to the decreased sugar consumption. However even though growth was considerably slower than in an ammonium-based medium, the mere ability of this yeast to utilize nitrogen, present it with an advantage in challenging environments such as wine (de Barros Pita et al. 2011, 2013).

Prior investigation into the physiology of *B. bruxellensis* revealed a negative Pasteur effect, when cells were previously grown aerobically. It was also noted that acetic acid accumulation is pH sensitive. *B. bruxellensis* cells cultured aerobically in a glucose containing medium, only produced CO_2, ethanol, and acetic acid. At a pH of 6.4 only acetic acid was produced, not from the sugar but from the ethanol. At a low pH (for example pH 3 - 4.5), some acetic acid was converted by oxidation into CO_2 (Skinner 1947).

It is clear that published data on the various growth parameters of *B. bruxellensis* are somewhat inconsistent and that these contradictory results could possibly be attributed to the different strains and different growth conditions that were used in each case. This could demonstrate a very large intra species diversity.

2.1.3.2. Morphological observations in *B. bruxellensis*

Another peculiar feature of *B. bruxellensis* is its ability to adapt its cell morphology in conjunction with environmental changes. However, in literature, morphological changes in *B. bruxellensis,* due to an environmental adaptation are limited and controversial, and would require further investigation. The typical morphology of the genus *Brettanomyces* was described as being ogival, (i.e. pointed cells of gothic shape). Poorly developed pseudomycelium and the lack of "blastospore apparatus", was seen on occasion, by Custers in 1940 (as cited by Skinner 1947). Aguilar Uscanga et al. (2000) noted that the general morphology of the cells was pseudomycelium-like, and that morphological changes were observed in ammonium sulphate-, and yeast extract-limiting media. Cells were noticeably more oval in shape without ammonium sulphate and a more spoon-shaped cell was indicative of yeast extract availability being limited (Aguilar Uscanga et al. 2000). The pseudomycelium formation may be primitive, consisting mainly of elongated cell chains, or may be more progressive by producing extensive, well-developed pseudomycelium structures (Lodder 1974; Conner and Beuchat 1984). The development of pseudomycelium appears to be associated with cell division mechanisms becoming impaired, while the cell maintains the ability to produce new cellular material (Morris 1958). *S. cerevisiae* is another species that may produce pseudomycelium, under certain conditions. In this species, it appears that pseudomycelium formation is strain dependent (Conner and Beuchat 1984).

Table 2. Summary of physiological characteristics of 35 *Brettanomyces* strains, the frequency indicative of the amount of the 35 strains positive for characteristics tested and the isolates indicative of the percentage of strains positive (Conterno et al. 2006).

Character tested	Frequency	Isolates (%)
Carbon source assimilation		
Arginine, cellulose, proline, tartrate	0	0
Adonitol	2	6
Arabinose, citrate, starch	3	9
Lactose, mannitol, raffinose	4	11
Ethanol	9	26
Glycerol	10	29
Lactate	12	34
Succinate	13	37
Malate	14	40
Galactose	28	80
Cellobiose, maltose	32	91
Trehalose	34	97
Sucrose	35	100
Nitrogen source assimilation		
Nitrate	25	71
Arginine, proline	35	100
Temperature growth		
At 37°C	13	37
At 10°C	11	31
Alcohol Tolerance		
> 10%	35	100
Sulphite tolerance		
>30 mg/L at pH 3.4	17	49
pH growth		
At pH 2.0	33	94
4-EP and 4-EG (µg/L) production		
High (>2000 4- EP; > 1500 4-EG)	17	49
Medium (1000-2000 4-EP; 700-1500 4-EG)	6	17
Low (< 50 4-EP; <60 4-EG)	7	20
None (<4.0 4-EP and 4-EG)	7	17

Curtin et al. (2012a) reported that the OrthoMCL cluster OG5_126579 was significantly expanded in *B. bruxellensis*. This cluster in *S. cerevisiae* contains ORF: *FIG2, FLO1, FLO5, FLO9, HKR1, HPF1*, *MSB2, MUC1, PRM7, YIL169C* and *YMR317W* which includes plasma membrane and cell wall proteins involved in cell wall budding, adhesion, and pseudohyphal growth (Curtin et al. 2012a). *B. bruxellensis* morphology in literature is still very controversial. These discrepancies could possibly be explained with current knowledge on the diversity associated with these yeast and the complexities of their genomes.

2.2. *Brettanomyces bruxellensis* genome reveals unique complexities

2.2.1. Genomic size, strain comparison and genomic differences

B. bruxellensis despite its economic importance and physiological interest has mostly remained unstudied at the genomic level, and therefore the physiological capabilities of *B. bruxellensis* remains largely unknown or poorly understood. A partial genome analysis of strain CBS 2499 was performed for the first time in 2007 (Woolfit et al. 2007). Studies by Hellborg and Piškur in 2009, using karyotyping, an electrophoretic chromosome analysis technique which is commonly used to distinguish between related yeast species and yeast strains by determining the size and number of a strain's chromosomes, showed the extensive variability in the karyotypes of *B. bruxellensis* strain. The number of chromosomes ranging between 4 and 9 and their size varying between 1 and 6 Mb, which is very unusual (Hellborg and Piškur 2009), while genome sizes fluctuate from under 20 Mb to over 30 Mb (Siurkus 2004; Woolfit et al. 2007)

Strain CBS 2499 used for genome analysis was shown to have an estimated genome size of 19.4 Mb (Woolfit et al. 2007), however this was revised by Piškur et al. (2012) and deduced by sequencing to be 13.4 Mb, considerably smaller than initially estimated by Woolfit et al. (2007) (Piškur et al. 2012).This revised genome size of CBS 2499 is comparable to the Australian AWRI 1499 strain with a genome size of 12.7 Mb (Curtin et al. 2012a). 2606 (partial or complete) sequences, of protein-coding genes with orthologs in *S. cerevisiae* and 277 genes without were identified. It was also observed that genes of *B. bruxellensis* have an overall higher GC content, than those of *S. cerevisiae* (Table 3).

Table 3. GC % value of *B. bruxellensis* genes compared to that of *S. cerevisiae* gene values (adapted from Woolfit et al. 2007).

Genomic region	*B. bruxellensis*	*S. cerevisiae*
Introns, genome	40.2	38.3
Introns, coding	42.9	39.7
Introns, GC3	44.2	37.0
Introns, intergenic	39.1	33.2
Introns, intronic	34.7	33.8

Nardi et al. (2010a) investigated nine genes of *B. bruxellensis* that showed similarity to well-characterized stress genes of *S. cerevisiae*. Some genes (i.e., *ATP1, ERG6, VPS34*) had unusual expression patterns in *B. bruxellensis*, compared to *S. cerevisiae*, while other genes were indicative that general regulations to stress responses are present between the two yeast,

(i.e. for *MSN4, SNF1, HSP82, NTH1*). The authors indicated that the latter genes were present during different stages of alcoholic fermentation, compared to what was observed for *S. cerevisiae*. This suggests that *B. bruxellensis* has both conserved and unique mechanisms to respond to stressful conditions (Nardi et al. 2010a). From reports in literature, *B. bruxellensis* indeed shows greater diversity among strains in chromosome number and ploidy than compared to *S. cerevisiae* (Hellborg and Piškur 2009).

2.2.2. *Brettanomyces bruxellensis* specificities at genomic level

Variations in physiological capabilities such as production of *Brettanomyces*-induced flavours and dissimilarities in its growth ability (Vigentini et al. 2008) between *B. bruxellensis* strains and even other yeast species are assumed to be due to differences on a genetic level in gene complementation and regulation in these species (Wedral et al. 2010). The presence of genes coding for *β*-glucosidase (lactase) and *L*-xylulose reductase (Verho et al. 2004) correlated to the study by Conterno et al. (2006) that showed the ability of *B. bruxellensis* to grow on lactose or arabinose. A gene cluster that encodes for the nitrate assimilation pathway was identified in *B. bruxellensis* and seems to play a role in the utilization of nitrate as a nitrogen source in these yeasts, however this gene cluster seems to be strain depended (Conterno et al. 2006). The gene cluster consists of five genes: nitrate transporter (*YNT1*), nitrate reductase (*YNR1*), nitrite reductase (*YNI1*) and two Zn(II)2Cys6 type transcription factors (*YNA1* and *YNA2*)(Woolfit et al. 2007; de Barros Pita et al. 2013). The ability of *B. bruxellensis* to utilize nitrate as a nitrogen source, will provide the yeast with an advantage during fermentations, and potentially allow it to outcompete *S. cerevisiae*, which is unable to assimilate nitrate as a nitrogen source (de Barros Pita et al. 2011, 2013; Blomqvist et al. 2012).

The number of lipid metabolism genes appeared to be enhanced in the *B. bruxellensis* genome (Woolfit et al. 2007). The latter authors were only able to sequence approximately 40% of the genome, indicating the complexity associated with this genome (Woolfit et al. 2007). However, due to the incomplete nature of this data, the genomic arrangement for this species was for the most part still unclear. Reanalysis of the same set of data by Hellborg and Piškur (2009) revealed that the initial assumption that the CBS 2499 strain was haploid, was incorrect, and actually appeared to be diploid or may even be polyploid (Piškur et al. 2012). This could be the result of the increase in ploidy in genes (Curtin et al. 2012a). The latter authors proposed that the genome of *B. bruxellensis* AWRI 1499 consisted from a heterozygous diploid genome, in addition to a divergent haploid genome, as this would possibly explain the presence of three alleles, they observed in some genes. They also suggested that the genomic assembly of the

AWRI 1499 strain, revealed a triploid genome, in agreement with speciation through inter-specific hybridisation and an asexual lifestyle.

Phylogenomic analyses, using various approaches (a Maximum Likelihood analysis of 347 protein families, with one-to-one orthologs, in all species considered and an identical topology was obtained from a super-tree method combining all trees in *B. bruxellensis* phylome) supported a topology where *B. bruxellensis* is a sister-group to *Pichia pastoris*, an aerobic and poor ethanol-producing yeast (De Schutter et al. 2009), contradictory to what is known about *B. bruxellensis* and *S. cerevisiae* (Piškur et al. 2012). A similar topology was shown in recent studies performed by Curtin et al. (2012a) (Figure 1). Significant heterozygosity was observed in the assembly of the genome and it was noted that for some genes up to three alleles were present (Hellborg and Piškur 2009). This led the author to the conclusion, that the *B. bruxellensis* genome is comprised of both a heterozygous diploid genome and a divergent haploid genome. This data in combination with observations made by Hellborg and Piškur (2009) indicated that *B. bruxellensis* genome resulted from the hybridisation of two closely related species (one diploid and one haploid) (Curtin et al. 2012a).

In the study conducted by Conterno et al. (2006), *B. bruxellensis* isolates of various geographic origins were compared using genetic and physiology aspects. Their results showed that some of the physiological parameters tested (Table 2), were related to the genetic groups, and established by comparing the 26S rDNA gene sequences. They were able to obtain a relative degree of separation of the strains, based on the 26S rDNA groupings, the production of 4-EP and 4-EG, the ability to grow at 37ºC, to metabolize ethanol, maltose, succinic acid, citrate, soluble starch, and glycerol (Conterno et al. 2006).

It is evident from what is currently observed in literature, that the *B. bruxellensis* genome is more complex than initially assumed. Its intricate genome has been shown to directly correlate to the variability observed and what is associated with *B. bruxellensis* strains, in terms of genome size, growth parameters and production of compounds, however it is also the main obstacle researchers is currently facing. The triploid genome makes analysis difficult and limiting sequence data from different strain inhibits the progress needed to better compare strains and understand this evolving yeast.

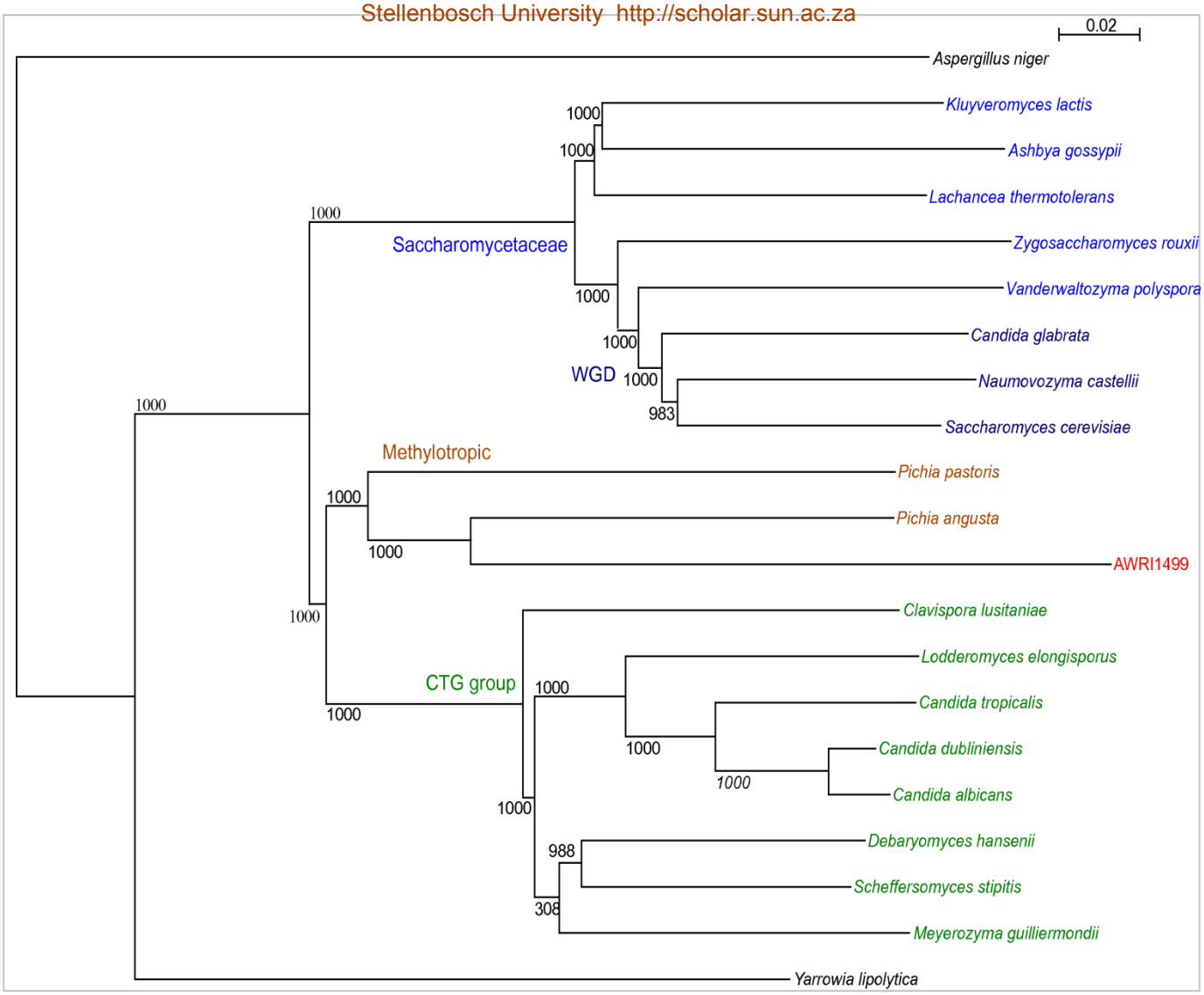

Figure 1. Phylogeny for species most represented for *B. bruxellensis* AWRI 1499 proteins. This topology was generated from an amino-acid alignment of 542 putative orthologous proteins (Curtin et al. 2012a).

2.3. Spoilage characteristics of *Brettanomyces* spp. associated with wine

2.3.1. Production of acetic acid and fatty acids

Brettanomyces spp. are renowned for the production of various compounds associated as off-flavours. One such compound is acetic acid, responsible for the increase in wine volatile acidity and aromas of nail polish remover (Scheffers 1961; Freer 2002), which is indicative of the amplified Custers effect associated with this yeast (Scheffers 1979).

Brettanomyces spp. have also been associated with the production of various short and medium chain volatile fatty acids (C_3-C_{14}). These fatty acids have been shown to contribute to the aroma profile of wine (Rozès et al. 1992; Malfeito-Ferreira et al. 1997; Licker et al. 1998). The most prevalent volatile fatty acid produced by *B. bruxellensis* strains, as observed by Licker et al. (1998) in wine, is isovaleric acid (IVA; 3- methylbutanoic acid). This metabolite is associated with odours of sweat, putrid or stale cheese (Ferreira et al. 2000; Kotseridis and Baumes 2000). Related medium-chain fatty acids include octanoic, dodecanoic (Rozès et al. 1992), isobutyric, and 2-methylbutyric acids (Fugelsang 1997), produced by *Brettanomyces* spp. (Fugelsang and Zoecklein 2003). They are also responsible for similar unpleasant aromas (Rozès et al. 1992; Fugelsang et al. 1993; Malfeito-Ferreira et al. 1997).

2.3.2. Formation of volatile phenols

Phenolic acids are natural components found in grape must and wine and are usually released, by specific esterase activities, as hydroxycinnamic precursors primarily *p*-coumaric acid, caffeic acid, and ferulic acid (Smit et al. 2003; Suárez et al. 2007) and to a lesser extent sinapic acid (Heresztyn 1986; Edlin et al. 1995). Various microorganisms associated with wine are able to metabolize these free phenolic acids (Chatonnet et al. 1992; Edlin et al. 1995) into 4-vinyl and 4-ethyl derivatives collectively referred to as volatile phenols, contributing to the aroma of wine. *B. bruxellensis* strains are also notorious for their ability to produce volatile phenols in wine (Chatonnet et al. 1995) and are associated with the pungent odour formation of the ethylphenols. The production of these volatile phenols is often perceived as ‘medicinal’, ‘barnyard-like’, ‘inky’, ‘sweaty leather’ and ‘Band-aid’ (Chatonnet et al. 1992; Rodriquez et al. 2007). Three main phenolic compounds are produced and responsible for the perceived off-flavours: 4-ethyl-phenol (4-EP), 4-ethyl-guaiacol (4-EG) and 4-ethyl-catechol (4-EC). These compounds are produced as a result of a two-step enzymatic conversion (Figure 3) of the free acids. The initial decarboxylation step is catalysed by the phenolic acid decarboxylase (PAD) enzyme, with the formation of vinyl phenol intermediates (4-vinylguaiacol, 4-vinylphenol and 4-vinylcatechol) (Chatonnet et al. 1992; Chatonnet et al. 1993; Edlin et al. 1995). These

intermediates are then reduced to 4-EG, 4-EP and 4-EC by the vinyl phenol reductase (VPR) enzyme (Chatonnet et al. 1992; Suárez et al. 2007; Harris et al. 2009).

2.3.3. *PAD1/POF1* identification in *S. cerevisiae* and *B. bruxellensis*

The *S. cerevisiae PAD1* gene is a single copy gene, present on chromosome IV and encodes a phenyl acrylic acid decarboxylase (Clausen et al. 1994; Shinohara et al. 2000). The *PAD1* gene seems to be steadily transcribed. However its encoded product, Pad1p, demonstrates minimal enzymatic activity (Clausen et al. 1994).

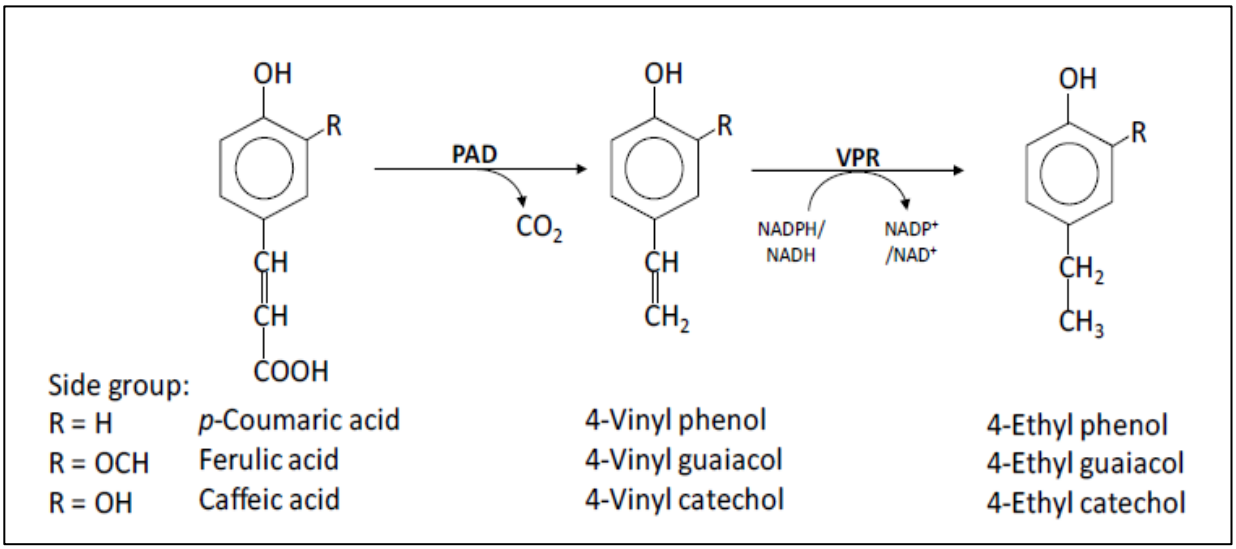

Figure 3. The enzymatic conversion of hydroxycinnamic precursors by the PAD and VPR enzymes into volatile phenolic compounds, from Edlin et al. (1998), Oelofse (2008),Tchobanov et al (2008), Benito et al. (2009) and Harris et al. (2009), Duckitt (2012).

In a study done by Shinohara et al. (2000) it was reported that phenolic off-flavour production did not correlate with the tolerance to the phenolic acids, dissimilarly to what was observed by Baranowski et al. (1980) and Gooder and Tubb (1982). Overexpression of the *PAD1* gene does not significantly increase the functionality of the Pad1 enzyme according to a study performed by Smit et al. (2003). However, this is in disagreement to what was reported by Larsson et al. (2001) since, according to the latter authors, overexpressed Pad1p transformants indicated a ten-fold increase in activity (Larsson et al. 2001; Smit et al. 2003). Conversely, the phenolic acid decarboxylase gene (*PADC*) of *Bacillus subtilis* (Cavin et al. 1998) and the *p*-coumaric acid decarboxylase gene (*PDC*) of *Lactobacillus plantarum* demonstrate elevated enzyme activity (Cavin et al. 1998). Overexpression of these bacterial gene constructs in *S.*

cerevisiae indeed showed an substantial increase in volatile phenol development as compared to that of the *PAD1* gene. Wines produced using recombinant strains were indicative of elevated levels of volatile phenol concentrations, confirming that the presence of the bacterial genes caused an increase in the formation of these volatile phenols (Smit et al. 2003).

The ability to decarboxylate ferulic acid seems to be due to the ferulic acid decarboxylase gene (*FDC1*). Interestingly, *FDC1* is located in close proximity to *PAD1* on chromosome IV of *S. cerevisiae*. Mukai et al. (2010) showed in their studies that both *PAD1* and *FDC1* are essential for the decarboxylation of cinnamic acid in *S. cerevisiae,* where *p*-coumaric acid is a hydroxy derivative of cinnamic acid. They also showed that although Pad1p is homologous to *UbiX* from *Escherichia coli* and Fdc1p is homologous to *UbiD* from *E. coli*, the biosynthesis of ubiquinone was not affected in *Δpad1*, *Δfdc1*, and *Δpad1Δfdc1* mutants (Mukai et al. 2010). The isofunctional genes, *UbiX* and *UbiD*, are of significant importance in the ubiquinone synthetic pathway in *E. coli* (Zhang and Javor 2003). Ubiquinone is essential for electron transport in the mitochondrion and mutants that cannot produce ubiquinone become respiratory deficient. Therefore further studies are required to clarify the relationship between *PAD1* and *FDC1* (Mukai et al. 2010). In *B. bruxellensis* no homolog of the *S. cerevisiae* protein Pad1p was found in either the partial sequence of *B. bruxellensis* CBS 2499 (Woolfit et al. 2007), or in the genomic assembly of AWRI 1499 according to Curtin et al. (2012a). However these latter authors were able to identify a hypothetical putative protein, DbPad, which showed greater homology to bacterial phenolic acid decarboxylase proteins, than to *S. cerevisiae* Pad1 protein its function has yet to be verified. The *FDC1* also needs to be identified in *B. bruxellensis* and the function verified. The exisitance of the *PAD1* and *FDC1* could possibly suggest a third gene (caffeic acid decarboxylase gene), which has yet to be found.

2.3.4. Enzymatic properties of Pad1 and role in volatile phenol production

Studies on the decarboxylation of cinnamic acid by *S. cerevisiae*, showed that the *PAD1* gene is responsible for the recovery of cinnamic acid resistance and Padp1 activity in cinnamic acid sensitive strains that lack Padp1 activity (Mukai et al. 2010). The Padp1 enzyme from *B. bruxellensis* was purified and characterized by Godoy et al. (2008) and indicated that the Pad1 enzyme has an estimated molecular mass of 21 kDa. This enzyme had optimal activity at a temperature of 40°C and a pH of 6.0. For *p*-coumaric acid, the Km value and Vmax were 1.22±0.08 mM and 98±0.15 µmol/min mg, respectively (Godoy et al. 2008).

2.3.5. *VPR* enzyme

The vinylphenol reductase (VPR) enzyme, putatively responsible for the second step in the two-step enzymatic reaction. This enzyme is not associated with *S. cerevisiae*. The *VPR* enzyme was never isolated until a study done by Godoy et al. (2008). These authors were able to purify and characterize this enzyme in *B. bruxellensis.* The VPR enzyme was shown to have an estimated molecular mass of 37 kDa. The Km value was 3.37±2.05 mM and its Vmax was 107.62±50.38 µmol/min/mg for NADPH used as a cofactor. Enzyme activity was indicated to be both stable at pH 3.4 and in the presence of ethanol (Godoy et al. 2008), but the optimal pH was at pH 6 and a temperature of 25˚C. Nevertheless, despite the study by Godoy et al. (2008), the gene sequence of the *VPR* enzyme of *B. bruxellensis* has yet to be found. Although the full genome has been sequenced and annotated, Curtin et al. (2012a) could not identify this gene. Considering that *PAD* of *B bruxellensis* is very distant from that of *PAD1 S. cerevisiae*, it could be hypothesized that the sequence of *VPR* is associated with a unique ORF in the *B. bruxellensis* genome, that will need to be functionally screened to identify the *VPR*. Further investigation into the specificity of these enzymes to particular hydroxycinnamic acids could be of significant interest, in the battle against *B. bruxellensis* off-flavours.

2.4. Importance of sulphur dioxide in wine-making

2.4.1. Chemical overview of the role of SO_2 in wine

To eliminate spoilage microorganisms such as *B. bruxellensis,* sulphur dioxide (SO_2) has been used in wine-making for centuries. SO_2 is a strong antimicrobial and anti-oxidant agent, inhibiting growth of spoilage organisms and preventing oxidative browning in white and red wine by binding to H_2O_2 as well as the inhibition of enzymatic oxidation (Main and Morris 1991; Gomez et al. 1995; Bradshaw et al. 2001; Li et al. 2008; Duckitt 2012). SO_2 is usually added to wine in either a potassium or sodium metabisulphite form. Once sulphur dioxide is added, it dissociates into three molecular species as illustrated.

$$SO_2 + H_2O \leftrightarrow SO_2.H_2O \text{ (Molecular } SO_2)$$

$$SO_2.H_2O \leftrightarrow HSO_3^- + H^+ \text{ (bisulpite)}$$

$$HSO_3^- \leftrightarrow SO_3^{2-} + H^+ \text{ (sulphite)}$$

2.4.2. The antimicrobial mechanisms and its effects on *B. bruxellensis* and other microorganisms

Molecular SO_2 ($SO_2.H_2O$) is the active antimicrobial species of SO_2 against microorganisms (Schimz 1980). The other two species are bisulphite (HSO_3^-) and sulphite (SO_3^{2-}), with the chemical equilibrium between the three species being dependent on the pH of the wine. Molecular SO_2 is most prevalent from pH 0 to 2 (pK_1 = 1.81), the bisulphite anion from pH 2 to 7 (pK_2 = 6.91) and sulphite from pH 7 to 10, yet in general, the pH of wines varies between 3 and 4, making the bisulphite anion the dominant SO_2 species in wine (Figure 4).

pK1= 1.9499 + (T- 20) x 0.0322 + (EtOH% - 10) x 0.01971

T (Temperature˚C)

EtOH% [Concentration of Ethanol in % eg. (5%-10)]

(Usseglio-Tomasset 1984)

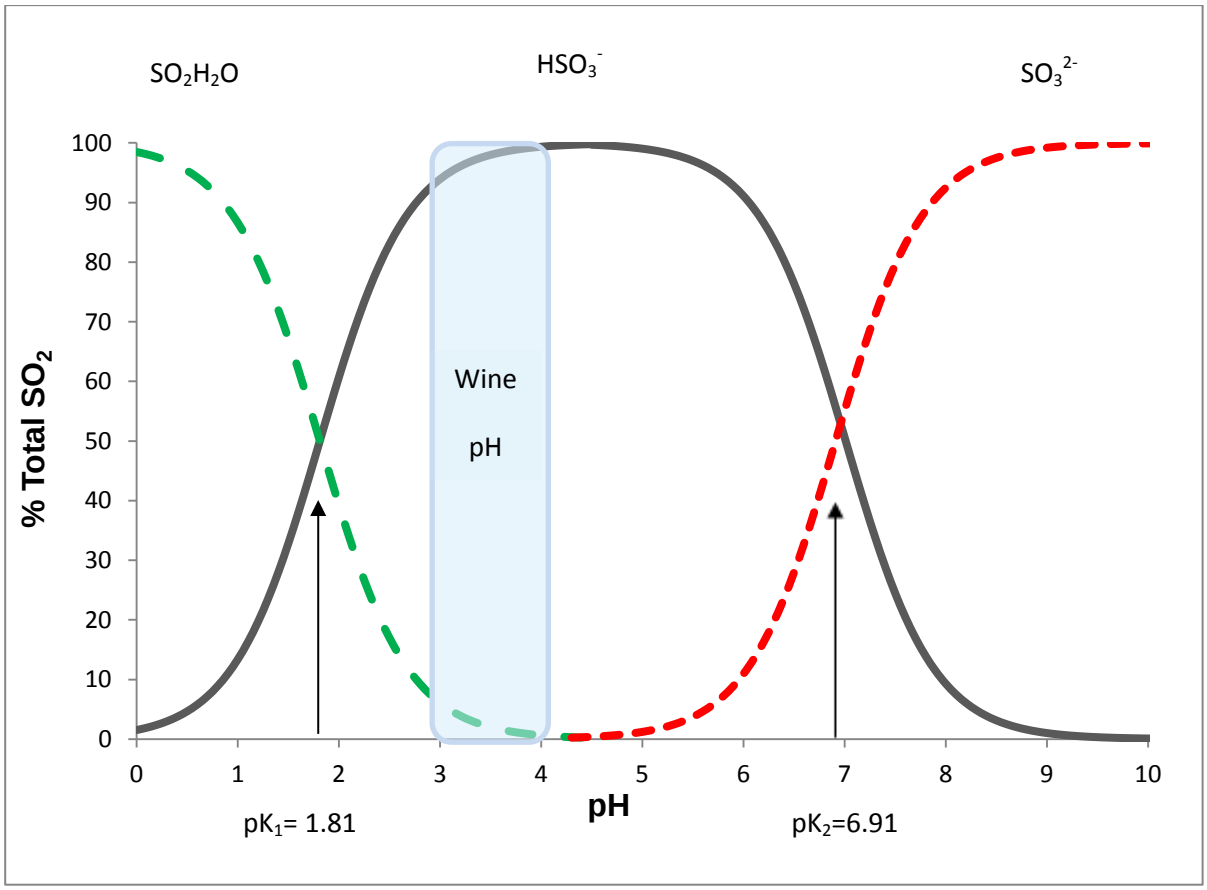

Figure 4. The three SO_2 species and their expected concentrations across the pH range and the pH of wine.

B. bruxellensis yeasts are particularly well adapted to survive the winemaking process due to their relative resistance to the SO_2 concentrations normally used in wine, superior ethanol tolerance and growth in nitrogen limited conditions (Licker et al. 1998).

2.4.3. SO_2 tolerance

Throughout literature it has been shown that *S. cerevisiae* tolerance to SO_2 is highly strain dependent (Schimz and Holzer 1979), this is also true for SO_2 tolerance in *B. bruxellensis* (Conterno et al. 2006; Curtin et al. 2012b). The impact of sulphur dioxide on cell growth, sporulation and recovery after exposure (Baldwin 1951), were indicated in early studies not to have the same inhibiting effect during different growth phases. Cells in late stationary phase showed an increased tolerance compared to cells in exponential phase (Ventre 1934 as reported in Divol et al. 2012). SO_2 can potentially induce a VBNC state in *B. bruxellensis* (Du Toit et al. 2005; Agnolucci et al. 2010). This phenomenon was then reaffirmed by Serpaggi et al. (2012), that reported the entry into the VBNC state after the addition of various concentrations of SO_2, and also indicated the ability of the cells to exit this VBNC state, once the SO_2 stress was removed (Serpaggi et al. 2012).

In literature, SO_2 tolerance in *B. bruxellensis* is extremely controversial. Curtin et al. (2012b) reported the first genotype-dependent sulphite tolerance of *B. bruxellensis* strains. They analysed 41 strains from different genotype groups for sulphite tolerance. These authors were able to observe a genotype-dependent sulphite tolerance phenotype across numerous representative isolates. Figure 5 summarises the maximal mean of SO_2 tolerance observed across the various ribotypes (26S rRNA) and genotypes (amplified length polymorphism). The strains could be separated into three main genotypes (a-c) and eight corresponding ribotypes (I-VIII), from previous work done by Curtin et al. (2007). The 41 strains were further divided based on their maximal mean of SO_2 tolerance. From Figure 5 it is clear that genotype c is the most prevalent with five of the eight ribotypes associated with it. The associated maximal mean of tolerance to SO_2 for genotype c varied between 0.35 mg/L – 0.60 mg/L molecular SO_2. Genotype b has two associated ribotypes with their three corresponding SO_2 maximal means of tolerance, varying from 0.21 mg/L – 0.31 mg/L. Genotype a is more uncommon with only a few strains associated under this genotype with low tolerance to SO_2, values in the range of 0.08 to 0.19 mg/L molecular SO_2.

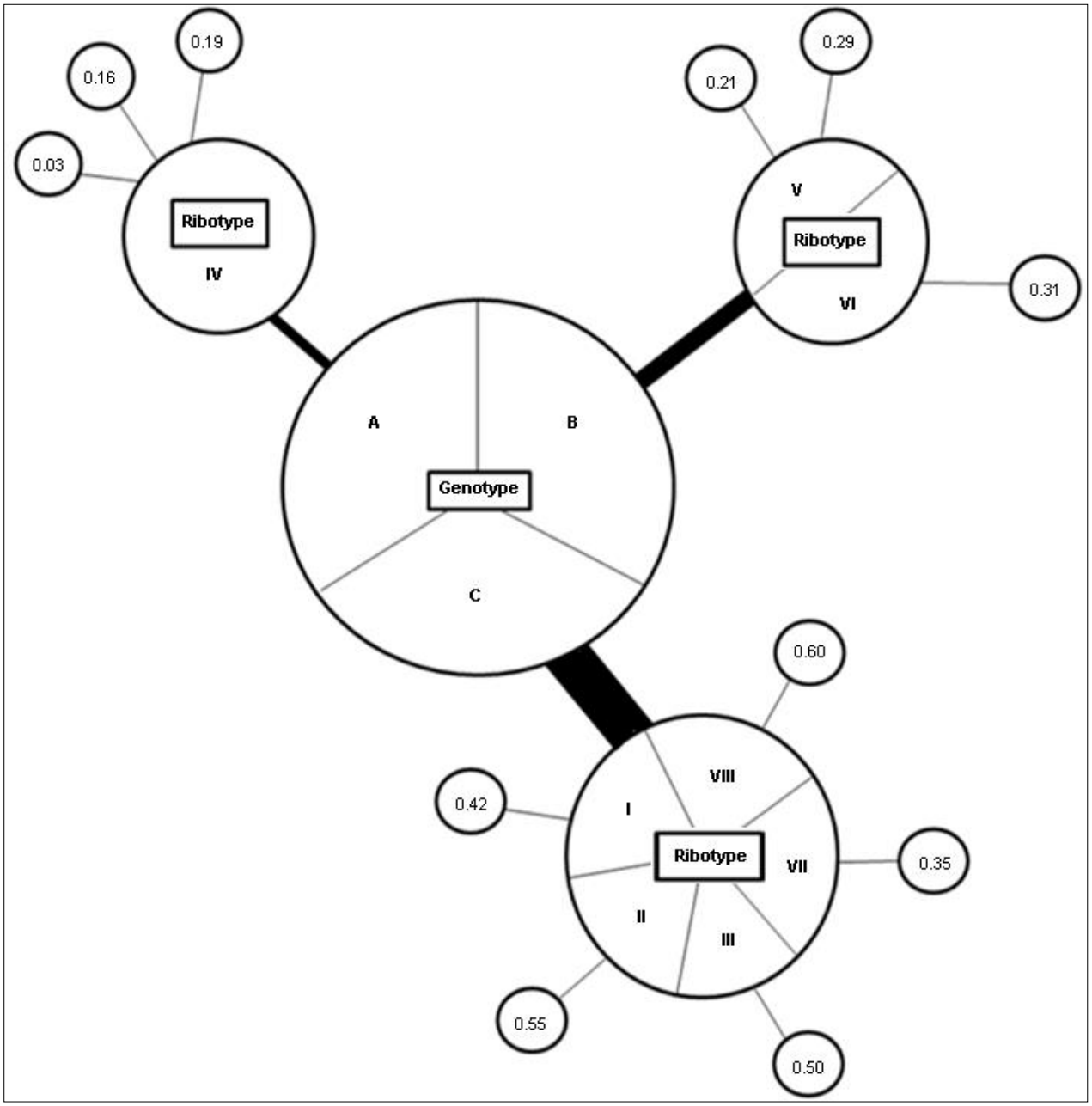

Figure 5. Relationship between the sulphite tolerance and *Dekkera bruxellensis* genotypes and ribotypes, as defined by Curtin et al. (2007). It clearly illustrates the three genotypes (A-C) and the corresponding eight ribotypes (I-VIII), with their mean maximal sulphite tolerance (mg/L molecular SO_2). The widths of the lines are indicative of the frequency of that extension. The thicker the line the greater the amount of strains associated with that extension, adapted from data Curtin et al. (2012b).

From their results the conclusion can be made that SO_2 tolerance in *B. bruxellensis* is highly strain dependent and also seems to be associated with an particular genotype and ribotype. This was confirmed by Vigentini et al. (2013), where numerous *B. bruxellensis* strains were tested for SO_2 tolerance and grouped according to their degree of tolerance. This highlights the

high level of phenotypic polymorphism within *B. bruxellensis* species (Vigentini et al. 2008, 2013; Curtin et al. 2012b).

2.4.4. Cellular response to SO_2 stress

As previously mentioned, *S. cerevisiae* has been extensively used to determine the yeast response to SO_2 (Park and Bakalinsky 2000) and it was shown that various cellular responses to the presence of SO_2 exists: (1) sulphur reduction (Yoshimoto and Sato 1968; Kobayashi and Yoshimoto 1982), (2) sulphur oxidation (Heimberg et al. 1953; Beck-Speier et al. 1985, (3) acetaldehyde production (Stratford et al. 1987), (4) glutathione sulphitolysis (Mannervik et al. 1974; Kåtgedal et al. 1986) and (5) sulphite efflux (Park and Bakalinsky 2000) (Figure 3). Refer to Divol et al. (2012) for a full review on all the responses, as only two aspects (acetaldehyde production and sulphite efflux) will be further discussed.

Acetaldehyde is an intermediate metabolite, highly volatile and reactive compound that binds to various compounds in wine such as SO_2. Acetaldehyde production in yeast is regarded as a leakage product (Cheng et al. 2003). This leakage product has a strong affinity for unbound SO_2: one mole of acetaldehyde binds one mole of SO_2, forming hydroxysulphonate. This results in a decrease in the sulphite stress on yeast such as *B. bruxellensis*. Increase of the SO_2 leads to the increased production and leakage of acetaldehyde in *S. cerevisiae* (Casalone et al. 1992; Divol et al. 2006). This was corroborated by the study of Duckitt (2012). Acetaldehyde production upon SO_2 exposure was assessed for *B. bruxellensis* and *S. cerevisiae* strains in various media. *B. bruxellensis* strains showed elevated levels of acetaldehyde production, in accordance to the increasing amount of SO_2, however the acetaldehyde concentration decrease when the metabolism resumes. This trend was not significantly observed in *S. cerevisiae* strains (Duckitt 2012). The overproduction of acetaldehyde could be a programmed stress response due to the presences of SO_2 or simply be a side effect of the enzymatic inhibition caused by the SO_2 stress, however further investigation would be required in order to establish the precise occurrence mechanism. Vigentini et al. (2013) reported that the most significant metabolic response to SO_2, was a decrease in the cytoplasmic levels of polyols and an increase in concentration of various amino acids: alanine, glutamic acid, glycine, proline, 5-oxoproline, serine and valine (Vigentini et al. 2013).

Another SO_2 stress associated response was identified by Park and Bakalinsky (2000), the active efflux of SO_2 by the sulphite pump, which is encoded by the *SSU1* gene in *S. cerevisiae* (Park and Bakalinsky 2000). The Ssu1p protein plays a significant role in maintaining a low intracellular sulphite level and allows the cell to survive in an environment with high levels

of extracellular SO_2 (Nardi et al. 2010 b). The gene belongs to the dicarboxylate transporter (TDT) family and is positively regulated by a putative five zinc finger based transcription factor *FZF1* (Avram et al. 1999; Aranda et al. 2006). *SSU1* is not only associated with *S. cerevisiae*, but has been found in various other fungal species (Léchenne et al. 2007). It was therefore assumed that such a transporter existed in *B. bruxellensis* (Duckitt 2012). Curtin et al. (2012a) reported that *B. bruxellensis* AWRI 1499 strain (genome fully sequenced) seems to possess one single ORF, whose corresponding protein displays a certain degree of homology to the *S. cerevisiae* sulphite efflux transporter (Ssu1p) however the function of the protein is yet to be verified. It was shown that in *S. cerevisiae*, the *SSU1* gene is duplicated. Strains associated with tolerance to high levels of sulphite, may involve the intricate expression of one or more *SSU1*-R (resistant) alleles (Goto-Yamamoto et al. 1998), for a comprehensive review refer to Divol et al. (2012). This phenomenon could be linked to the diversity observed among *B. bruxellensis* strains, with regards to SO_2 tolerance, however further comparisons of both transcriptomic and genomic data of numerous *B. bruxellensis* strains will be necessary to establish if the molecular mechanisms are comparable between the two species (Curtin et al. 2012b). A comparative study by Duckitt (2012) between *S. cerevisiae* and *B. bruxellensis* strains with regards to SO_2 tolerance and SO_2 intracellular efflux ability, yielded unexpected results. The author reported that SO_2 tolerance did not correlate with the ability to actively excrete intracellular SO_2. Strains associated with a high tolerance to SO_2 did not show high levels of SO_2 efflux, contradictory to what would be expected. It can therefore be deduced that the active efflux of SO_2 from the cells, is not the main mechanism involved in SO_2 tolerance observed in strains that would suggest an alternative mechanism not yet investigated. Figure 6 gives a summary of the abovementioned responses to SO_2. For a comprehensive review on these mechanisms refer to Divol et al. (2012).

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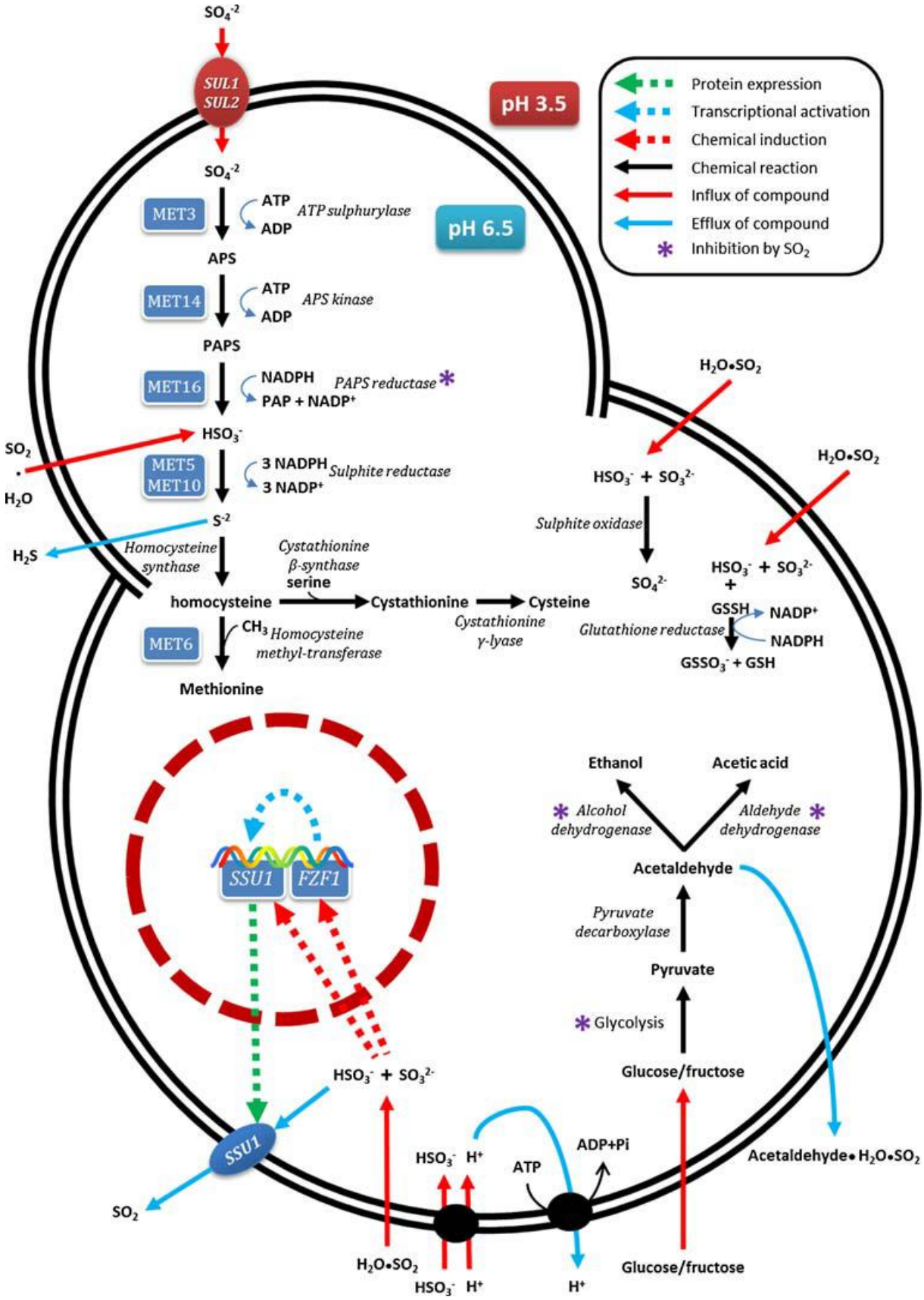

Figure. 6 A summary of the sulphate assimilation pathway and the cellular and molecular responses of *S. cerevisiae* to the presence of SO_2. (SAAB sulphur amino acid biosynthesis, SR sulphur reduction) (Divol et al. 2012).

2.5. Conclusion

The wine environment proves to be extremely challenging for most organisms, due to its high ethanol, high SO_2 concentration and lack in nutrients, however *B. bruxellensis* is commonly associated with the spoilage of red wines. *B. bruxellensis* yeasts are able to endure these hostile environments, due to the ability to tolerate high levels of ethanol, SO_2 and to utilize an extensive range of alternative carbon and nitrogen sources. Other physical characteristics include being facultative anaerobic organisms with an amplified Custers effect. Numerous inconsistencies with regards to *B. bruxellensis* growth and physical parameters have been noted, which are indicative of high strain variability. These inconsistencies are also prevalent with regards to morphological changes observed amongst strains, with the cells in some strains adopting a pseudomycelium like shape. The reasons behind these morphological changes are still unclear, but are proposed to be associated with stress response. Further investigations into the morphological changes associated with some *B. bruxellensis* strains might generate invaluable data, that could be further extrapolated to the variability observed amongst *B. bruxellensis* strains and emphasise the diversity and complexities associated with these organisms' genome.

B. bruxellenis indeed exhibit greater diversity in chromosome number and genome size, compared to what is normally observed for yeast like *S. cerevisiae*. High degree of heterozygosity is observed in *B. bruxellensis*, with some degree of homology between *B. bruxellensis* and *S. cerevisiae* genes. However, *B. bruxellensis* possesses numerous unique gene sequences. This is in agreement to phylogenomic studies that reported the distant relationship between *S. cerevisiae* and *B. bruxellensis*.

The spoilage characteristics of these yeasts include production of acetic acid, fatty acids and formation of volatile phenols. These volatile phenols are the major spoilage mechanism, where phenolic compounds are produced, through a two-step enzymatic conversion of hydrocinnamic acids. The *PAD1* gene in *S. cerevisiae* confers phenolic acid decarboxylase activity. In *B. bruxellensis,* a *DbPAD* was found to possibly possess the same activity, but the gene shows greater homology to bacterial gene than to *S. cerevisiae.* The VPR enzyme is not found in *S. cerevisiae* strains, as these yeasts lack the ability to convert the vinyl intermediates into phenolic compounds. In *B. bruxellensis* the VPR protein has been isolated and purified, but the gene sequence has yet to be found. Future studies into these specific genes, could explain the discrepancies observed in literature, with regards to SO_2 tolerance variance among strains.

Further spoilage characteristics can include the ability of the yeast to tolerate high concentrations of SO_2 and ability to enter a VBNC state. Various coping mechanisms for the resistance to SO_2 have been identified. These include: sulphur reduction, sulphur oxidation, acetaldehyde production and active sulphur efflux. Acetaldehyde is a leakage product, that binds any free intra- or extracellular SO_2, decreasing the SO_2 stress on yeast. In *S. cerevisiae,* a

cell wall associated sulphite pump, Ssu1p, has been shown to actively efflux SO_2 from the cell. In *B. bruxellensis* only a single ORF was identified to have some homology to the Ssu1p from *S. cerevisiae.* This highlights the distant relationship between these two yeast species and the difficulty in identifying spoilage mechanisms in *B. bruxellensis.*

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Chapter 3

Research results

Brettanomyces bruxellensis: the chameleon among yeasts

3. *Brettanomyces bruxellensis*: the chameleon among yeasts

3.1 Abstract

Pseudohyphae formation in *Brettanomyces bruxellensis* has been poorly investigated and literature regarding the induction mechanism in this yeast lacks clarity as results published are contradictory. This study elucidates this phenomenon among strains from geographically different areas. Environmental cues were investigated to attain a better understanding of this mechanism and its importance. Sulphur dioxide (SO_2) was reported to induce this morphology change however the results obtained in this study did not support this reported SO_2 induced morphology and instead highlighted the detrimental effects of SO_2 on cells. These included cell size, with cells displaying an 81% decrease in length, delayed growth, with a significantly prolonged lag phase in the presence of SO_2 and membrane integrity. Fluorescent probes and microscopy demonstrated a decrease in fluorescence and the appearance of an inclusion body-like structure in the cells following exposure to SO_2.

3.2 Introduction

Preventing growth of spoilage microorganisms presents a continuous challenge to winemakers. Numerous yeast species indeed possess the ability to spoil wine thereby negatively affecting its quality (Loureiro and Malfeito-Ferreira 2003). Spoilage yeasts are responsible for the formation of biofilms, sediments, cloudiness (Mansfield et al. 2002; Dias et al. 2003; Tchobanov et al. 2008), gas and off-flavour compounds production (Chatonnet et al. 1992, 1995, 1997; Echeverrigaray et al. 2013). These yeasts belong to several genera including *Hansenula, Candida, Pichia, Dekkera/Brettanomyces, Zygosaccharomyces, Schizosaccharomyces*, and even some *Saccharomyces* strains (Echeverrigaray et al. 2013), among which *Brettanomyces bruxellensis* is one of the most destructive, causing spoilage by production of off-flavour compounds (Boulton et al. 1996; Loureiro and Malfeito-Ferreira 2003; Suárez et al. 2007; Echeverrigaray et al. 2013).

B. bruxellensis possesses various characteristics that confer this yeast the advantage to survive in wine and actively spoil it (Woolfit et al. 2007; Hellborg and Piškur 2009). Some of

these characteristics include tolerance to high concentrations of ethanol (Dias et al. 2003), varying levels of sulphur dioxide (SO_2) (Licker et al. 1998) and ability to utilize an extensive range of carbon sources (Conterno et al. 2006). Another possible key-adaptation is the morphological change occurring in the cells of *B. bruxellensis*. Pseudomycelium development was indeed observed in some *B. bruxellensis* isolates as early as 1958 (Morris, 1958; Lodder 1974, Conner and Beuchat 1984), but observations were scattered and contradictory to one another. Since then, this morphological change has received little interest in this yeast, even though it was and still is extensively studied in other yeasts such as *Candida albicans* (Sudbery 2011) and *Saccharomyces cerevisiae* (Gancedo 2001). Recent studies have once again reported the observation of this phenomenon in *B. bruxellensis*, but results are still inconsistent among authors and seem to be highly strain dependent (Echeverrigaray et al. 2013; Vigentini et al. 2013). Pseudohyphae development has been reported in other yeasts, such as *Saccharomyces cerevisiae,* to be due to nutrient (especially nitrogen) deprivation and also as a result of an oxidative stress respons (Lo and Dranginis 1997; Zaragoza and Gancedo 2000; Gancedo 2001). In *Saccharomyces cerevisiae*, during nitrogen starvation, the *FLO11* gene is responsible for the production of a cell wall protein which is required for pseudohyphae development (Lo and Dranginis 1997). In the latter yeast species, it has been reported that two signalling pathways (MAP kinase cascade and the cAMP-dependent) are involved in the morphogenetic switch between yeast-shaped-like cells to pseudohyphae (Gancedo 2001). However, these two signalling pathways are not conserved among yeast species. These signalling pathways may help elucidate the genetic aspects involved in the formation of pseudohyphae in *B. bruxellensis*, as they are currently unknown in this yeast.

This study investigates the cell morphology associated differences from *B. bruxellensis* strains isolated from geographically different areas, under typical growing and stress conditions connected to the presence of SO_2.

3.3 Materials and Methods

3.3.1 Yeast strains

B. bruxellensis strains used during the course of this study are listed in Table 1. All yeast strains were routinely maintained on yeast peptone dextrose (YPD) with 1.5% agar added (Biolab Diagnostics, Wadeville, South Africa), incubated at 30°C and stored at 4°C.

Table 1. Yeast strains used during this study.

Collection	Strain Number	Source	Reference
IWBT	Y121	MLF, Cabernet Sauvignon, old barrel, 2004	Oelofse (2008)
ITV	LO2E2	IIsolated from Burgundy red wine	Serpaggi et al. (2012)
AWRI	1499	Isolated from McLaren Vale red wine	Curtin et al. (2012a)

MLF: malolactic fermentation; IWBT: Institute for Wine Biotechnology, Stellenbosch University, South Africa; AWRI : Australian Wine Research Institute, Adelaide, Australia, ITV : Institut Technique de la Vigne et du Vin, Beaune, France.

3.3.2 *B. bruxellensis* growth conditions and sampling, for growth in the presence of sulphur dioxide and in the absence of sulphur dioxide

A single colony was inoculated into 5 ml YPD and incubated for 24 h. One-hundred-milliliter YPD (pH 3.5) Erlenmeyer flasks were inoculated to a cell concentration of $1x10^6$ cells per ml, from the 5 ml pre-cultures. The YPD medium was adjusted to pH 3.5 with tartaric acid. Flasks were saturated with nitrogen gas in order to ensure an anaerobic environment. Cultures were grown with shaking (130 rpm) at 25°C. Additional parameters were tested in the absence of SO_2. These include the YPD (pH 6.5) with pH not adjusted to 3.5, and aerobic conditions. All experiments were carried out in triplicate, and the analysis on each sample was performed in triplicate. Cultures were sampled anaerobically every 4 h for a total of 100 h. Two-milliliter samples were taken at each sampling interval, centrifuged for 5 min at 13,200 rpm, and the supernatant stored at −20°C for further analysis. Another 1 ml sample was taken for microscopy work. The same culture conditions were maintained for the SO_2 stress experiment. SO_2 was calculated using the following formula (binding capacity of SO_2 in YPD was taken into consideration, as determined by Duckitt 2012) and added to the culture to obtain a final concentration of 0.6 mg/L molecular SO_2, after the flasks have been saturated with nitrogen gas and samples taken anaerobically every 4 h for 180 h. Samples were further processed as described above with one addition, another 1 ml sample was taken biomass determination.

$$[\text{Molecular } SO_2] = \frac{[\text{Free } SO_2]}{1 + 10^{\text{pH-pK1}}}$$

pK1= 1.9499 + (T - 20) x 0.0322 + (EtOH% - 10) x 0.01971

T: Temperature in ˚C

EtOH%: Concentration of Ethanol in % v/v

(Usseglio-Tomasset 1984)

3.3.3 Sample analysis and microscopy

Cell growth was estimated spectrophotometrically at 600 nm using a Lambda 25 UV/VIS spectrophotometer (Perkin Elmer). D-Glucose concentrations were quantified from the supernatant using the Arena 20XT automated enzymatic kit robot (Thermo Electron Oy, Finland), with the following enzymatic kit: Enytec ™ *Fluid* D-Glucose Id-No: 5140 (Thermo Fisher Scientific Oy, Finland). Cells were visually inspected with the Olympus IV81 Widefield Fluorescent Microscope Imagining Station at 100x magnification and pictures taken were analysed with the Olympus Cell^R Imaging Software, scale bar set at 20 µm. Measurements in µm were performed for length, width and diameter of the cells, using the same imaging software (CAF- Central Analytical Facility).

3.3.4 Viability assay

Two fluorochromes were used to count and discriminate viable and dead cells by microscopy. Cell viability was determined by staining living cells with fluorescein diacetate (FDA, Sigma-Aldrich, St. Louis, Missouri, USA) and dead cells with propidium iodide (PI, Sigma-Aldrich, St. Louis, Missouri, USA). FDA is a lipophilic, non-fluorescent substrate that is cleaved by cellular esterase within living cells, releasing green fluorescence. Cells with intact membranes are able to retain the green fluorescence (Schnürer and Rosswall, 1982). PI is an intercalating agent that enters cells whose membrane is damaged and binds to the nucleic acids. It can then be visualized as red fluorescence. Cells were stained for 15 min in the dark with FDA (10mM in acetone), at a final concentration of 15 µM, in FDA buffer (0.5 M disodium phosphate pH 7.4 and 0.5 M sodium phosphate pH 7.0). 100 µl cells in 900 µl 0.5 M sodium phosphate buffer at pH 7.0 were stained for 15 min in the dark with 3 µl FDA, at a final concentration of 15 µM. PI staining was immediate and cells were stained, with 10 µl of 50 µg/ml PI stock solution (phosphate-buffered saline, pH 7.2, containing 0.09% sodium azide), immediately before analysis. Cells were visually inspected with the Olympus IV81 Widefield Fluorescent Microscope

Imagining Station at 20x magnification, using FITC and TxRed (to visualize red and green fluorescence). Pictures taken were further analysed with the Olympus Cell^R Imaging Software, scale bar set at 100 µm (CAF- Central Analytical Facility).

3.3.5 Statistical analysis

Statistica (Statsoft) was used to perform statistical analysis on data. Distribution of data was determined with basic statistics, which indicated that data was not normally distributed and had to therefore be further analysed using the ANOVA non-parametric test equivalent Kruskal-Wallis test.

3.4 Results and Discussion

3.4.1 Growth of Yeast strains

The growth of three *B. bruxellensis* strains was investigated in order to establish whether the 3 strains originating from different geographical areas have similar growth characteristics. Figure 1A clearly illustrates that this was not the case. The French strain LO2E2 demonstrated the fastest growth rate of the three, while the Australian strain AWRI 1499 growth was protracted and the South African strain IWBT Y121 was the intermediate between the former two. Strain LO2E2 indeed reached stationary phase within approximately 40 h, followed by the IWBT Y121 within 50 h and the AWRI 1499 within just over 60 h. The growth curves are not perfectly sigmoidal: there are distinctive up and down points in the growth curve of all three strains and the large standard deviations show the significant variations between replicates. These growth curves were repeated several times in replicates each time and similar trends were systematically observed.

These unusual variations form the sigmoidal curve were further investigated to explain this reoccurring trend. Glucose concentrations correlated well with the growth curves for each strain (Figure 1B), with glucose depletion corresponding with the onset of stationary phase.

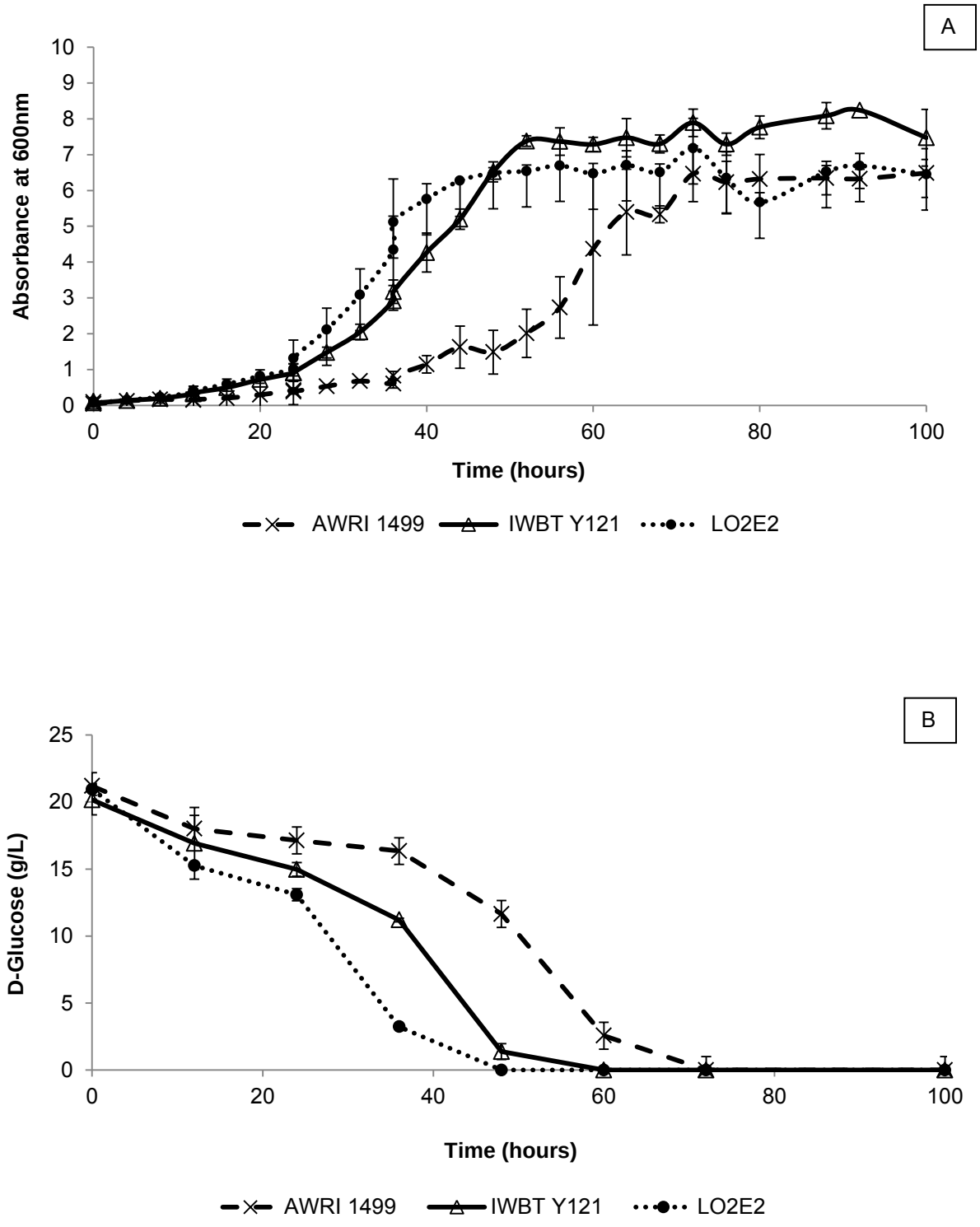

Figure.1 **A**: *B. bruxellensis* growth, estimated by optical density (OD 600nm). Yeast strains LO2E2, IWBT Y121 and AWRI 1499 were monitored over 100 hours. **B**: *B. bruxellensis* utilization of D-Glucose (g/L) over time.

Microscopic analysis revealed fascinating morphological differences among the three *B. bruxellensis* strains (Figure 2). LO2E2 presented the most significant changes, from typical yeast shape-like cells (Figure 2C) at time 0 (lag phase), to complex elongated pseudomycelium structures (Figure 2I) at a time 72 h (stationary phase). The AWRI 1499 strain had an altered morphology, from yeast shape-like cells (Figure 2A) at time 0 (lag phase), to chain-like structures as observed at 72 h (stationary phase) (Figure 2G). These chain-like structures have been previously described as a more primitive adaptation in comparison to pseudohyphae formation (Lodder 1974, Conner and Beuchat 1984). The IWBT Y121 strain exhibited some elongation of the cells, similar to what was initially observed for the LO2E2, but little to no pseudohyphal formation was present. The onset of these morphological changes commenced as early as 8 hours after inoculation. Table 2 highlights the cell dimensions of the three different *B. bruxellensis* strains used in this study. LO2E2 decreased in cell width and significantly increased in length over time, with a corresponding increase in cell area. Some elongation was also observed for the IWBT Y121 cells but not nearly as pronounced as in the LO2E2. The AWRI 1499 retains its basic cell dimensions over time. The same growth trend and morphological characteristics were observed during aeration and pH of 6.5 (data not shown).

We hypothesized that these morphological alterations to the cells could be due to depletion of some nutrient in the medium. This hypothesis was further corroborated by the conversion of the cells back to their yeast shape-like morphology within 30 min after culture samples taken at time 50 h (early stationary phase), were transferred to fresh medium. This was observed in YPD and synthetic wine medium (simulating wine conditions) (data not shown). A study performed by Aguilar Uscanga et al. (2000) indeed reported changes in cell morphology due to lack of nutrients, such as ammonium sulphate and yeast extract. The glucose consumption was monitored to establish whether it contributed to the onset of cell morphology changes, but the results (Figure 1B) however did not support this hypothesis, as morphological changes did not coincide with the depletion of glucose. Additionally, the growth curve experiment was performed in YPD without adjusting the pH to 3.5, to determine if the low pH was responsible for the change observed in cell morphology The results were similar to those obtained in the initial growth curve experiment in YPD pH 3.5, thereby unruling a potential influence of acidity on cell morphology. Yeast assimilated nitrogen was measured, with the robot and the ammonium sulphate was also measured using a manual kit, but neither compounds could not be accurately calculated in YPD due to a technical error. Therefore further investigation would be required to verify results reported by Aguilar Uscanga et al. (2000).

It is also interesting to note that the cells of the AWRI 1499 and LO2E2 strains appeared to clump together (Figure 2: G, I). The clumping of the cells was also macroscopically observed after 100 h of growth, as a biofilm formed on the surface in the flasks, similar to the flor formed on the surfaces of ageing Sherry wines. These morphological changes and flocculation

phenomenon certainly explain the up and down increment observed in the growth curves, as growth was measured by means of optical density.

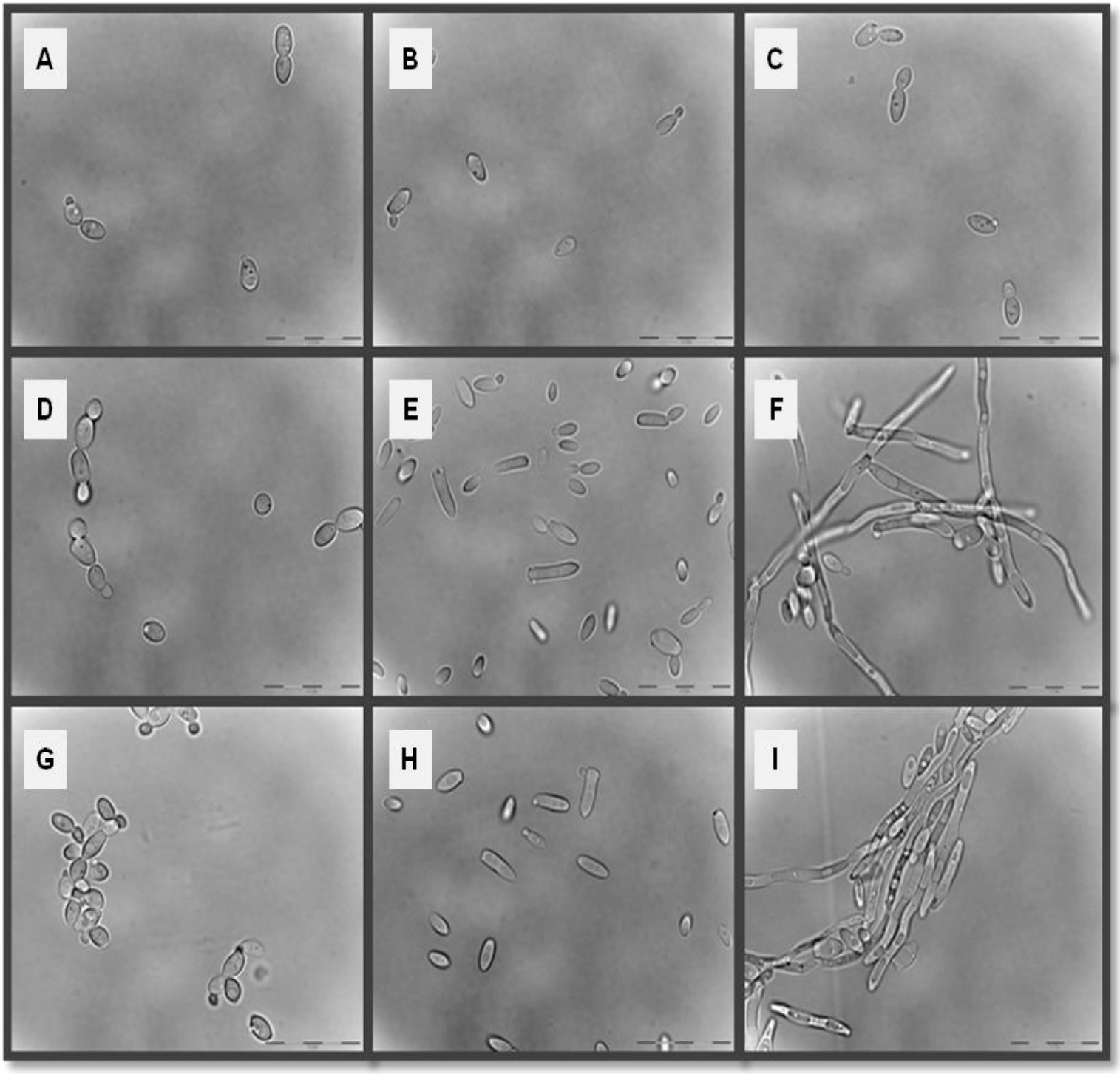

Figure 2. Cell morphology presented by *B. bruxellensis* strains. Time 0 hours **A**: AWRI, **B**: IWBT, **C**: LO2E2; Time 48 hours **D**: AWRI, **E**: IWBT, **F**: LO2E2; Time 72 hours **G**: AWRI, **H**: IWBT, **I**: LO2E

3.4.2 Sulphur dioxide growth conditions

The growth of *B. bruxellensis* strain LO2E2 (most dramatic alteration in cell morphology) was further investigated in the presence of SO_2. Growth in the absence of SO_2 was used as a reference. Figure 3A illustrates that in the presence of 0.6 mg/L molecular SO_2, growth is dramatically halted, with a lag phase of 70 h after which a sudden spike in growth resembling that of the control was observed. This delayed growth in the presence of SO_2 was observed in 1959 by Schanderl. It can be speculated that as the cells adapt to the presence of SO_2, through various mechanisms such as production of acetaldehyde (Stratford et al. 1987; Cheng et al. 2003; Divol et al. 2012), activation of a sulphite pump and reduction of SO_2 (Yoshimoto and Sato 1968; Kobayashi and Yoshimoto 1982; Divol et al. 2012), counteracting the toxic effect of the SO_2 on the cells, growth can resume with the concurrent utilization of glucose (Figure 3B). Biomass production (Figure 3B) correlated with the optical density measurements for both the control and the cells exposed to SO_2, illustrating the negative effect SO_2 has on the ability of the cells to proliferate at the same rate compared to the control.

Higher concentrations (0.8 mg/L or 1.2 mg/L) of molecular SO_2 were also tested, as the LO2E2 strain demonstrated a high degree of tolerance to SO_2 when in stationary phase (data not shown). However, growth was not observed after 180 h at either of these two SO_2 concentrations, suggesting that these concentrations are too high to allow for growth and was therefore excluded from the current study, the 0.6 mg/L molecular SO_2 was therefore the highest concentration tested.

Cells were visualised under microscope throughout the growth period (Figure 4). An interesting phenomenon was observed for the cells growing in the presence of SO_2. Pseudohyphal growth was significantly delayed and less pronounced for these cells (only observed after 120 h) in comparison to the control exhibiting pseudohyphal development as early as 8 h after inoculation. This result is therefore contradictory to what was observed by Vigentini et al. (2013). The latter authors reported pseudohyphae development only in SO_2 treated cultures (Vigentini et al. 2013). In our study, less pronounced pseudohyphae development was observed in the presence of SO_2 and a more prominent pseudohyphal formation in the absence of SO_2. This could suggest that pseudohyphal formation is solely linked to growth, as growth is retarded by SO_2, consequently pseudohyphal formation is delayed. These discrepancies could be as a result of using different strains of *B. bruxellensis* and different SO_2 concentrations used, as great variations are observed between strains of *B. bruxellensis*. It could also be as a result of using different medium composition.

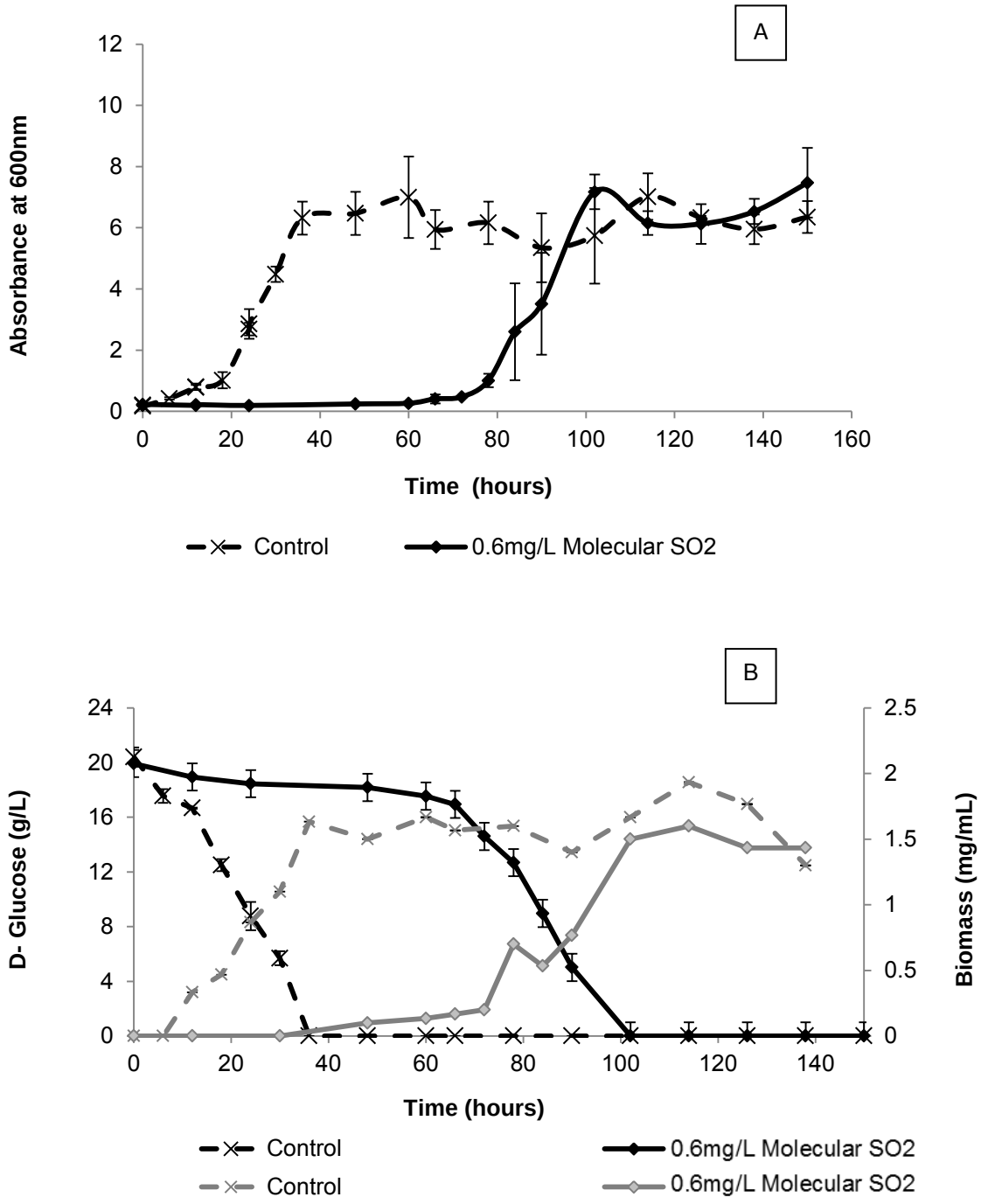

Figure.3 **A**: Comparison of *B. bruxellensis* growth, estimated by optical density (OD 600nm), monitored for the control (no SO_2 added) (dotted line) up to 150 hours and in the presence of 0.6 molecular SO_2 (solid line) up to 150 hours. **B**: *B. bruxellensis* utilization of D-Glucose (g/L) (in black) and biomass production (mg/L) (in grey) over time. Yeast strain LO2E2, control and 0.6 mg/L Molecular SO_2 were monitored till full depletion of D-Glucose was obtained.

Another interesting observation was made. The cells appeared more granular (exhibiting round structures resembling inclusion bodies, typically observed in bacteria such as *E. coli*), in comparison with the cells from the control (Figure 2 C, F, I). The presence of these structures resembling inclusion bodies seemed to increase over time (as indicated by the arrows in Figure 4 A and B). The appearance of these intracellular structures possibly suggests either an adaptation of the cells to SO_2 over time, or the increasing damage caused by the presence of SO_2 over time. However, it is the first time that this phenomenon is reported and extensive investigation is required to identify these cellular structures, their occurrence among other strains of *B. bruxellensis* and the reason behind their formation.

The full effect that SO_2 has on the cell in *B. bruxellensis* is not fully understood. In order to establish whether a size decrease was induced in the presence of SO_2 the cell dimensions (length, width and area) were measured. These three parameters were chosen in order to better portray the three dimensional cell as a whole. A minimum of ten cells were used for each parameter and for each strain. The averaged values are presented in Table 2. Only three time points were considered: lag phase (0 h), exponential phase (24 h) and stationary phase (48 h). LO2E2/SO_2 (LO2E2 strain with SO_2 added) is in lag phase for all three time points. Time points after 48 h were excluded, based on the LO2E2 cells being beyond the scope measurable. The results showed a 73% length decrease at time 24h and an 81% length decrease at time 48h for the cells grown in the presence of SO_2 compared to those grown in the absence thereof. This confirms reports from literature in which a 22% decrease in cell size was reported (Serpaggi et al. 2012). Decrease in cell size also reported for other yeasts and bacteria (Divol and Lonvaud Funel 2005). This observation further suggests that SO_2 critically impact cell growth.

Statistical analysis of this data (summarised in Table 2) revealed that the area is statistically the same for all three strains and time points. The width and length were statistically the same for all strains at time 0 h. The width and length of LO2E2 and IWBT Y121 were statistically different from the AWRI 1499 and LO2E2/SO_2 (LO2E2 strain with SO_2 added) at 24 h. At 48 h, the length of the LO2E2 strain was statistically different from all the other strains.

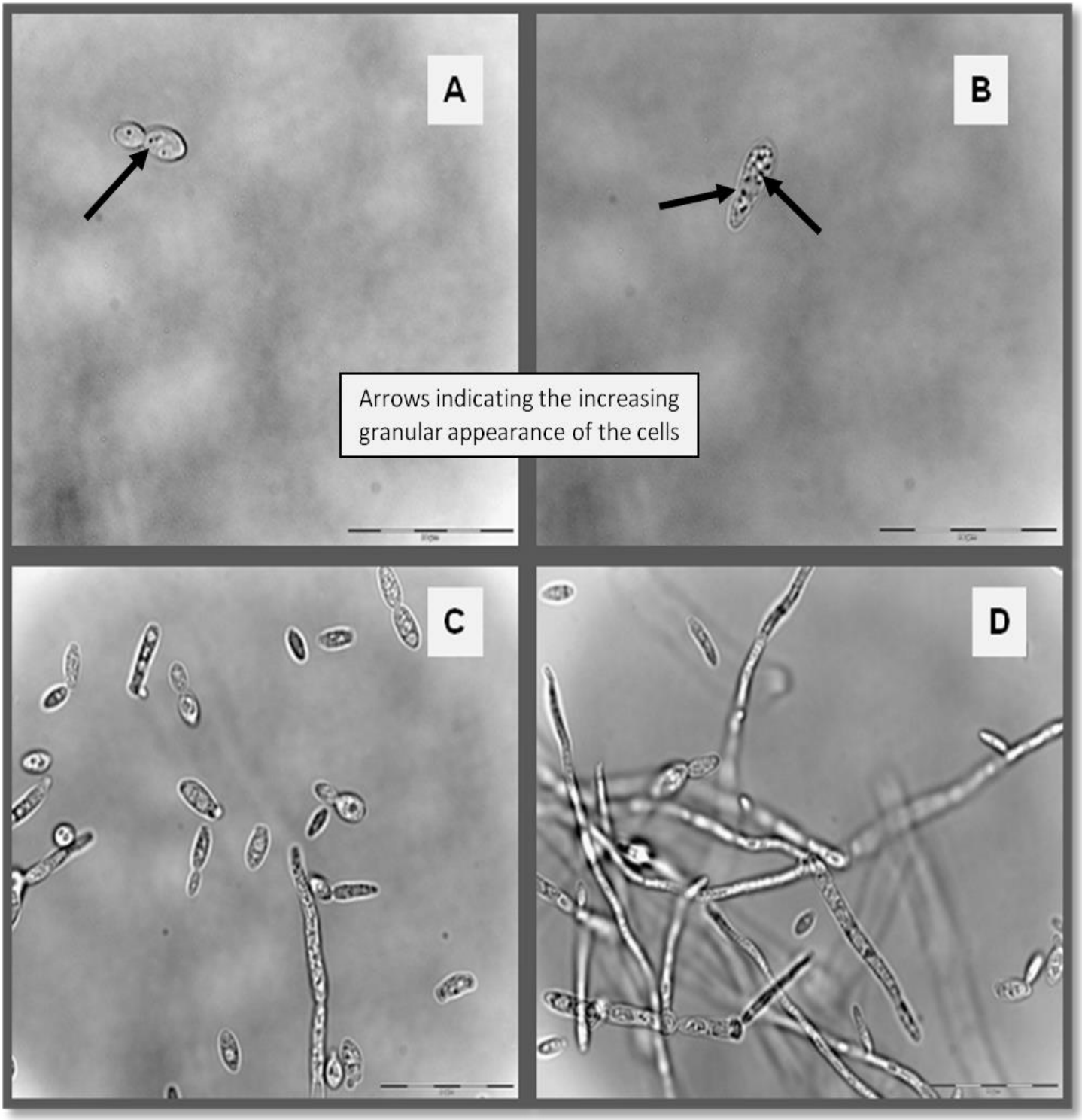

Figure 4. Cell morphology presented by *B. bruxellensis* strain LO2E2 in the presence of 0.6 mg/L molecular SO_2. **A**: time 0 h, **B**: 48 h, **C**: 120 h, **D**: 168 h.

3.4.3 Viability assay results

The viability of the cells exposed to SO_2 was investigated using two fluorescent probes, using untreated LO2E2 cells as control. Figure 5 illustrates the vast differences observed for treated and untreated cells at time 0 h (lag phase). SO_2 exposed cells indicated an immediate and significant decrease in fluorescence as can be observed in figure 5B. A decrease in fluorescence has also been previously reported by Divol and Lonvaud-Funel (2005) and Salma et al. (2013) in *S. cerevisiae* as well as other yeast species following exposure to SO_2. This decrease is indicative of a decrease in the hydrolysis of FDA by intracellular esterases. This can possibly suggest a decrease in the general metabolic activity of *B. bruxellensis*. PI staining revealed no dead cells at this time, suggesting that the membranes of SO_2 treated cells were still intact.

To further highlight this observation, the fluorescence intensities (FI) of the cells were measured over time and normalized against the background intensity (baseline) (Figure 6). The baseline is reported as the average background noise of the instrument. From the results obtained, it is evident that the control group has a significantly higher FI, compared to what is observed for the SO_2 cells. The fluorescence of these cells were so quenched that they were barely detectable above instrument noise up until the onset of the exponential phase, after which they increased in FI.

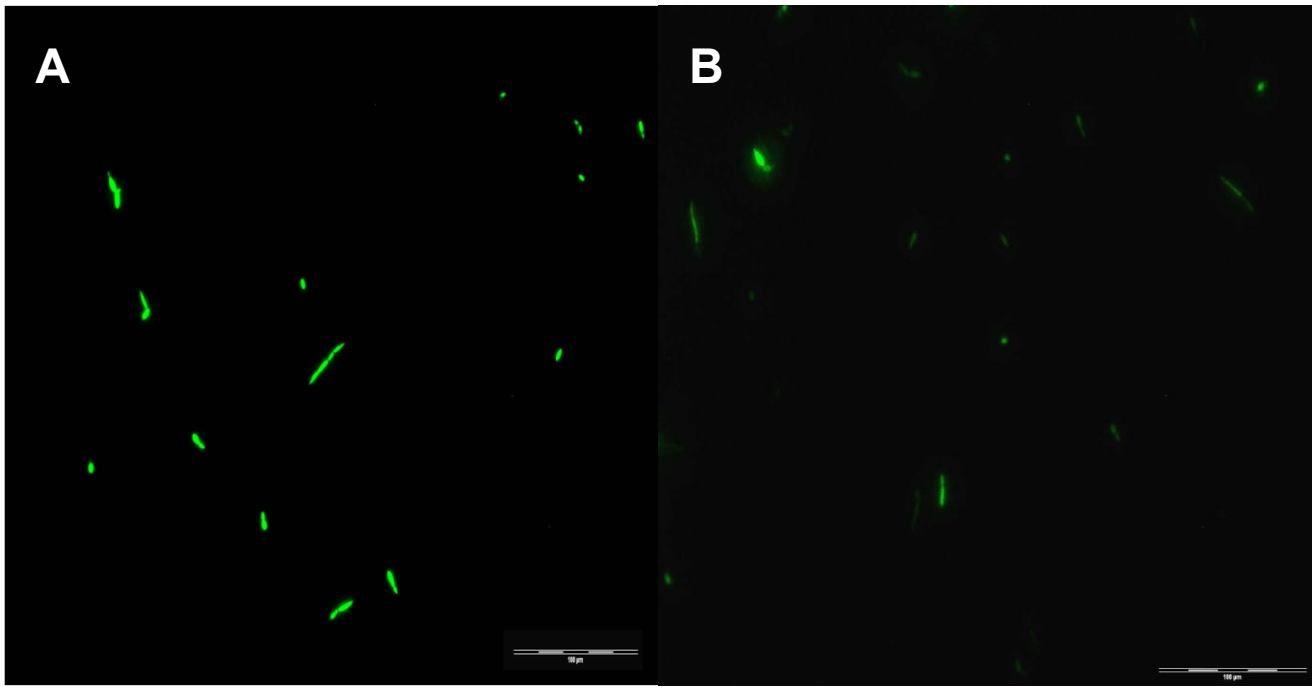

Figure 5. Fluorescence intensity (FI) of *B. bruxellensis* strain LO2E2 time 0 h. A: Control (No SO_2 added) B: SO_2 exposed cells (0.6mg/L molecular SO_2).

Table 2. Average cell dimensions (length and width in µm as well as area in μm^2), were measured over three time points (0, 24h and 48h) for three different strains (AWRI 1499, IWBT Y121, LO2E2) as well as LO2E2 after addition of SO_2 (LO2E2/SO_2). Statistical differences among strains at the same time point, are indicated (with a, b) for parameters (length and width).

	AWRI 1499			IWBT Y121			LO2E2			LO2E2/SO_2		
Time	Length	Width	Area	Length	Width	Area	Length	Width	Area	Length	Width	Area
0	5.47[a]	3.80[a]	16.18	5.49[a]	2.74[a]	13.15	5.80[a]	3.37[a]	16.34	5.65[a]	3.30[a]	14.67
24	6.09[a]	3.91[a]	18.97	9.31[b]	2.69[b]	18.00	24.99[b]	2.70[b]	27.32	6.74[a]	3.59[a]	19.75
48	5.53[a]	3.79[a]	17.02	6.70[a]	2.73[a]	17.31	40.34[b]	2.85[b]	21.16	7.67[a]	3.46[a]	21.71

This FI increase during the exponential phase suggests that the inhibiting effects that the SO_2 exerted on the cells were diminished as it corresponds to where growth resumed.

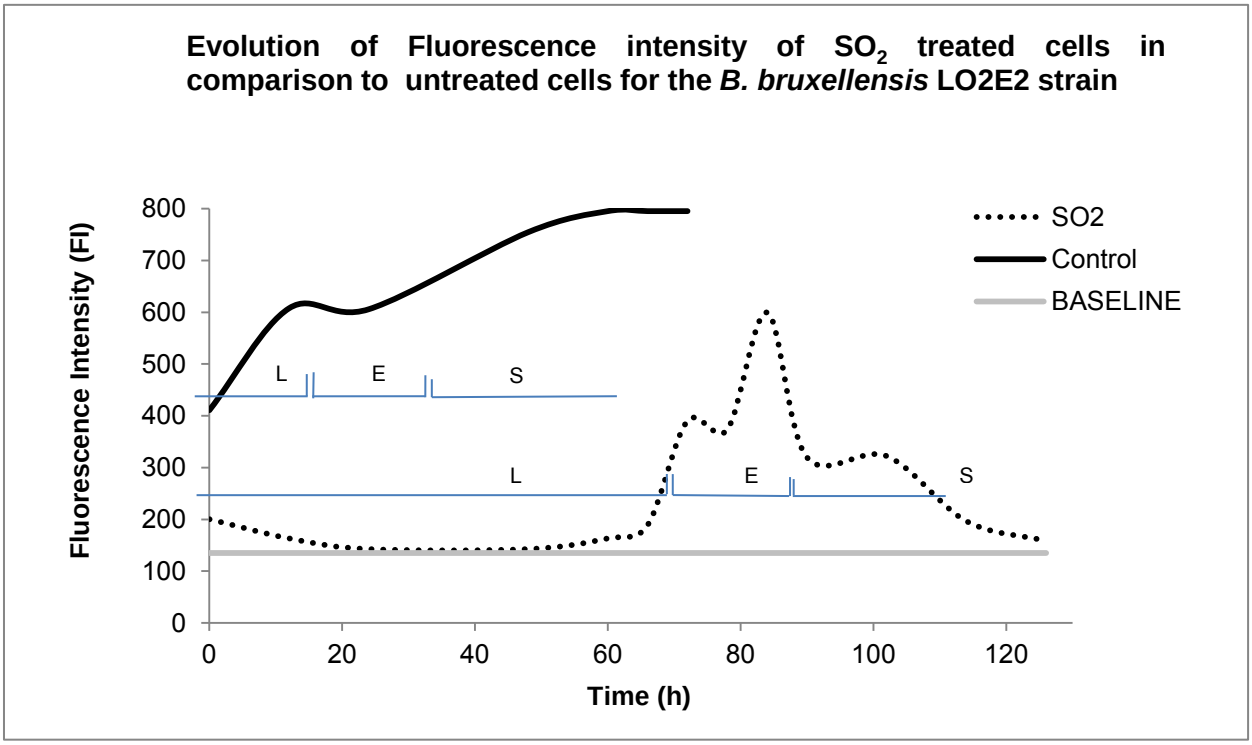

Figure 6. Average green fluorescence intensities (FI) of SO_2 exposed cells during lag, exponential and stationary phase, compared to unexposed cells (control) of *B. bruxellensis*. The baseline is indicative of the average background noise. Symbols refer to growth phase. L- lag phase, E- exponential phase, S-stationary phase.

However, this increase does not seem to be sustainable as the FI starts to decrease during the stationary phase to almost undetectable values, not observed in the control It was also observed that numerous cells did not exhibit any fluorescence (red, yellow or green) (data not shown). We hypothesized that these unstained cells were in a quiescent state, exhibiting no esterase activity but full membrane integrity. However, this hypothesis could not be corroborated using plate assays, as cells remained culturable. It is also interesting to note that there was a small increase in the number of dead cells over time as well as an increase in "intermediate" cells (cells that display both green and red fluorescence) during stationary phase in the presence of SO_2. This was not observed for the control. These intermediate cells suggest that even though the cells are still viable, their membranes were no longer intact allowing PI to enter the cells, suggesting that the cells are dying.

3.5 Conclusion

This study provides fascinating results that demonstrate clear differences among *B. bruxellensis* strains with regards to cell morphology. These morphological differences confirm the diversity reported in literature associated with this spoilage yeast. Observed aggregation could explain the unsuccessful treatment of spoiled wine, as the flor mass could protect cells in strains adopting this behaviour. The factors inducing pseudohyphae formation in *B. bruxellensis* are still unclear, with inconsistent results throughout literature. The current study excluded some of the reported inducing mechanisms (pH, SO_2, aeration) regarding the onset of pseudohyphal growth. However, these were preliminary results and further investigation into the various contributing factors is required. The growth of SO_2 exposed cells was significantly retarded, with dramatic decreases in cell size, as observed for the LO2E2 strain. Pseudohyphae formation was delayed and a minimished appearance was observed, after the addition of SO_2. Fluorescence intensity was lowered, suggesting a decrease in metabolic activity of cells, as previously reported. This study therefore not only highlights the effect that SO_2 has on several aspects such as cell development, proliferation, viability and cell size and a shared morphological/physiological feature associated with bacteria, but also the distinctive morphological features identified among strains of this yeast.

3.6 References

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Chapter 4

Research results

Coping mechanisms during sulphur dioxide induced stress in *Brettanomyces bruxellensis*

4. Coping mechanisms during sulphur dioxide induced stress in *Brettanomyces bruxellensis*

4.1 Abstract

Spoilage of wine can be caused by a diversity of microorganisms, but *Brettanomyces bruxellensis* is renowned for being one of the most destructive red wine spoilage yeast. Spoilage caused by this yeast involves the phenolic acid decarboxylase enzyme that is responsible for the production of off-flavours. Sulphur dioxide is commonly applied as a treatment to prevent growth of detrimental microorganisms. However, *B. bruxellensis* strains are known for their varying degrees of tolerance to sulphur dioxide. Sulphite has been demonstrated by numerous studies in *S. cerevisiae* not to act as an inducer of *SSU1* transcription, a gene encoding a sulphite efflux pump, but was recently reported to exhibit an inducible effect in some strains. The preliminary results of this study indicate that sulphite has a significant effect on the expression of not only the *SSU1* gene but also the *PAD* gene in *B. bruxellensis*. The study further highlights the strain dependant involvement of the secondary metabolite acetaldehyde as a metabolic response to the presence of molecular SO_2 and the co-involvement of both genes and produced metabolite to diminish the detrimental effects of SO_2.

4.2 Introduction

Brettanomyces bruxellensis is one of the most complex and fascinating red wine spoilage microorganisms. This diploid/triploid yeast (Curtin et al. 2012a; Piškur et al. 2012) actively results in the spoilage of wine through the production of various compounds. These compounds include acetic acid (Scheffers 1961; Freer 2002), fatty acids (Rozès et al. 1992; Malfeito-Ferreira et al. 1997; Licker et al. 1998) and ethylphenols (Smit et al. 2003; Suárez et al. 2007), but other compounds are also produced to a lesser extent (Oelofse et al. 2009). The production of these latter volatile compounds (perceived as off-flavours with descriptors such as barnyard and medicinal) is the main spoilage mechanism associated with this yeast (Chatonnet et al. 1992; Rodriquez et al. 2007). The formation of ethylphenols occurs through a two-step enzymatic reaction that involves the transformation of the hydroxycinnamic precursors *p*-coumaric, caffeic and ferulic acids (that naturally occur in wine and grape must). The first step involves the formation of vinyl phenol intermediates (4-vinylguaiacol, 4-

vinylphenol and 4-vinylcatechol respectively) (Chatonnet et al. 1992, 1993; Edlin et al. 1995) through the decarboxylation catalysed by the phenolic acid decarboxylase (Pad) enzyme. The vinyl phenol reductase (VPR) enzyme then converts these intermediates to their corresponding volatile compounds (4-ethyl-phenol, 4-ethyl-guaiacol and 4-ethyl-catechol respectively) (Heresztyn 1986; Lauritsen et al. 1991; Chatonnet et al. 1992; Suárez et al. 2007; Harris et al. 2009). Generally to reduce spoilage caused by microorganisms such as *B. bruxellensis*, sulphur dioxide (SO_2) is commonly added to grape must and wine in order to inhibit their growth. Unfortunately, *B. bruxellensis* strains have been shown to have varying degrees of SO_2 tolerance among strains, ranging from as low as 0.08 mg/L to 0.8 mg/L molecular SO_2 (Curtin et al. 2007, 2012b; Vigentini et al. 2008, 2013; Duckitt 2012). A genotype-dependent phenotype was observed by Curtin et al. (2012b), further supporting SO_2 tolerance to be a highly strain dependent characteristic, that allows it to remain viable and able to produce off-flavours (Curtin et al. 2007, 2012b).

An increase in the production of volatile compounds in the presence of SO_2, reported by Agnolucci et al. (2010), is of further concern, as this leads to an increase in spoilage. Although a number of mechanisms for SO_2 resistance have been described for *S. cerevisiae* (Divol et al. 2012), those specific to *B. bruxellensis* have not yet been identified. Some of these cellular responses include the production of acetaldehyde, a metabolite that has a high affinity for unbound molecular SO_2. It has been reported in literature that an increase in the SO_2 concentration results in over production of acetaldehyde in *S. cerevisiae* (Casalone et al. 1992; Divol et al. 2006) and in *B. bruxellensis* (Duckitt 2012). Another important cellular response to the presence of SO_2 is the active efflux of SO_2 by a sulphite pump, encoded by the *SSU1* gene in *S. cerevisiae* (Park and Bakalinsky 2000). The sequence of the latter gene has been found in the genome of *B. bruxellensis* (Curtin et al. 2012a) but its function has not been verified. It can therefore be speculated that *B. bruxellensis* may potentially possess similar mechanisms, compared to *S. cerevisiae* to deal with SO_2.

This study investigates the production of metabolites, in the presence of SO_2 and demonstrates for the first time the expression profiles of the *SSU1* and *PAD* genes in *B. bruxellensis*.

4.3 Materials and Methods

4.3.1 Microbial strains, media and growth conditions

All yeast strains used during the course of this study are summarized in Table 1. They were routinely maintained on yeast peptone dextrose (YPD) with 1.5% agar added (Biolab Diagnostics, Wadeville, South Africa) plates at 30°C. *Escherichia coli* DH5*α* was used for cloning purposes and cultured in Luria-Bertani broth (Biolab Diagnostics) at 37°C.

Table 1. Strains used during this study

Collection	Strain Number	Source	Reference
IFI[a]	CB63	Isolated from red wine Madrid, Spain	
ISA[b]	1649[T]	Isolated from lambic beer	
IWBT[c]	Y121	MLF, Cabernet Sauvignon, old barrel, 2004	Oelofse (2008)
IFV[d]	LO2E2	Isolated from Burgundy red wine	Serpaggi et al. (2012)
AWRI[e]	1499	Isolated from McLaren Vale red wine	Curtin et al. (2012a)

MLF= malolactic fermentation; T = type strain; a = Instituto de fermentaciones industrials, Madrid, Spain; b = Instituto Superior de Agronomia, Lisbon, Portugal; c = Institute for Wine Biotechnology, Stellenbosch University, South Africa; d = Institut Français de la Vigne et du Vin, Beaune, France; e = Australian Wine Research Institute, Adelaide, Australia.

4.3.2 DNA extraction

Genomic DNA was extracted using mechanical cell disruption and phenol-chloroform-isoamyl acetate extraction as previously described (White et al. 1990).

4.3.3 Amplification of the *SSU1* and *PAD* genes by PCR and agarose gel electrophoresis

PCR reaction was performed for both the *SSU1* and *PAD* genes. The PCR reaction consisted of 36 µL milliQ water, 1 µL (10 mM) dNTP, 10 µL 5X Phusion Buffer high fidelity, 0.25 µL (100 mM) gene specific forward primer and , 0.25 µL (100 mM) gene specific reverse primer (Table 2), 50- 250ng template DNA and 0.5 µL Phusion DNA polymerase in a final volume of 50 µL. The PCR reaction was performed in an ABi 2720 Thermal Cycler (Applied Biosystems) using the following program settings: 98°C for 30 seconds (Initial denaturing), 30 cycles of 98°C for 10 seconds (denaturing), 50°C for 30 seconds (annealing) and 72°C for 20 seconds (extension), with a final extension step at 72°C for 7 minutes. Agarose gel electrophoresis procedure consisted of agarose gels (1%), stained with ethidium bromide, run at 100V-120V in 1X TAE buffer for 25 - 60 min. Gels were visualised using a G:BOX ultraviolet illuminator imaging system (Syngene, Cambridge, England). Bands of interest were excised from the gel using Zymoclean™ Gel DNA recovering Kit (The Epigenetics Company, Zymoresearch, Irvine, US).

4.3.4 Blunt-end Vector based cloning and DNA sequencing

Blunt-ended PCR products required the addition of a poly-A tail to allow cloning into the vector system pGEM®-T Easy (Promega, Madison, WI, USA) using manufacturer's instructions. Transformation was performed using *E. coli* DH5α, plated onto LB (Luria Bertani) Ap (Ampicillin) (100 mg/L) – X-gal (5-bromo-4-chloro-3-indolyl-β-galactoside 3%) agar plates. Plasmid extractions were carried out according to the manufacturer's instructions, using the GenElute™ Plasmid miniprep kit (Sigma-Aldrich). DNA sequencing was performed in an ABi 3130XL Genetic Analyser at the Central Analytical Facility (Stellenbosch University, South Africa) using gene specific primers for each gene (Table 2). Gor4 (http://npsa-pbil.ibcp.fr/cgi-bin/) secondary structure prediction software (NPS - network protein sequence analysis) was used to predict secondary structures.

4.3.5 Carbon energy metabolism flux over a 3-week period

All *B. bruxellensis* strains were pre-cultured in 5 ml YPD broth and then adapted until early stationary phase in YPD broth, pH adjusted to 3.5 and supplemented with 5% v/v ethanol), before inoculations into 200 mL synthetic wine medium (SWM). SWM consisted of 6.7 g/L yeast nitrogen base (Difco), 2.5 g/L D-glucose, 2.5 g/L D-fructose, 5 g/L glycerol, 5 g/L tartaric acid, 0.5 g/L L-malic acid, 0.2 g/L citric acid, 4 g/L L-lactic acid, 0.12 g/L NH_4Cl, 0.02 g/L uracil, 5 mg/L oleic acid, 0.5 mL/L Tween 80 and 15 mg/L ergosterol, 0.18 g/L peptone

(Vigentini et al. 2008). The medium was adjusted to pH 3.5 with NaOH. After filter sterilization, it was supplemented with 10% (v/v) ethanol and *p*-coumaric (10 mg/L) and ferulic acid (10 mg/L). The 250-mL side-ported Erlenmeyer flasks containing 200 mL SWM were made anaerobic using Nitrogen gas. Total SO_2 was added in the form of sodium metabisulphite (Sigma-Aldrich) 1h after inoculation to obtain different molecular SO_2 concentrations ranging from 0 – 1.4 mg/L. Molecular SO_2 was calculated using the following equation:

$$[\text{Molecular } SO_2] = \frac{[\text{Free } SO_2]}{1 + 10^{\text{pH-pK1}}}$$

pK1= 1.9499 + (T- 20) x 0.0322 + (EtOH% - 10) x 0.01971

T : Temperature in ˚C)

EtOH%: concentration of Ethanol in % v/v

(Usseglio-Tomasset 1984)

Samples were taken anaerobically every two days up to day 17. The 2 mL samples were centrifuged at 13,200 rpm for 5 minutes and the supernatant was then transferred to 2-mL microcentrifuge tubes and stored at -20°C. The supernatants-free cells were stored at -80°C. Enzymatic analysis of D-Glucose, D-Fructose, acetic acid and acetaldehyde concentrations were quantified from the supernatant using the Arena 20XT automated enzymatic kit robot (Thermo Electron Oy, Finland), with the following enzymatic kits: Enzytec ™ *Fluid* D-Glucose Id-N°: 5140, Enzytec™ Fluid Acetic acid Id-N°: 5226, Enytec™ *Fluid* D-Fructose Id-N°: 5120 (Thermo Fisher Scientific Oy, Finland), Acetaldehyde enzymatic bio analysis/food analysis kit code: 10668613035 (Roche, AEC, Amersham). One-millilitre samples were centrifuged at maximum speed and the cell mass dried for 48 h at 100°C. The dry mass was weighed and used for normalisation of the acetaldehyde data generated by the enzymatic metabolite quantification.

4.3.6 Total RNA extraction and cDNA synthesis

Total RNA was extracted from samples by adding 1.5 mL EA (6.6 ml Na-Acetate 50mM and 8 ml EDTA 10mM for 1x EA buffer) buffered Acid Phenol:Chloroform (25:1) and 200 µl acid washed glass beads to cell pellet. The mixture was vortexed for 1 min 30 sec and incubated at 65˚C for 4 min, followed by incubation in 100% ethanol (stored at -80˚C) bath till phenol

crystals formed. The suspension was centrifuged at 13,000 rpm for 5 min at 4˚C and the aqueous phase was transferred to a new Falcon™ tube. To the aqueous phase, 0.08 volume of Na-Acetate (0.3M) and 2 volumes of 80% ethanol were added and the samples were incubated at -20˚C overnight. Thereafter, RNA samples were centrifuged at 13,000 rpm for 20 min at 4˚C. The precipitated RNA pellet was washed twice with ice-cold 70% ethanol and then air-dried. The RNA samples were re-suspended in 30 µL of ice-cold RNase-free water. One microlitre of Ribolock (Thermo scientific) was added to the sample. The total RNA was then purified from contaminants using the RNeasy kit (Qiagen) following the clean-up protocol provided in the manufacturer's instructions. The RNA concentration was determined using a Nanodrop® (ND- 1000, Wilmington, Delaware USA). RNA quality was tested by electrophoresis on 1.5% agarose gel. DNase I (Fermentas) treatment was performed on all RNA samples according to the manufacturer's instructions.

cDNA was synthesized from a total of 1 µg of RNA using ImProm-II™ Reverse Transcription System (Promega) according to the manufacturer's instructions using the supplied Oligo(dT)$_{15}$ primers (Promega). cDNA was stored at -20˚C for further analysis.

4.3.7 Gene expression analysis by using real-time quantitative PCR

The expression levels of *B. bruxellensis' SSU1* and *PAD* genes were determined using real-time PCR. All real-time PCRs were performed in the ABi 7500 (Applied Biosystems). The KAPA SYBR® Fast qPCR Kit Master Mix (2x) Universal (KAPABiosystems) and ROX Low dye was used according to the manufacturer's instructions. Optimized reactions were performed in 96-well MicroAmp optical plates (Bio-Rad), each 20 µl reaction mixture contained 200 nM of Fw and Rev primers, 1x KAPA SYBR® FAST qPCR Master Mix (2x) Universal, 0.4 µl ROX Low, 1-2 µl cDNA and PCR-grade water. PCR primers (Table 2) specific for each gene were used. Primer efficiency for *SSU1* gene was 96.49% and the primer specificity for the PAD gene was 96.69%. PCR efficiency was determined using *B. bruxellensis* IWBT Y121 gDNA. Lambda DNA was used to normalise data, instead of housekeeping genes, as Lambda DNA results in better quantification of data, according to Ruthledge and Stewart (2010). One hundred femtogram Lambda gDNA (BioLab) was used as reference and data calibrated using LRE (Linear Regression Efficiency) software (Ruthledge and Stewart 2010). The qPCR experiments were carried out using two biological repetitions (independent fermentations). Real-time PCR was repeated twice for each sample (technical repetitions).

Table 2. Primers used during this study

Primer	Sequence (5'-3')	Target organism	Expected size (bp)
BbSSU1fw	GGATCCATGAGCCGAGCAAGCAAA	*B. bruxellensis*	1353
BbSSU1rev	CTCGAGTCAGTTTTCGGTAACCTTTGTG	*B. bruxellensis*	1353
BbSSU1fwd(INT)	GTTGGTTGGGGAGTCACATT	*B. bruxellensis*	800
BbSSU1rev(INT)	GGTGGCAATCCGTGTATGAA	*B. bruxellensis*	800
BbPADfwd	ATGAAACTCCCTTGCTATCA	*B. bruxellensis*	532
BbPADrev	CTAAAAGGTAATTGCATCAGG	*B. bruxellensis*	532
F1	AGACGAATGCCAGGTCATCTGAAACAG	Lambda	151
R1	CTTTTGCTCTGCGATGCTGATACCG	Lambda	151

4.4 Results

4.4.1 Metabolic Experiment

The molecular response that *B. bruxellensis* strains exhibit in the presence of SO_2 was investigated in SWM (pH 3.5, 10% v/v ethanol), which simulates wine conditions where limited nutrients and elevated amounts of ethanol are present. A range (0.4 mg/L – 1.4 mg/L) of molecular SO_2 concentrations were tested to determine which concentrations resulted in the most significant metabolic response for each strain, as SO_2 tolerance was highly strain dependent. The addition of SO_2 was performed one hour after inoculation, under anaerobic conditions. The production of acetaldehyde and acetic acid was assessed and the utilization of both D-glucose and D-fructose was monitored throughout the course of the experiment. Biomass production was also determined by means of dry mass. During preliminary experiments to optimize SO_2 concentrations, it became abundantly clear that the metabolic responses for each strain differed for each metabolite investigated, resulting in different metabolic profiles being generated.

These metabolic profiles seemed to have a reproducible trend among different fermentation batches but presented too much variation among replicates for a single fermentation to consider the data for further analysis, while others still required further optimization of SO_2 concentration to obtain an inhibitory effect on sugar consumption and an increase in acetaldehyde production. Therefore, these strains were excluded from the final fermentation experiment, due to time constraints and only one *B. bruxellensis* strain (IWBT

Y121) was used. The utilization profiles of D-glucose (Fig 1A) and D- fructose (Fig 1B) strongly suggest that the addition of SO_2 has a halting effect on the consumption of these two sugars, in comparison to the continued consumption observed for the negative control (no SO_2 added) during preliminary results. This stalling effect becomes more prominent as the concentration of molecular SO_2 increases, with maximal inhibition at 1.2 mg/L molecular SO_2 (Fig 1). The subsequent resumption of sugar consumption can most likely be attributed to the adaptation of cells to the presence of SO_2. (Fig 1) demonstrates a sharp decline in biomass production due to the initial addition of SO_2, after which a steady condition is observed where a basal level of biomass is maintained. Acetic acid production was minimal (0.068 g/L- 0.175 g/L) under the SWM anaerobic conditions over a 3-week period (data not shown). Acetaldehyde production was markedly affected by the addition of SO_2 as showed in figure 2, where an increase in acetaldehyde production is observed, following increments of molecular SO_2. The acetaldehyde seems to peak at day 10 of the fermentation for all tested concentrations of molecular SO_2, with the most elevated levels in acetaldehyde at a 1.2 mg/L molecular SO_2. It is unclear why there is a small increment of acetaldehyde from day 0 to day 8. It is assumed that the all the free SO_2 is bound at this point, as growth has resumed, but would need to be confirmed.

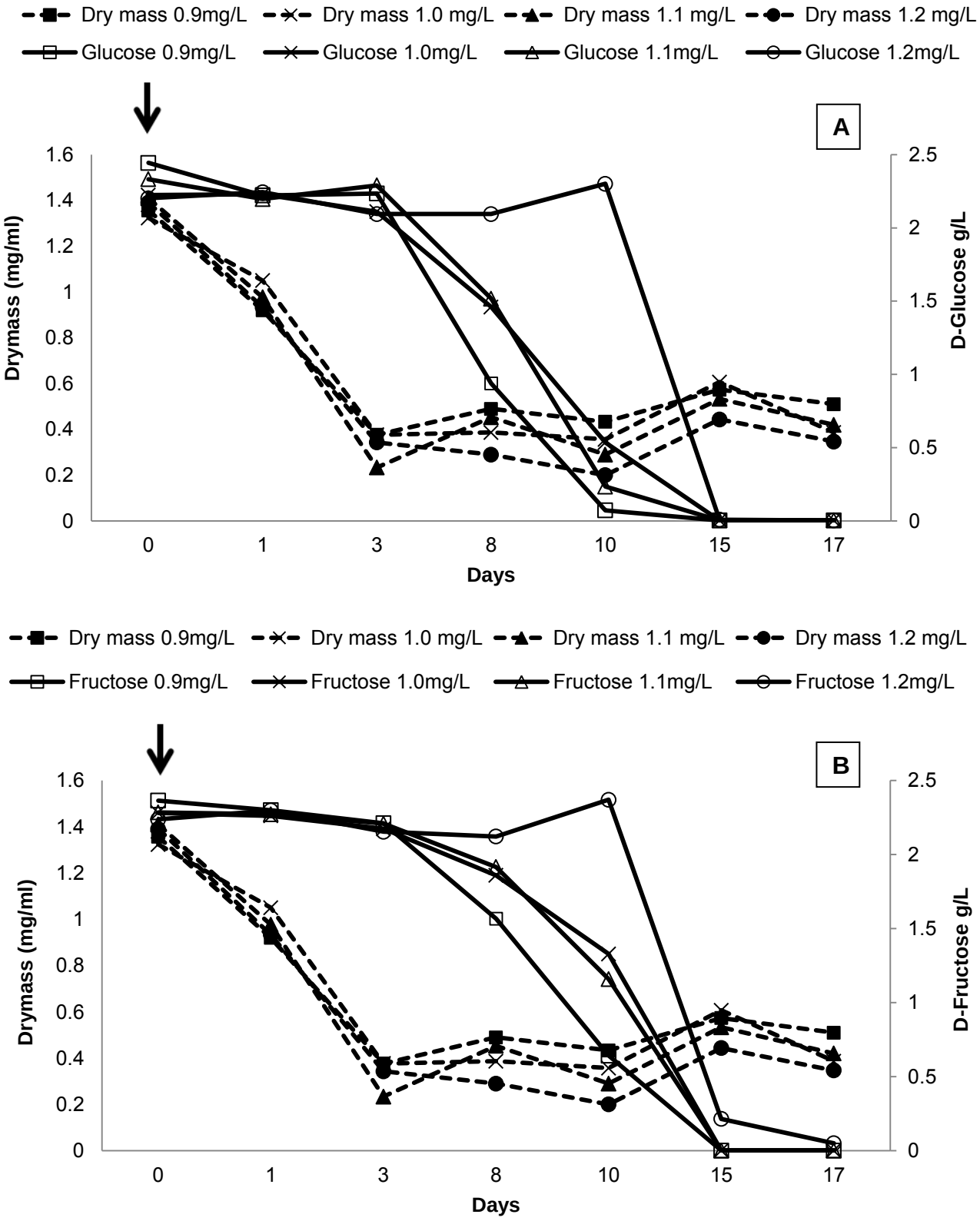
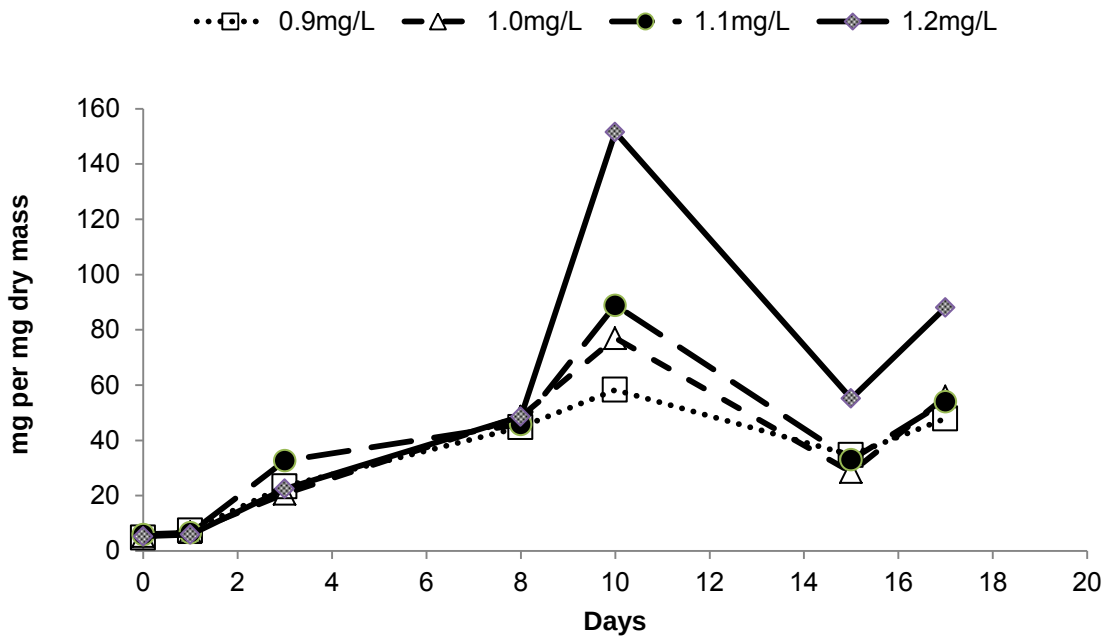


Fig. 1 The effect that 4 different molecular SO_2 concentrations have during fermentation in SWM on sugar consumption and biomass production. A: D-Glucose consumption (solid lines), biomass production (dashed lines) and B: D-Fructose consumption (solid line), biomass production (dashed lines) monitored over 17 days. The arrow indicates the addition of SO_2



Acetaldehyde production over a 17 day period, as a metabolic response to the addition of 4 different molecular concentrations of SO_2 to *B. bruxellensis* IWBT Y121. Values are normalised against 1ml dry mass.

4.4.2 Gene sequences

The genes were sequenced in all strains used in this study in order to establish whether these genes are present in all strains. The nucleotide sequences (Addendum A) of the *SSU1* and *PAD* gene PCR products and subsequent sequence alignments were performed using the ClustalW program. The aligned nucleotide sequences had numerous SNPs for both genes. All the strains have the common allele (*BbSSU1*-1, addendum A) for the *SSU1* gene and another allele was only found in the IWBT Y121 and LO2E2 (*BbSSU1*-2, addendum A). In the *SSU1* gene, only two of the single base mismatches result in amino acid changes. These amino acid changes were investigated using Gor4 secondary prediction software to establish whether the observed amino acids resulted in changes in the secondary structure of the protein. According to the prediction software used, the first point mutation (position 143) is in the middle of a helix and the second point mutation (position 207) in the middle of a loop. Neither of these two mutations results in conformational changes of the secondary structure of the *SSU1* gene (Fig. 3). In the case of the *PAD* gene, the common allele (*BbPAD*-1, addendum A) was found in all the strains, except for the AWRI 1499. The AWRI 1499 exhibited two different alleles (*BbPAD*-2, *BbPAD*-3, addendum A) from what is observed for the other strains. Four point mutations result in amino acid changes. The latter (positions 111, 387, 420 and 510) all result in modifications in the predicted secondary structure of the protein.

SSU1 gene

Alignment of amino acid sequences:

```
BbSsu1-1p       MSRASKHNSKTEMDIESQIRVNENDDGVVSGCEERCSSINDDNDTLTASPTSSVLGHQET
BbSsu1-2p       MSRASKHNSKTEMDIESQIRVNENDDGVVSGCEERCSSINDDNDTLTASPTSSVLGHQET
                ************************************************************

BbSsu1-1p       RKCFQVLDIILDNLHPMHFVITMGVGITSGILYNFPIECIRHGSRYLGIAYFYINLTCFI
BbSsu1-2p       RKCFQVLDIILDNLHPMHFVITMGVGITSGILYNFPIECIRHGSRYLGIAYFYINLTCFI
                ************************************************************

BbSsu1-1p       VIHLLFIMKYFFLCDRYKTSFKDVLYDHRLNVFLGCEVMGTSTLINMIYFMRPDWYVFVY
BbSsu1-2p       VIHLLFIMKYFFLCDRYKTSFKNVLYDHRLNVFLGCEVMGTSTLINMIYFMRPDWYVFVY
                **********************:*************************************

BbSsu1-1p       VLWWINVAFSILVGWGVTFLMFACNKIRPEDINATILLPIVTLTVVASTGSLISVSFMDN
BbSsu1-2p       VLWWINVAFSILVGWGVTFLMFACNKITPEDINATILLPIVTLTVVASTGSLISVSFMDN
                ************************** ******************************

BbSsu1-1p       PKWQISSNIITFLLFANAVVLSIFIVSVYFERLFIHGLPPKPAIYTCFIPIGILGQGGWA
BbSsu1-2p       PKWQISSNIITFLLFANAVVLSIFIVSVYFERLFIHGLPPKPAIYTCFIPIGILGQGGWA
                ************************************************************

BbSsu1-1p       IQLNYKDVGHFIAHHSGLKWMGLGTDYSQEILLSIESVLNFFGVCIALVLASVGVCFTVI
BbSsu1-2p       IQLNYKDVGHFIAHHSGLKWMGLGTDYSQEILLSIESVLNFFGVCIALVLASVGVCFTVI
                ************************************************************

BbSsu1-1p       SFMSVIYCGKPPTFIRTMWASTFPLGTMALSFNEMFKTTNIQGFHIVGTIYSVMLFLITT
BbSsu1-2p       SFMSVIYCGKPPTFIRTMWASTFPLGTMALSFNEMFKTTNIQGFHIVGTIYSVMLFLITT
                ************************************************************

BbSsu1-1p       YCLINTVIFEIPFGKIRNVCHQDATKVTEN
BbSsu1-2p       YCLINTVIFEIPFGKIRNVCHQDATKVTEN
                ******************************
```

PAD gene

Alignment of amino acid sequences:

```
BbPad-1p        MKLPCYQNNTPLDPSFDDDLKDVHLVYDYDATDSNGRPEKWRYEIWFFSENKIVYAIHGG
BbPad-2p        MKLPCYQNNTPLDPSFDDDLKDVHLVYDYDATDSNGKPEKWRYEIWFFSENKIVYAIHGG
BbPad-3p        MKLPCYQNNTPLDPSFDDDLKDVHLVYDYDATDSNGKPEKWRYEIWFFSENKIVYAIHGG
                ************************************:***********************

BbPad-1p        PMAGRINYQTVAYQCVRPGEIWQINWLEETGTVVSIVYDIVNKTVNGLLCFSKGHWENSE
BbPad-2p        PMAGRINYQTVAYQCVRPGEIWQINWLEETGTVVSIVYDIVNKTVNGLLCFSKGHWENSE
BbPad-3p        PMAGRINYQTVAYQCVRPGEIWQINWLEETGTVVSIVYDIVNKTVNGLLCFSKGHWENSE
                ************************************************************

BbPad-1p        AAHGDKRNPDDFARWRNLAKQGIQTDRFVLVERAHILKSFKGQGDLEPIEPDAITF-
BbPad-2p        AAHGDKRNPDDFARWRNLARQGIQTDRFVLVERAHILKSFKGQGDLEPIKPDAITF-
BbPad-3p        AAHGDKRNSDDFARWRNLAKQGIQTDRFVLVERAHILKSFKGQGDLEPIKPDAITF-
                ********.**********:****************************:******
```

Figure 3. The predicted amino acid sequence for the *SSU1* and *PAD* gene respectively. Highlighted regions indicate amino acid changes due to point mutations. *SSU1*: Common allele in all 5 strains, other allele in only IWBT Y121 and LO2E2. *PAD*: Common allele in all strains except for AWRI 1499, which possesses two different alleles (i.e. *PAD-2* and *PAD-3*).

4.4.3 *SSU1* and *PAD* genes expression levels from the *B. bruxellensis* IWBT Y121 at different SO_2 concentrations

The gene expression was investigated at two different time points, for two concentrations of molecular SO_2, during the fermentation in synthetic wine medium. The time points represent day 8 (1) and day 15 (2) of the two fermentations (0.9 mg/L and 1.2 mg/L molecular SO_2) (Fig 4). Due to complications during the course of this fermentation, the expression level of the control (no SO_2 added) could not be included and time constraints prevented the inclusion of additional time points, therefore the lower SO_2 concentration serves as a hypothetical control. The results obtained are therefore only speculative, as the lack of a genuine negative control and a day 0 for both fermentations prevents drawing conclusive remarks. The observed expression of the *SSU1* gene only tentatively suggests that the gene could be inducible in presence of SO_2 over time. At day 8, the 0.9 mg/L molecular SO_2 concentration indeed exhibits the highest expression level for the *SSU1* gene for the two time points and fermentations. Even though both SO_2 concentrations for day 8 had elevated levels, compared to day 15, the 1.2 mg/L molecular SO_2 concentration had a 45% lower expression profile in comparison to the 0.9 mg/L molecular SO_2 expression level. A decrease (for both SO_2 concentrations) were observed (Fig 4) at day 15 in comparison to the day 8 time point. It is interesting to note that at this time point the 1.2 mg/L molecular SO_2 concentration displayed a 45% higher expression level in comparison to the 0.9 mg/L molecular SO_2 for the same time point.

The expression pattern of the *PAD* gene was also investigated for the same set of samples during the same two sampling stages (Fig. 4). Again the proper controls could not be included. The results obtained are therefore speculative and should merely serve as a preliminary indication to potentially inducible patterns, as a result of SO_2. When the two concentrations of SO_2 were compared with each other at day 8, the 1.2 mg/L molecular SO_2 resulted in a 44% higher expression level than the 0.9 mg/L molecular SO_2. Furthermore at day 15, minimal variation was observed in the expression profile for 0.9 mg/L molecular SO_2 in comparison to the expression at day 8 and a 61% decrease in the expression level for the 1.2 mg/L molecular SO_2 was noted.

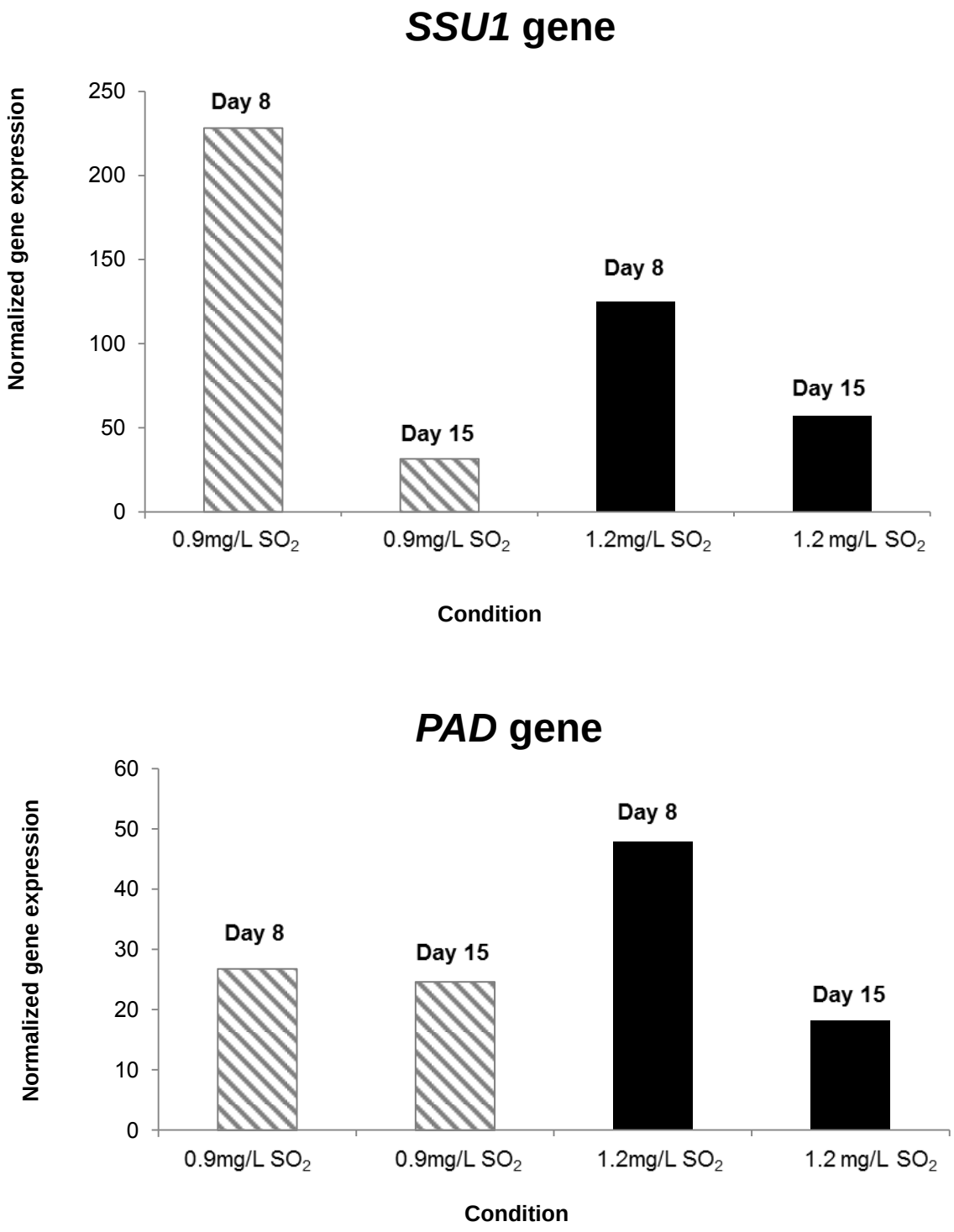


Fig. 4 Expression levels of the *SSU1* and *PAD* genes at two different stages during SWM fermentation (1- Day 8) (2- Day 15) for two different molecular SO_2 concentrations (0.9mg/L and 1.2mg/L molecular SO_2). Samples are identified by molecular SO_2 concentration and fermentation. Samples are normalized against Lambda genomic DNA using LRE software.

4.5 Discussion

The metabolic experiment indicates a clear delay observed in the fermentation at a 1.2 mg/L molecular SO_2. There is also a decrease in the biomass after the addition of SO_2. This decrease in biomass has been reported in literature, for other yeasts to be due to many contributing factors, such as nutrient deficiencies (nitrogen) and presence of inhibitory substances that result in a decrease in pH and inhibition of key enzymes that may consequently lead to a decrease in biomass (Alexandre and Charpentier 1998). In this study, nitrogen deficiency involvement can be excluded, but biomass decrease could still be due to yet unidentified inhibiting substances. It is also plausible that the cells decrease in size or are completely autolyzed, which in turn would result in a decrease in biomass however it cannot be excluded that the observed decrease may also be as a result of a technical error incurred during measurements of biomass. Only further investigation would be able to clarify these speculations made regarding the observed decrease in biomass. Acetaldehyde production is significantly elevated at a high SO_2 concentration, and correspondingly, a decreased acetaldehehyde production was observed for lower SO_2 concentrations. It can therefore be speculated that the yeast cell's primary mechanism to remove SO_2 is the upregulation of the *SSU1* gene expression, at lower SO_2 concentrations. However, at very high molecular SO_2 concentrations, the increased stress upon the cell indirectly impedes the cells' ability to express the *SSU1* gene. Under these conditions the cell reverts to alternative mechanisms, such as acetaldehyde production (an involuntary process most probably due to the inhibition of key glycolytic enzymes by SO_2). This is correlated to what is observed from the data, with an increase in this secondary metabolite that peaks at day 10 and stays elevated until day 15. The acetaldehyde peak at day 10 directly correlates with the onset of growth and corresponding utilization of sugars, indicating that the SO_2 stress and inhibition on glycolytic enzymes has been sufficiently reduced to allow growth to commence. These metabolic response results obtained during this study are supported by observations made by Duckitt (2012) who reported similar results with a few slight variations. These variations are most likely due to differences in the strains used. Variations in *SSU1* expression have also been reported in other wine yeasts (*S.cerevisiae, S. uvarum, S. fermentati, S. bayanus and S. italicus*) (Yuasa et al. 2004, 2005; Goto-Yamamoto et al. 1998; Perez-Ortin et al. 2002; Townsend et al. 2003; Park and Hwang 2008).

The *SSU1* gene expression profile of *B. bruxellensis* seems to correspond to results obtained by Nardi et al. (2010). The latter authors indeed reported that the *SSU1* gene showed an inducible behaviour in the presence of SO_2 for *S. cerevisiae*. However, these

authors demonstrated that this induction was highly strain specific (Nardi et al. 2010). The results obtained for the *SSU1* gene expression of *B. bruxellensis* were inconclusive, due to the lack of proper negative controls, but these results prove to be of some interest, as they could indicate a constitutively expressed gene and possible inducible behaviour in the presence of SO_2, but would need to be confirmed in future studies. The elevated expression levels observed for this gene seem to not correlate directly with the increase in the molecular SO_2 concentration, as a higher expression level was observed for the lower (0.9 mg/L) SO_2 concentration tested in comparison to the 1.2 mg/L SO_2. This observation could possibly be explained by a correlation with the production and accumulation of secondary metabolites such as acetaldehyde. Results from the metabolic response to SO_2, supports this hypothesis. The analysis of the *PAD* gene's expression profile showed an increase at a 1.2 mg/L molecular SO_2 concentration compared to the lower SO_2 concentration (0.9 mg/L). It can be hypothesized that the *PAD* gene is upregulated in the presence of high molecular SO_2 concentrations but this assumption will need to be supported by future work, where the required standards are included. An increase in the expression of this gene, in the presence of SO_2, potentially proposes the involvement to maintain the cellular redox balance by increased oxidation of NADH/NADPH to NAD^+/$NADP^+$, as one mole of NADH per mole substrate is generated. Previous studies that reported an increase in ethyl phenols (Tchobanov et al. 2008; Harris et al. 2009; Oelofse et al. 2009; Agnolucci et al. 2010; Duckitt 2012) are supportive of this hypothesis.

The nucleotide sequences could potentially indicate the presence of different alleles present for these genes that could help elucidate variations reported in literature regarding the production of phenolic compounds and SO_2 tolerance for this yeast. This proposition is supported by the presence of both the *SSU1* and *SSU1-R* genes in *S. cerevisiae* strains (Novo et al. 2009, Perez-Ortin et al. 2002). The ploidy in different strains would therefore need to be further investigated in *B. bruxellensis* that exhibit different heterozygosity

4.6 Conclusion

In conclusion, this study investigated the expression profile of the *SSU1* and *PAD* genes as well as the effect that SO_2 has on the respective expression levels of these genes. Concrete conclusions cannot be made, as there were numerous drawbacks that inherently affected the scientific nature of the results obtained. This study therefore reports conjecture reviews. These include elevated expression levels for these genes in the presence of molecular SO_2, which to our knowledge has never before been shown for *B. bruxellensis,* and would need to be confirmed with future work. Furthermore, results on a metabolic level indicated elevated production of certain metabolites that allowed for correlations between metabolic responses and possible gene expression. The combined response observed from both gene and metabolite in the presence of SO_2, will in future work, further our knowledge regarding sulphite sensing and signalling in order to better control sulphite resistance in *B. bruxellensis.*

4.7 References

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4.8 Addendum A

SSU1 gene- Alignment of nucleotide sequences:

```
BbSSU1-1        ATGAGCCGAGCAAGCAAACACAATTCAAAAACTGAAATGGATATAGAGAGTCAAATTCGT
BbSSU1-2        ATGAGCCGAGCAAGCAAACACAATTCAAAAACTGAAATGGATATAGAGAGTCAAATTCGT
                ************************************************************

BbSSU1-1        GTGAATGAAAATGATGATGGTGTTGTTTCAGGGTGCGAAGAACGATGTTCAAGTATAAAT
BbSSU1-2        GTGAATGAAAATGATGATGGTGTTGTTTCAGGGTGCGAAGAACGATGTTCAAGTATAAAT
                ************************************************************

BbSSU1-1        GATGATAACGATACCCTTACCGCATCACCCACTAGTTCAGTATTGGGCCATCAAGAAACA
BbSSU1-2        GATGATAACGATACCCTTACCGCATCACCCACTAGTTCAGTATTGGGCCATCAAGAAACA
                ************************************************************

BbSSU1-1        CGCAAATGTTTTCAGGTGCTAGATATAATACTTGACAATCTTCACCCCATGCATTTTGTG
BbSSU1-2        CGCAAATGTTTTCAGGTGCTAGATATAATACTTGACAATCTTCACCCCATGCATTTTGTG
                ************************************************************

BbSSU1-1        ATAACCATGGGTGTTGGGATTACATCTGGTATCCTATACAACTTCCCGATTGAGTGTATA
BbSSU1-2        ATAACCATGGGTGTTGGAATTACATCTGGGATCCTATACAACTTCCCGATTGAGTGTATA
                ***************** ********** ******************************

BbSSU1-1        AGACACGGTTCAAGATACTTGGGTATTGCGTACTTCTACATTAACCTAACATGCTTTATT
BbSSU1-2        AGACACGGTTCAAGATACTTGGGTATTGCGTACTTCTACATTAACCTAACATGCTTTATT
                ************************************************************

BbSSU1-1        GTGATTCACCTACTGTTCATAATGAAATATTTCTTTCTTTGTGACAGATACAAGACCTCG
BbSSU1-2        GTGATTCACCTACTGTTCATAATGAAATATTTCTTTCTTTGTGACAGATACAAGACCTCG
                ************************************************************

BbSSU1-1        TTTAAAGACGTACTTTATGATCATCGTTTGAATGTATTCCTCGGATGCGAAGTCATGGGC
BbSSU1-2        TTTAAAAACGTACTTTATGATCATCGTTTGAATGTATTCCTCGGATGCGAAGTCATGGGC
                ****** ***************************************************

BbSSU1-1        ACATCTACCTTGATCAACATGATCTATTTCATGAGACCGGACTGGTACGTATTTGTCTAT
BbSSU1-2        ACATCTACCTTGATCAACATGATCTATTTCATGAGACCGGATTGGTACGTATTTGTCTAT
                ****************************************** ******************

BbSSU1-1        GTTCTCTGGTGGATAAATGTTGCTTTTAGTATCCTTGTTGGTTGGGGAGTCACATTTCTC
BbSSU1-2        GTTCTCTGGTGGATAAATGTTGCTTTTAGTATCCTCGTTGGTTGGGGAGTCACATTTCTC
                ********************************** ************************

BbSSU1-1        ATGTTTGCGTGTAATAAGATCAGGCCAGAGGATATAAATGCCACTATTCTTCTACCAATC
BbSSU1-2        ATGTTTGCGTGTAATAAGATCACGCCAGAGGATATAAATGCCACTATTCTTCTACCAATC
                ********************** ************************************

BbSSU1-1        GTGACTTTGACGGTTGTTGCTTCAACTGGATCTCTAATCTCTGTTTCTTTCATGGACAAT
BbSSU1-2        GTGACTTTGACCGTTGTTGCTTCAACTGGATCTCTAATCTCTGTTTCTTTCATGGACAAT
                *********** ************************************************

BbSSU1-1        CCCAAATGGCAGATCTCATCAAACATAATCACATTTCTGCTTTTCGCTAATGCTGTTGTA
BbSSU1-2        CCCAAATGGCAGATCTCATCAAACATAATCACATTTCTGCTTTTCGCTAATGCTGTTGTA
                ************************************************************

BbSSU1-1        CTCTCAATATTCATCGTTAGCGTCTATTTTGAACGACTTTTCATACACGGATTGCCACCG
BbSSU1-2        CTCTCAATATTCATCGTTAGCGTCTATTTCGAGCGACTTTTCATACACGGATTGCCACCG
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BbSSU1-1        AAGCCGGCAATTTACACCTGCTTTATTCCAATAGGTATTCTCGGCCAAGGCGGTTGGGCA
BbSSU1-2        AAGCCGGCAATTTACACCTGCTTTATTCCAATAGGTATTCTCGGCCAAGGCGGTTGGGCA
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BbSSU1-1        ATTCAATTAAATTACAAGGATGTTGGCCATTTTATAGCTCACCACAGTGGTCTAAAATGG
BbSSU1-2        ATTCAATTAAATTACAAGGATGTTGGCCATTTTATAGCTCACCACAGTGGTCTAAAATGG
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BbSSU1-1        ATGGGATTGGGCACAGATTACTCTCAAGAGATATTGTTAAGCATTGAATCAGTACTTAAC
BbSSU1-2        ATGGGATTGGGCACAGATTACTCTCAAGAGATATTGTTAAGCATTGAATCAGTACTTAAC
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BbSSU1-1        TTTTTCGGTGTTTGCATTGCTTTGGTTTTAGCATCTGTCGGTGTCTGCTTTACAGTTATC
BbSSU1-2        TTTTTCGGTGTTTGCATTGCTTTGGTTTTAGCATCTGTCGGTGTCTGCTTTACAGTTATC
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BbSSU1-1        TCTTTCATGTCTGTCATTTACTGTGGAAAGCCGCCAACATTCATAAGAACAATGTGGGCA
BbSSU1-2        TCTTTCATGTCTGTCATTTACTGTGGAAAGCCGCCAACATTCATAAGAACAATGTGGGCA
                ************************************************************

BbSSU1-1        AGCACCTTCCCCCTTGGTACCATGGCGTTGTCTTTTAACGAGATGTTCAAGACTACAAAC
BbSSU1-2        AGCACCTTCCCCCTTGGTACCATGGCGTTGTCTTTTAACGAGATGTTCAAGACTACAAAC
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BbSSU1-1        ATACAAGGATTCCACATTGTGGGTACAATATATTCGGTTATGCTTTTTCTCATAACAACA
BbSSU1-2        ATACAAGGATTCCACATTGTGGGTACAATATATTCGGTTATGCTTTTTCTCATAACAACA
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BbSSU1-1        TATTGCCTGATCAACACCGTCATATTTGAAATTCCTTTCGGCAAGATCAGGAATGTTTGC
BbSSU1-2        TATTGCCTGATCAACACCGTCATATTTGAAATTCCTTTCGGCAAGATCAGGAATGTTTGC
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BbSSU1-1        CACCAGGACGCCACAAAGGTTACCGAAAACTGA
BbSSU1-2        CACCAGGACGCCACAAAGGTTACCGAAAACTGA
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PAD gene -Alignment of nucleotide sequences:

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 BbPAD-1        ATGAAACTCCCTTGCTATCAAAACAATACGCCCCTTGATCCTTCCTTCGATGATGACCTG
 BbPAD-2        ATGAAACTCCCTTGCTATCAAAACAATACGCCTCTTGATCCTTCCTTCGATGATGACCTG
 BbPAD-3        ATGAAACTCCCTTGCTATCAGAACAATACGCCCCTTGATCCTTCCTTCGATGATGACCTG
                ******************** *********** ****************************

 BbPAD-1        AAGGACGTTCATCTCGTCTATGATTATGATGCCACAGATTCGAACGGAAGACCAGAAAAA
 BbPAD-2        AAGGACGTTCATCTTGTCTATGATTATGACGCCACAGACTCGAACGGAAAACCAGAAAAA
 BbPAD-3        AAGGACGTTCATCTTGTCTATGATTATGACGCCACAGACTCGAACGGAAAACCAGAAAAA
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 BbPAD-1        TGGAGGTATGAAATATGGTTTTTCTCAGAAAATAAAATTGTTTATGCGATTCATGGTGGT
 BbPAD-2        TGGAGGTATGAAATATGGTTTTTCTCAGAAAATAAAATTGTTTATGCGATTCATGGTGGT
 BbPAD-3        TGGAGGTATGAAATATGGTTTTTCTCAGAAAATAAAATTGTTTATGCGATTCATGGTGGT
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 BbPAD-1        CCAATGGCAGGAAGGATTAATTATCAAACGGTTGCTTATCAATGTGTACGCCCTGGAGAA
 BbPAD-2        CCAATGGCAGGAAGGATTAATTATCAAACAGTTGCTTATCAATGTGTACGCCCTGGAGAA
 BbPAD-3        CCAATGGCAGGAAGGATTAATTATCAAACAGTTGCTTATCAATGTGTACGCCCTGGAGAA
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 BbPAD-1        ATATGGCAGATAAATTGGCTTGAAGAAACAGGCACTGTTGTGTCAATAGTTTATGACATT
 BbPAD-2        ATATGGCAGATAAATTGGCTTGAAGAAACAGGCACTGTTGTGTCAATAGTTTATGACATT
 BbPAD-3        ATATGGCAGATAAATTGGCTTGAAGAAACAGGCACAGTTGTGTCAATAGTTTATGACATT
                ********************************** ************************

 BbPAD-1        GTGAATAAAACGGTAAACGGACTTCTATGCTTTTCTAAGGGACATTGGGAAAATTCTGAA
 BbPAD-2        GTGAATAAAACGGTAAACGGACTTCTATGCTTTTCTAAGGGACATTGGGAAAATTCTGAA
 BbPAD-3        GTGAATAAAACGGTAAACGGACTTCTATGCTTTTCTAAGGGACATTGGGAAAATTCTGAA
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BbPAD-1         GCTGCTCATGGGGATAAAAGAAACCCAGATGACTTTGCCCGTTGGAGAAATTTGGCCAAG
BbPAD-2         GCTGCTCATGGGGATAAAAGAAACCCAGATGACTTTGCTCGTTGGAGAAATTTGGCCAGG
BbPAD-3         GCTGCTCATGGGGATAAAAGAAATTCAGATGACTTTGCTCGTTGGAGAAATTTGGCCAAG
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BbPAD-1         CAGGGCATTCAAACCGATCGTTTCGTCTTGGTTGAAAGAGCCCATATATTGAAATCATTT
BbPAD-2         CAGGGCATTCAAACCGATCGTTTCGTCTTGGTTGAAAGGGCCCATATATTGAAATCATTT
BbPAD-3         CAGGGCATTCAAACCGATCGTTTCGTCTTGGTTGAAAGGGCCCATATATTGAAATCATTT
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BbPAD-1         AAAGGTCAGGGTGATTTGGAACCGATCGAACCTGATGCAATTACCTTTTAG
BbPAD-2         AAAGGTCAGGGTGATTTGGAACCGATCAAACCTGATGCAATTACCTTTTAG
BbPAD-3         AAAGGTCAGGGTGATTTGGAACCGATCAAACCTGATGCAATTACCTTTTAG
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The green highlighted areas are indicative of the snp among the strains tested. The red highlighted and boxed areas refer to the snp that leads to amino acid changes.

Chapter 5

General discussion and conclusions

5. General discussion and conclusion

5.1 Conclusion

Elimination of *B. bruxellensis* in contamined wineries is extremely difficult. Even though wine exhibits an extremely challenging living environment, with high ethanol levels, high SO_2 concentration and shortage of available nutrients, some *B. bruxellensis* strains are able to survive in these harsh conditions, due to strain dependent traits that allows for tolerance to SO_2 and ethanol (Dias et al. 2003; Woolfit et al. 2007; Hellborg and Piškur 2009). This yeast also exhibits the ability to utilise an extensive range of carbon and nitrogen sources (Conterno et al. 2006; Aguilar Uscanga et al. 2007; de Barros Pita et al. 2011; de Barros Pita et al. 2013). Spoilage can occur as a result of the production of numerous compounds, including fatty acids (Rozès et al. 1992; Malfeito-Ferreira et al. 1997; Licker et al. 1998), acetic acid (Scheffers 1961; Freer 2002) and volatile phenols (Chatonnet et al. 1992; Edlin et al. 1995). However, *B. bruxellensis* is most commonly associated with the production of volatile phenols (Chatonnet et al. 1995) that negatively affect the wine bouquet when present above a certain threshold.

Comparative studies have highlighted the complex and inconsistent nature of this yeast as results among authors are at times extremely contradictory to one another. Numerous inconsistencies with regards to *B. bruxellensis* growth, physical parameters, morphological changes, SO_2 tolerance and production of volatile phenols have been reported in literature. This study was aimed at investigating specific metabolic, physiological and genetic responses to SO_2 induced stress in strains from different wine producing areas, to ascertain a basis for the identification of spoilage mechanisms specific to *B. bruxellensis*.

Distinctive morphological characteristics were identified amongst three different strains investigated. Strains were observed to have unique growth rates. Pseudohyphae formation was prominent in only one strain and a primitive form was present in another (Morris 1958; Lodder 1974, Conner and Beuchat 1984). The ability of some strains to undergo these morphological changes strongly suggests an adaptation mechanism, as it was reported that pseudomycelium formation was due to nitrogen limitation or a stress response (Lo and Dranginis 1997; Zaragoza and Gancedo 2000; Gancedo 2001). Morphological changes in the presence of molecular SO_2 were indicative of the inhibiting effects SO_2 has on the cell, with retarded pseudomycelium formation, which suggests that pseudomycelium formation occurs independently from addition of SO_2. Glucose utilisation was inhibited in the presence of SO_2 indicating the negative effect on glycolysis and length of the cells were significantly

reduced in the presence of molecular SO_2. The metabolic response of *B. bruxellensis* yeast in synthetic wine media at different SO_2 concentrations further highlighted the inhibiting effect on sugar utilisation and production of metabolites in the case of acetaldehyde. A synthetic wine medium was used to simulate real wine conditions with only 2 g/L glucose and fructose. However it does present some limitations. For instance, ethanol concentration was only 10%, lower than the concentrations usually observed in real wine and no polyphenolic compounds were added.. These limitations should be thoroughly considered when interpreting the results. The peak for the secondary metabolite acetaldehyde plays an instrumental role in binding to unbound molecular SO_2, allowing for the onset of sugar utilization in this yeast however the production of acetaldehyde could also merely be attributed to the inhibitory effect on glycolytic enzymes, and would require further research. These results nevertheless confirm previous observations made by Duckitt (2012), at least for the one strain that was investigated. Future research would need to investigate the correlation between the onset of glucose utilization and the peak in acetaldehyde production, as acetaldehyde peaked at the same time point irrespective of the concentrations of SO_2 added.

Strain variation became abundantly clear during the course of this study with strains being tolerant to different concentration ranging from 0.4-1.4 mg/L of molecular SO_2. The LO2E2 strain appeared to be the most tolerant strain and the AWRI 1499 was the least tolerant strain used in this study. Each strain exhibited independent acetaldehyde production profiles, suggesting that this mechanism is a strain dependent characteristic. Conclusions regarding the expression of the *PAD* and *SSU1* genes are conjecture. The results suggest that the expression of both genes might be inducible in the presence of SO_2, but they need to be confirmed as difficulties incurred during the course of this metabolic experiment, with regards to considerable variation among replicates for some strain and lack of proper negative controls prevented from drawing final conclusions. Therefore the resulting quantitative real time PCR results would require reanalysis of data with appropriate negative controls (no SO_2 added) and additional time points. The preliminary results are nevertheless promising as they would indicate for the first time in this yeast the potentially inducible effect that SO_2 has on these genes and the importance thereof in the management of spoilage of this yeast. This study also indicates the presence of different alleles for these genes. The *PAD* gene seems to have two different alleles, present in only the AWRI 1499 strain, in comparison with all other strains tested only possessing the common allele, not present in AWRI 1499. The *SSU1* gene seems to only have a common allele and a second allele present in the IWBT Y121 and AWRI 1499 strains. These results would need to be further analysed by allelic discrimination using qRT-PCR.

This study confirmed the vast number of mechanisms that are affected in the presence of SO_2, and the contributing roles of each individual aspect and the combined response in the cell. Electron microscopy would shed light on the effect of SO_2 on the cell membrane and intracellular associated structure. Further in-depth analysis of gene expression would be required to facilitate further downstream experiments. Allelic discrimination would elucidate and confirm the presence of different alleles present for these genes. Finally, combining our current knowledge with future transcriptomic and proteomic analyses would provide a holistic view on the role of SO_2 on the cell.

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