Investigating the impact of sulphur dioxide on *Brettanomyces bruxellensis* at a molecular and cellular level

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12/12/2011

Summary

The yeast *Brettanomyces* was isolated from beer in 1904 and associated with wine thereafter. A sporulating form, *Dekkera*, was discovered later. *Brettanomyces bruxellensis* produces high levels of volatile phenol off-flavours in wine. Sulphur dioxide (SO_2) is the most widely used chemical preservative in wine. Yeasts have several mechanisms to cope with the SO_2, namely Ssu1p, a membrane bound SO_2 transporter; sulphite reduction, sulphite oxidation and acetaldehyde production. In unfavourable environmental conditions, certain yeasts can enter a viable-but-non-culturable (VBNC) state which is characterised by reduced metabolic rate, inability to reproduce on solid media and a reduction of cell size. VBNC can be triggered by chemical stress such as high SO_2 levels. The objectives of this study were to examine the SO_2 tolerance of *B. bruxellensis* and *Saccharomyces cerevisiae*, to quantify their rates of SO_2 accumulation and efflux, determine the effect of SO_2 on their energy metabolism and investigate if *B. bruxellensis* possesses an orthologue to *S. cerevisiae SSU1.*

In this study, the identity of a number of *Brettanomyces/Dekkera* strains was confirmed using 5.8S rDNA-ITS RFLP analysis and DNA sequencing. Sporulation assays were used to confirm whether these strains belonged to the *Dekkera* or *Brettanomyces* genus. A method to accurately quantify SO_2 in laboratory conditions was optimised. Molecular SO_2 tolerance was tested by spotting fresh yeast cultures on media with SO_2 and/or ethanol. Tolerance to SO_2 and/or ethanol showed highly strain dependent results with *S. cerevisiae* showing the highest tolerance levels while *B. bruxellensis* tolerated SO_2 and ethanol poorly but certain strains grew well with only SO_2. The SO_2 accumulation and efflux rates of 3 *S. cerevisiae* strains and 3 *B. bruxellensis* strains were determined. It was shown that the *S. cerevisiae* strains followed the same trends as previously found in literature whereas *B. bruxellensis* strains showed similar trends but displayed highly variable strain-dependent results.

B. bruxellensis CB63 and *S. cerevisiae* VIN13 were investigated for their response to SO_2 in two different media, TA and SWM, over a 48-hour and 32-day period respectively. Acetic acid, acetaldehyde, D-glucose, D-fructose (only in SWM) and ethanol (only in TA) were regularly monitored over the time course of each experiment. SO_2 had the greatest impact on *B. bruxellensis* with decreased rates of glucose consumption and ethanol production as well as increased acetic acid. Acetaldehyde peaked shortly after SO_2 addition with the subsequent restarting of sugar consumption for certain samples. This suggests that sufficient acetaldehyde was produced to bind free SO_2 to reduce SO_2 stress. Volatile phenols were quantified for day 32 of the SWM experiment. An increase of 4-ethyl guaiacol was correlated to higher molecular SO_2 levels. SO_2 negatively affected both yeasts energy metabolism, forcing the yeasts metabolism to adapt to ensure survival.

In general, SO_2 was shown to have a negative impact on all aspects of a yeasts growth and metabolism and that SO_2 tolerance is highly strain dependent and a far more complicated characteristic than currently understood.

Opsomming

Die gis *Brettanomyces* is in 1904 uit bier geïsoleer en daarna met wyn geassosieer. 'n sporulerende vorm, *Dekkera*, is later ontdek. *Brettanomyces bruxellensis* produseer hoë vlakke van vlugtige fenol afgeure in wyn. Swaweldioksied (SO_2) is die mees gebruikte chemiese preserveermiddel in wyn. Giste het verskeie meganismes om SO_2 te hanteer, naamlik Ssu1p, 'n membraan-gebonde SO_2 transporter, sulfietvermindering, sulfiet-oksidasie en asetaldehiedproduksie. In ongunstige omgewingstoestande kan sekere giste 'n lewensvatbare, maar nie-kultiveerbare (LMNK)-toestand aanneem wat gekenmerk word deur verlaagde metaboliese tempo, onvermoë om voort te plant op soliede media en 'n vermindering van die selgrootte. LMNK kan veroorsaak word deur chemiese stres, soos hoë SO_2-vlak. Die doelwitte van hierdie studie was om die SO_2 -bestandheid van *B. bruxellensis* en *Saccharomyces cerevisiae* te ondersoek, hul spoed van SO_2 -opneming/akkumulasie en -uitskeiding te kwantifiseer, die invloed van SO_2 op energiemetabolisme te bepaal en te ondersoek of *B. bruxellensis* oor ‘n soortgelyke geen as die *S. cerevisiae SSU1* beskik.

In hierdie studie is die identiteit van 'n aantal *Brettanomyces/Dekkera*-stamme bevestig deur 5.8S rDNA-ITS RFLP-analise en DNA-opeenvolging te gebruik. Sporulasietoetse is gebruik om te bevestig of hierdie stamme aan die genus *Dekkera* of *Brettanomyces* behoort. 'n Metode om SO_2 onder laboratoriumtoestande akkuraat te kwantifiseer, is geoptimiseer. Molekulêre SO_2-bestandheid is getoets deur vars giskulture op media met SO_2 en/of etanol te groei. Bestandheid teen SO_2 en/of etanol het stam-afhanklike resultate getoon, *S. cerevisiae* wat die hoogste toleransievlakke getoon het, terwyl *B. bruxellensis* SO_2 en etanol swak tolereer, maar sekere stamme het goed gegroei met slegs SO_2. Die SO_2-akkumulasie en -uitskeidingtempo van 3 *S. cerevisiae*-rasse en 3 *B. bruxellensis*-stamme is bepaal. Daar is gevind dat die *S. cerevisiae*-rasse dieselfde tendens soos voorheen in die literatuur beskryf, gevolg het, terwyl *B. bruxellensis*-stamme soortgelyke tendense getoon het,maar hoogs veranderlike stam-afhanklike resultate vertoon.

B. bruxellensis CB63 en *S. cerevisiae* VIN13 is ondersoek vir hul reaksie tot SO_2 in twee verskillende media, TA en SWM, oor 'n tydperk van 48-uur en 32-dae onderskeidelik. Asynsuur, asetaldehied, D-glukose, D-fruktose (slegs in SWM) en etanol (slegs in TA) is gereeld gemoniteer oor die verloop van elke eksperiment. SO_2 het die grootste impak op *B. bruxellensis* met ‘n verlaagde tempo van glukoseverbruik en etanolproduksie, sowel as verhoogde asynsuur. ‘n Asetaldehiedhoogtepunt is bereik kort na die SO_2-byvoeging met die daaropvolgende hervatting van suiker wat vir sekere monsters gebruik is. Dit dui daarop dat voldoende asetaldehied geproduseer is om vry SO_2 te bind om SO_2-stres te verminder. Vlugtige fenole is op dag 32 van die SWM-eksperiment gekwantifiseer. 'n Toename van 4-etiel-guajakol korreleer met hoër molekulêre SO_2-vlakke. SO_2 het beide giste se energiemetabolisme negatief beïnvloed, wat die gis dwing om sy metabolisme aan te pas om oorlewing te verseker.

Oor die algemeen het SO_2 'n negatiewe impak op alle aspekte van giste se groei en metabolisme, en SO_2-bestandheid is hoogs stam–afhanklik. Dit is ook 'n baie meer ingewikkelde kenmerk as wat tans verstaan word.

Acknowledgements

I would like to dedicate this work to my family for their love and support throughout my university studies.

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Abbreviation list

4-Ethyl phenol 4-EP
4-Ethyl guaiacol 4-EG
4-Ethyl catechol 4-EC
Gas Chromatography/Mass Spectrometry ..... GC/MS
Glyceraldehyde-3-phosphate dehydrogenase ..... GAPDH
Fluorescent *in situ* hybridisation ..... FISH
Molecular SO_2 ..... mSO_2
Phenolic acid decarboxylase ..... PAD
Potassium metabisulphite ..... PMB
Sulphur amino acid biosynthesis pathway ..... SAAB
Sulphur dioxide ..... SO_2
Vinyl phenol reductase ..... VPR
Viable but non-culturable ..... VBNC

CHAPTER 1

General introduction

1 General introduction

The modernisation of the wine fermentation process has greatly minimised the occurrence of spoiled wine product due to contamination by unwanted yeasts and bacteria. Practises such as cellar equipment and barrel sanitation and/or sterilisation, the maintenance of hygienic cellar conditions, minimisation of grape must oxygen contact, controlled fermentation conditions and correct molecular sulphur dioxide (SO_2) concentration maintenance can significantly reduce the growth of spoilage microorganisms such as *Acetobacter aceti*, *Pediococcus damnosus* and *Brettanomyces bruxellensis*.

In light of this, wine spoilage is still an economic burden on wine cellars. *B. bruxellensis* is a notorious red wine spoilage yeast due to its tolerance to harsh environmental conditions such as minimal nutrient availability, high ethanol and high SO_2 levels. It can survive throughout the wine fermentation, as well as filtration and bottling in a state known as viable-but-non-culturable (VBNC) where the cell enters a state with a reduced metabolic output, reduced cell size and the inability to grow on routine solid microbiological growth media (Agnolucci *et al.* 2010; Coulon *et al.* 2011; Divol and Lonvaud Funel 2005; Serpaggi *et al.* 2011). When *B. bruxellensis* is in the VBNC state it is still capable of producing off-flavours such as the volatile phenols 4-ethyl phenol and 4-ethyl guaiacol (Serpaggi *et al.* 2011) and possibly acetic acid and iso-valeric acid as well.

SO_2, in the form of potassium metabisulphite, is the most commonly used chemical antimicrobial agent that is added to wine to assist in the control of unwanted microorganisms (Ribéreau-Gayon 2006). Many yeast and bacteria species will persist during the wine fermentation if the correct level of molecular SO_2 is not maintained. It has been shown by numerous authors that a 0.8 mg/L molecular SO_2 level is the optimum level to control almost all yeast and bacteria species (du Toit *et al.* 2005). However, *B. bruxellensis* has been shown to persist and spoil wines when the molecular SO_2 level is maintained at this concentration (Serpaggi *et al.* 2011). At the correct intracellular concentration, SO_2 negatively

impacts the cell metabolic processes by the inhibition of key metabolic enzymes such as glyceraldehyde-3-phosphate dehydrogenase, ATPase, alcohol dehydrogenase, aldehyde dehydrogenase and NAD^+-glutamate dehydrogenase. This results in a loss of ATP generation and NADH regeneration and ultimately cell death if sufficient enzyme inhibition occurs (Hinze and Holzer 1986; Macris and Markakis 1974; Maier *et al.* 1986; Pagano *et al.* 1990; Schimz 1980; Schimz and Holzer 1979).

It is therefore vital for the cell to be able to cope with the presence of high levels of intracellular SO_2 and yeast have been shown to have mechanisms in place to assist in the removal and detoxification of intracellular SO_2. In *S. cerevisiae* a membrane bound protein transporter, ssu1p, has been shown to actively efflux SO_2 out of the cell and that over- and under-expression of this protein resulted in increased or decreased tolerance to SO_2, respectively (Park and Bakalinsky 2000). Other SO_2 coping mechanisms are also presented such as the indirect production of acetaldehyde (Stratford *et al.* 1987), sulphite reduction (Kobayashi and Yoshimoto 1982 ; Yoshimoto and Sato 1968) and sulphite oxidation (Ingrid Beck-Speier *et al.* 1985; Feng *et al.* 2007; C. Friedrich *et al.* 2005; Heimberg *et al.* 1953; Ulrike Kappler and Christiane Dahl 2001).

It is crucial to further examine these cellular processes to enhance our knowledge on how to prevent growth or control *B. bruxellensis* in a wine fermentation. Insight into the least amount of SO_2 needed to prevent *B. bruxellensis* spoilage will not only have knock on effects such as an increase in wine quality by the reduction of SO_2-related negative sensorial attributes but also decrease the prevalence of consumer sulphite allergic reactions.

Project aims

This study is aimed at evaluating the impact of SO_2 at a molecular and cellular level in *B. bruxellensis*. The main objectives were the following:

a) To determine the tolerance to SO_2 of *B. bruxellensis* in comparison to *S. cerevisiae*;

b) To quantify the rate of intracellular accumulation and extracellular efflux of SO_2 of *B. bruxellensis* in comparison to *S. cerevisiae* and to assess whether this correlates to SO_2 tolerance;

c) To ascertain the response of *B. bruxellensis* energy metabolism to the presence of molecular SO_2 by monitoring the levels of metabolites such as acetaldehyde, acetic acid, ethanol and D-glucose/D-fructose in two different media simulating growth and survival conditions.

CHAPTER 2

Literature review:

Surviving in wine: a review of known cellular mechanisms – Does Brettanomyces bruxellensis *stand out?*

2 Literature review

2.1 *Brettanomyces/Dekkera* spp. and wine

2.1.1 History and taxonomy of *Brettanomyces/Dekkera* spp.

Brettanomyces was first isolated from British beer in 1904 (Andrews and Gilliland 1952; Claussen 1904; Gilliland 1961; Halcrow et al. 1966). It was thereafter also associated with wine in 1960 (Kunkee and Amerine 1970; Walt and Kerken 1960). Yeasts belonging to the *Brettanomyces* genus were later isolated by Kufferath *et al*. (1921) with the resultant creation of the genus in 1952 by Lodder *et al.* (1952). It was not regarded as a spoilage yeast in beer as it contributed to the beer flavour. However, the link between red wine spoilage and *Brettanomyces* presence in wine was made only much later by Chatonnet *et al.* (1995) when it was shown that *Brettanomyces* produced 4-ethyl-phenol, a potent off-flavour compound.

The *Brettanomyces* genus has undergone many revisions and reclassifications in the past. *Dekkera* was classified taxonomically by van der Walt in 1964 after it was observed that certain *Dekkera bruxellensis* strains sporulated (Van der Walt 1984).

Table 1
Teleomorphic and anamorphic species of *Dekkera* and *Brettanomyces*

Teleomorph (sporulating)	Anamorph (non-sporulating)
Dekkera anomala	*Brettanomyces anomalus*
Dekkera bruxellensis	*Brettanomyces bruxellensis*
n/a	*Brettanomyces naardenensis*
n/a	*Brettanomyces nanus*
n/a	*Brettanomyces custersianus*

The *Brettanomyces* and *Dekkera* genera jointly share the species *Brettanomyces anomalus* and *Brettanomyces bruxellensis.* The difference between the two genera is that *Brettanomyces* is the anamorphic (non-sporulating) and *Dekkera* the teleomorphic (sporulating) form. Teleomorphic forms of *Brettanomyces custersianus, Brettanomyces naardenensis* and *Brettanomyces nanus* have not been detected (Table 1). For the remainder of this thesis the genus *Brettanomyces* will be used and where confusion may arise between *Brettanomyces* and *Dekkera,* the distinction between the two will be detailed further.

2.1.2 *Brettanomyces* and off-flavour production

The off-flavours produced in wine by *Brettanomyces* species are often described as sweaty, mousy, medicinal, sewage, barnyard and smoky as well as combinations of these compounds (Chatonnet et al. 1992; Etievant et al. 1989). These odours are due to the production of volatile compounds such as 4-ethyl-phenol, 4-ethyl-guaiacol and 4-ethyl-catechol (4-EP, 4-EG and 4-EC respectively) which are described as smoky, medicinal, clove-like or spicy, the N-heterocycle family (such as 2-ethyl-3,4,6-tetrahydropyridine) which contribute to the mousy off-flavour (Costello and Henschke 2002; Grbin and Henschke 2000; Grbin et al. 2007) and isovaleric acid which is described as rancid (Romano *et al.* 2009). *Brettanomyces* is also a major contributor to volatile acidity by the production of acetic acid (Freer 2002) which is enhanced when increased oxygen is present in the environment (Aguilar Uscanga *et al.* 2003). Sensorially, the threshold level at which these volatile compounds impart negatively on a wine is dependent on consumer preference and wine style as summarised in Table 2.

Table 2
Threshold detection levels of off-flavours and their sensorial impact in wine. *model wine, **red wine, ***water. Adapted from Oelofse *et al* (2008).

Product	Precursor	Product concentration in red wine (µg/l)	Odour	Odour threshold (µg/l)
4-Vinyl-phenol	*p*-coumaric acid	8.8–4.3	Phenol, Medicinal	440*/600**
4-Vinyl-guaiacol	ferulic acid	0.2–15	Clove-like	33*/110**
4-Vinyl-catechol	caffeic acid	unknown	Phenol	unknown
4-Ethyl-phenol	4-Vinyl-phenol	118–3696	Smoky, medicinal,	30-60
4-Ethyl-guaiacol	4-Vinyl-guaiacol	1–432	Clove, spice	20***
4-Ethyl-catechol	4-Vinyl-catechol	27–427	Medicinal	10*
N-heterocyclic	D/L-Lysine	2.7-106	Mousy	0.1-1.6
Iso-valeric acid	L-Leucine	500-2000	Rancid	unknown

The precursors for 4-EP, 4-EG and 4-EC, *p*-coumaric acid, ferulic acid and caffeic acid respectively, occur naturally in wine grapes. During wine maturation, either in tank, bottle or barrel, *Brettanomyces* cell lysis will result in the release of the intracellularly located phenolic

acid decarboxylase (PAD) and vinyl phenol reductase (VPR) enzymes [It is important to note that *B. bruxellensis* and *B. anomalus* possess both PAD and VPR, whereas *B. nanus, B. naardenensis* and *B. custerianus* only possess VPR (Harris *et al.* 2009)]. This will result in the increased synthesis of volatile phenols due to the exposure of the enzymes to available hydroxycinnamic acids in the wine (Godoy et al. 2008). Synthesis of 4-EP, 4-EG and 4-EC occurs in a two-step enzymatic process as illustrated in Figure 1. An initial decarboxylation step is catalysed by PAD with the formation of vinyl phenol intermediates and the creation of one CO_2 (Chatonnet et al. 1992; Chatonnet et al. 1993; Edlin et al. 1995).

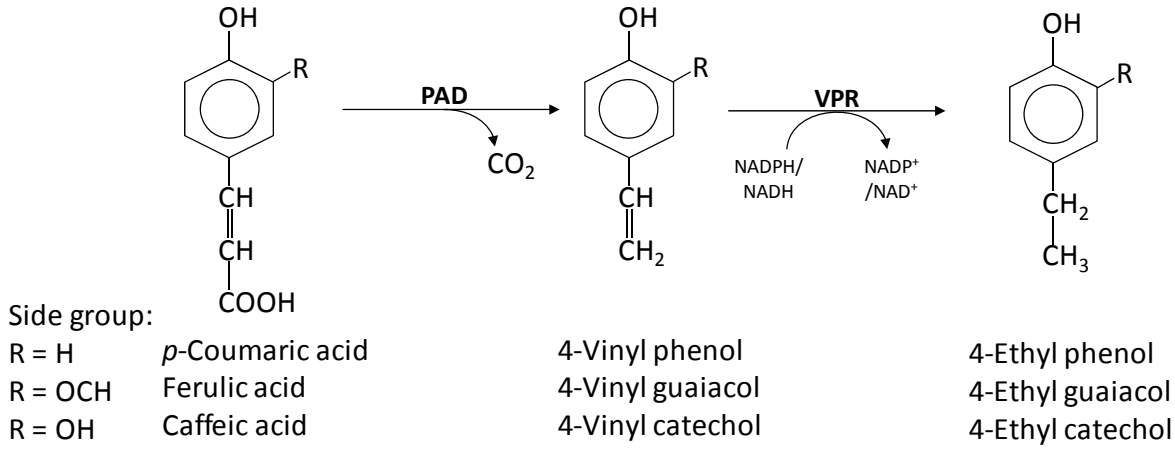

Figure 1
The synthesis of volatile phenolics from hydroxycinnamic acids. Edlin *et al*. (1998), Oelofse (2008), Tchobanov *et al*. (2008), Benito *et al.* (2009) and Harris *et al.* (2009).

These intermediates are then reduced by VPR (Godoy *et al.* 2009; Harris *et al.* 2009) while the co-factors NADPH/NADH are oxidised (Godoy et al. 2008). Unclear evidence is present in the literature detailing the preference of VPR to NADH or NADPH. According to Godoy *et al.* (2008), a 25% increase in VPR activity is seen when NADPH is oxidised compared to NADH where Tchobanov *et al.* (2008) found a 50-fold increase in VPR activity in the presence of NADH. The kinetic, optimal temperature and pH properties of PAD and VPR are summarised in Table 3.

Table 3
Kinetic and optimal temperature and pH properties of the PAD and VPR enzymes (Godoy *et al.* 2008).

Enzyme	K_m (mM)	V_{max} (µmol/min/mg)	Active pH	Optimum pH	Optimum °C
phenolic acid decarboxylase	1.22±0.08	98±0.15	3.0-8.0	6	40
vinyl phenol reductase	>3.37±2.05	107.62±50.38	3.0-10.0	6	25.0

The PAD and VPR enzymes of *Brettanomyces* were found to be inhibited at high ethanol levels (15-20% v/v), low pH (pH 1.75 – 2), moderate free SO_2 levels (9.6 – 20.2 mg/L, pH 3.5), high sorbic acid levels (900 – 1100 mg/L), high benzoic acid levels (150 – 200 mg/L) and low and high temperature ranges (0 – 15°C and 30 – 40°C) (Benito et al. 2009; Godoy et al. 2008).

2.1.3 Identification and quantification of *Brettanomyces/Dekkera*

Conventional microbiological methods such as selective growth media and biochemical testing have been used to isolate and identify species or genera for many decades (Yarrow 1998). These techniques, although highly important as an initial identification step, have been largely superseded by more recent molecular biology based identification techniques. Several molecular biology techniques have been developed in the past 30 years to accurately identify and detect the presence of microorganisms in complex environments such as food and beverages (refer to Ivey and Phister (2011) for a complete review). Along with the complex environmental matrix which these environments present, many chemical and physical inhibitors e.g. polyphenols and tannins, are also present in wine. These inhibitors can lead to false-negative identifications as well as the incorrect quantification of microorganism populations (Cocolin *et al.* 2004; Delaherche *et al.* 2004; Ibeas *et al.* 1996).

Molecular biology techniques such as mitochondrial DNA restriction analysis (Martorell et al. 2006), restriction fragment length polymorphism analysis (RFLP) (Dias 2003; Esteve-Zarzoso *et al.* 1999; Martorell *et al.* 2005; Zott *et al.* 2010), random amplified polymorphic DNA (RAPD) PCR (Agnolucci *et al.* 2009; Martorell *et al.* 2006; Mitrakul *et al.* 1999),

amplified fragment length analysis (AFLP) (Curtin et al. 2007; Esteve-Zarzoso et al. 2010), electrophoretic karyotyping (Mitrakul *et al.* 1999), quantitative real-time PCR (qRT-PCR) (Agnolucci *et al.* 2007; Andorrà *et al.* 2010; Delaherche *et al.* 2004; Phister and Mills 2003; Salinas *et al.* 2009; Tessonnière *et al.* 2009; Zott *et al.* 2010), intron splice site PCR amplification (ISS-PCR) (Vigentini *et al.* 2010), loop-mediated isothermal amplification (Hayashi et al. 2007), fluorescent-based detection (Roder *et al.* 2007) and flow-cytometry (Serpaggi et al. 2010) have all been used to successfully detect and classify prokaryotic and eukaryotic microorganisms. All these techniques have been used and/or optimised to identify, detect and/or enumerate *Brettanomyces* in wine (Table 4).

Identification of *Brettanomyces* at the strain level is a much more complex task compared to species identification due to the complex structure of *Brettanomyces* genome. Hellborg and Piskur (2009) analysed 30 strains of *Dekkera bruxellensis* from geographically distinct locations around the world. Analysis of the nuclear rDNA 26S D1/D2 domain and mitochondrial 15S rDNA confirmed that all strains belonged to *D. bruxellensis*. Variation in the number of 26S loci was detected, with two strains having two different copies of the 26S rDNA locus.

Karyotyping is 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. It has been used previously to distinguish between *Brettanomyces* strains (Miot-Sertier and Lonvaud-Funel 2007; Mitrakul *et al.* 1999). It is usually expected that the chromosome number does not vary between strains of a given species. However in the study by Hellborg and Piskur, it was shown that the number of chromosomes varied significantly between 4 and 9 and their size between 1 and 6 Mbp in the analysed *D. bruxellensis* strains.

Table 4
Summary of molecular biology identification techniques used to identify and quantify yeast

Method	Target organism	Source	Authors
Nested PCR	***Dekkera/Brettanomyces***	Sherry	*Ibeas et al.*(1996)
mtDNA and RAPD-PCR restriction	***Dekkera bruxellensis****; Pichia guilliermondii; Brettanomyces bruxellensis;*	wine	Agnolucci *et al.*(2009); Martorell *et al*.(2005);
ITS-RFLP-PCR;	***Dekkera bruxellensis****; Pichia guilliermondii*	wine	Dias (2003)
ITS-RFLP-PCR	*Candida* sp.*; Debaromyces* sp.*;* ***Dekkera* sp.***; Hansenula* sp.*; Issatchenkia* sp.*; Kluyveromyces* sp.*; Lodderomyces* sp.*; Metschnikowia* sp.*; Pichia* sp.*; Saccharomyces* sp.*; Saccharomycodes* sp.*; Schizosaccharomyces* sp.*; Torulaspora* sp.*; Zygoascus* sp.*; Zygosaccharomyces* sp.*;*	Food / beverages	Esteve-Zarzoso *et al* (1999); Martorell *et al.* (2005);
qRT PCR; ITS-RFLP-PCR	*Issatchenkia* sp.*; Metschnikowia* sp.*; Torulaspora* sp.*; Hanseniaspora* sp.*; Candida* sp.*; Saccharomyces* sp.*;*	wine	Zott *et al* (2010)
Real Time-PCR	***Dekkera bruxellensis***	wine	Agnolucci et al. (2007); Phister and Mills (2003);
	Dekkera bruxellensis*; Hanseniaspora uvarum;Candida zemplinina; Saccharomyces cerevisiae; Pichia anomala; Zygosaccharomyces bailii;*	wine	Andorrà *et al.* (2010)
	Saccharomyces cerevisiae	wine	Salinas *et al*. (2009)
	Brettanomyces bruxellensis*;*	wine	Delaherche *et al.* (2004)
RFLP Karyotyping; RAPD-PCR;	***Dekkera* sp.*;*** *Candida* sp.*; Hanseniaspora* sp.*; Pichia* sp.*; Saccharomyces* sp.*; Brettanomyces* sp.*;*	wine	Mitrakul *et al.* (1999)
ITS-RFLP-PCR; AFLP	***Dekkera* sp.*;***	wine	Curtin *et al*. (2007)
	Hanseniaspora uvarum; Candida zemplinina; Saccharomyces cerevisiae; Hanseniaspora vineae; Pichia anomala; Saccharomycodes ludwigii; Zygosaccharomyces bailii;	wine	Esteve-Zarzoso *et al*. (2010)
ISS-PCR	***Dekkera bruxellensis***	wine	Vigentini *et al.* (2010); Oelofse *et al.*(2009);
LAMP	***Brettanomyces/Dekkera* sp.**	wine	Hayashi *et al*. (2007)
(PNA) FISH	***Brettanomyces/Dekkera* sp.**	wine	Dias (2003); Millet and Lonvaud-Funel (2000); Roder *et al.* (2007); Stender *et al.* (2001);
Flow cytometry	***Brettanomyces/Dekkera* sp.**	wine	Serpaggi *et al.* (2010)
Chemiluminescent DNA sensor	***Brettanomyces bruxellensis***	wine	Cecchini *et al.* (2011)

2.1.4 The complexities of the *Brettanomyces bruxellensis* genome

B. bruxellensis has long been studied for its ability to spoil red wine but very little is known about *B. bruxellensis* at the genetic and genomic levels and where it fits into the yeast evolutionary tree based on current DNA sequences. Woolfit *et al.* (2007) sequenced approximately 40% of the *D. bruxellensis* petite positive (lacks a mitochondrial genome) mutant strain, Y1031. The strain was chosen because of its small estimated genome size of 19.4Mb (*B. bruxellensis* genome sizes have been shown to vary from 20 to 30Mb) (Siurkus 2004) and because mitochondrial DNA would not interfere with the sequencing. 40% of the genome was covered in the sequencing reaction and yielded 2606 complete or partial protein-coding gene sequences. These sequences were identified based on homology with *S. cerevisiae* genes. Approximately 3-7% of the genome shows duplication. 277 other genes were identified which showed orthology in other *Saccharomycetalous* yeasts.

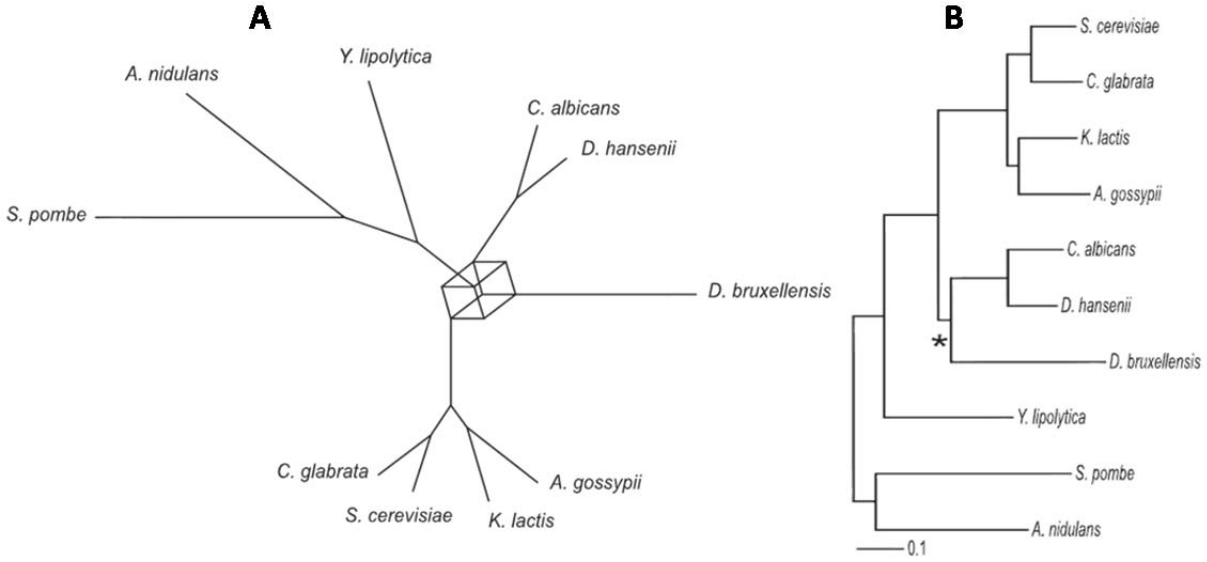

Figure 2
(**A**) Consensus network based on 396 protein sequences from *D. bruxellensis* and nine fungal species. (**B**) A phylogenetic topology inferred from (**A**), with *D. bruxellensis* position indicated with a *(Woolfit et al. 2007).

Woolfit *et al.* (2007) constructed a consensus network based on 396 protein sequences from *D. bruxellensis* and nine fungal species (Figure 2). The degree of incongruence between the protein sequences was calculated by generating a list of splits from the complete tree sets and weighting them according to how frequently they occurred. A box designates an

incompatible split in the tree. The incompatible split in Figure 2 (A) is joined by three branches in the tree and creates three possible phylogenetic topologies.

In Figure 2 (B) the most likely phylogenetic topology of the three possibilities deduced from Figure 2 (A) is illustrated. The topology was chosen as it was the best fit for the protein sequence data. However, it is only marginally better than the second choice but significantly better than the third topology choice. From the topology, one can deduce that *Dekkera* is closely related to *Candida* and *Debaryomyces* and not closely related to *Saccharomyces*. However, one criticism of such a protein sequence based phylogenetic topology is that the number of proteins analysed needs to represent a significant portion of the organism's proteome to which it is being compared. *Saccharomyces cerevisiae* has approximately 5800 genes and according to the Saccharomyces Genome Database (Mitrakul *et al.* 1999), 4928 open reading frames have been verified. Therefore the true phylogenetic relationship between *D. bruxellensis* and *S. cerevisiae* could only be elucidated if the full protein complement of *D. bruxellensis* was examined.

2.2 Sulphur dioxide and its role in wine making

2.2.1 History of sulphur dioxide in food and wine

SO_2 has been used throughout history as an antimicrobial agent to prevent unwanted spoilage microorganisms from growing in wine. It also acts as a strong anti-oxidant, preventing oxidative browning in white and red wine by binding to H_2O_2 as well as the inhibition of enzymatic oxidation (Bradshaw *et al.* 2001; Gomez *et al.* 1995; Li *et al.* 2008; Main and Morris 1991). It is commonly added to wine in the form of potassium metabisulphite (PMB) although other forms such as sodium sulphite can also be used.

Cellar equipment is sanitise/sterilised using liquid PMB whereas tanks and barrels are sanitise/sterilised through the burning of molecular sulphur. Unwanted yeasts and bacteria survive on the surfaces of most cellar equipment and it is therefore crucial to follow standard cellar hygiene practises to prevent the introduction of potential spoilage microorganisms into the wine (Malfeito-Ferreira 2005).

2.2.2 The chemistry of SO_2 in wine

Once sulphur is added to wine or any aqueous solution, in any of the commonly used forms, it dissociates into three molecular species namely molecular SO_2, the active antimicrobial species of SO_2 against microorganisms (Schimz 1980), ($SO_2.H_2O$), bisulphite (HSO_3^-) and sulphite (SO_3^{2-}) as illustrated below:

$$SO_2 + H_2O \leftrightarrow SO_2.H_2O$$
$$SO_2.H_2O \leftrightarrow HSO_3 + H^+$$
$$HSO_3^- \leftrightarrow SO_3^{2-} + H^+$$

The chemical equilibrium between each species is dependent on the pH of the wine. As seen in Figure 3, 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. In general, the pH of wines varies between 3 and 4 and therefore the dominant SO_2 species in wine is the bisulphite anion HSO_3^-. SO_2 behaviour in wine and its interaction with the various compounds in wine is however not as simple as depicted in Figure 3.

SO_2 is a strong reducing agent and anti-oxidant. When sufficient SO_2 is added to the wine, it can prevent the oxidation of compounds such as anthocyanins in red wines and reduces the effects of browning in white wines caused by oxidative enzymes. However when added to wine, excess SO_2 can cause bleaching of anthocyanins resulting in a loss of colour in red wine (Bakker et al. 1998) as well as negatively affecting the organoleptic properties of the wine. HSO_3^- and SO_3^{2-} are highly reactive and can bind many of the compounds present in wine. It is therefore said that SO_2 exists in “free” and “bound” forms.

The “free” species is the portion of HSO_3^- and SO_3^{2-} which is not already bound to compounds such as acetaldehyde, anthocyanins and organic acids present in the wine (Burroughs 1975; Ribéreau-Gayon 2006). The concentration of free SO_2 present in wine is critical as it is the only form of SO_2 which is available to bind the compounds which would otherwise oxidise important flavour and colour compounds in the wine.

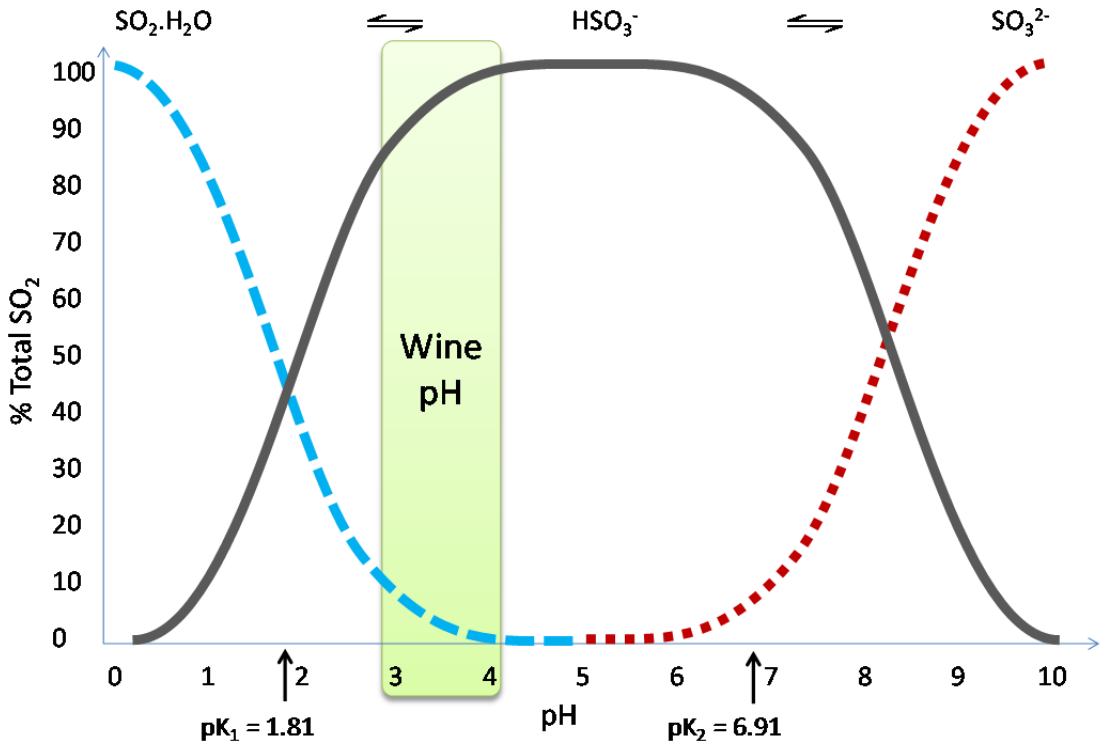

Figure 3
SO_2 species and their approximate concentration throughout the pH range.

2.2.3 Effect of SO_2 on growth of wine yeasts and *Brettanomyces* spp. in wine

The tolerance of natural grape microbial flora to SO_2 varies greatly and certain species can survive at SO_2 concentrations far higher than the legal limits allowed in wine. Yeasts such as *Schizosaccharomyces pombe* can grow at 300 mg/L SO_2 whereas other common wine yeasts such as *Hanseniaspora uvarum* and *Candida* spp. die off quickly in the presence of 50 mg/L SO_2 (Cocolin and Mills 2003; Yang 1975).

The main wine fermenter *Saccharomyces cerevisiae* tolerance to SO_2 is highly strain dependent: 64 mg/L SO_2 causes cell death in type-strains (Schimz and Holzer 1979) and *S. cerevisiae* wine strains have been specifically bred to tolerate SO_2 in wine and can grow at 256 mg/L total SO_2. Molecular SO_2 is the active antimicrobial SO_2 form and should be maintained at a concentration of 0.4 mg/L up to 0.8 mg/L to prevent growth of spoilage microorganisms (du Toit *et al.* 2005).

The concentration of molecular SO_2 in the wine can be calculated using the following formula:

$$[\mathbf{Molecular\ SO_2}] = \frac{[\mathbf{Free\ SO_2}]}{\mathbf{1 + 10^{pH-1.8}}}$$

Ribéreau-Gayon *et al.*(2006)

The pH of the wine should be maintained below 4.0 as this is crucial to ensure sufficient molecular SO_2 is present to prevent growth of unwanted microorganisms as seen in Fig. 3.

Cocolin and Mills (2003) carried out mixed culture fermentations with *S. cerevisiae, H. uvarum* and *Candida EJ1.* As seen in Figure 4, it was observed that after treating the must with 50 mg/L PMB on day 0, *S. cerevisiae* cell count decreased to almost 0 on day 1 and recovered fully to complete the fermentation. *H. uvarum* and *Candida EJ1* however did not recover at all after the initial SO_2 treatment. SO_2 therefore has a strong negative impact on cell viability.

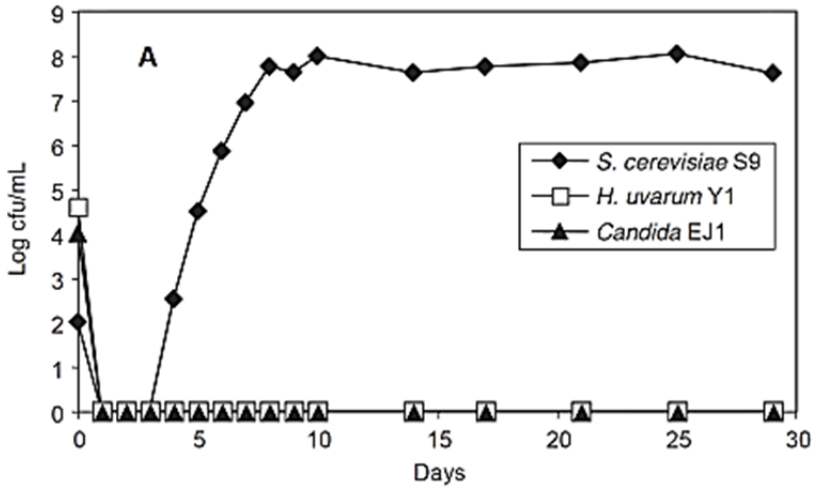

Figure 4
Colony forming unit analysis of a wine fermentation containing 50 mg/L added SO_2 (Cocolin and Mills, 2003).

Barata *et al.* (2008) inoculated dry red wine with 19 strains of *Brettanomyces*. The type strain ISA 1649 did not recover after inoculation into the wine. This could be due to the fact that ISA 1649 was isolated from lambic beer and therefore it is only adapted to low SO_2 levels and not the average wine environment. As seen in Figure 5, ISA 1703 and ISA 2173 did not recover

after the addition of 100 mg/L PMB, ISA 2298 and 2172 did recover. *Brettanomyces* tolerance to SO_2 is therefore strain dependent and the effect of SO_2 on the cell occurs quite rapidly.

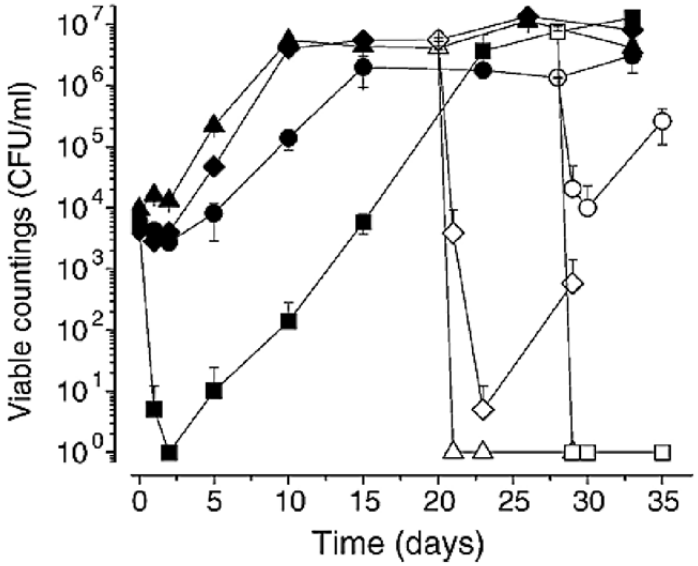

Figure 5
The effect of the addition of 100 mg/L potassium metabisulphite on the viability of several strains of *D. bruxellensis* grown in red wine with pH 3.5 and 12% ($^{v}/_{v}$) of ethanol. Symbols: (■, □) ISA 1703; (●, ○) ISA 2298; (▲, △) ISA 2173; (◆, ◇) ISA 2172. Before sulphite addition: filled symbols; after sulphite addition: open symbols (Barata *et al.* 2008).

2.2.4 Mechanism of SO_2 antimicrobial action

As previously mentioned, molecular SO_2 is the dominant species between pH 0 and 2. At common wine pH, molecular SO_2 contributes approximately 0-5% of the sulphur in wine. As molecular SO_2 has no charge, it passes easily through microbial cell membranes by simple diffusion (Stratford and Rose 1986). Once inside the cell, where the pH is approximately 5.5 - 6.5 (Imai and Ohno 1995), the molecule rapidly dissociates into bisulphite and sulphite anions. This decreases the intracellular molecular SO_2 concentration allowing more molecular SO_2 to enter the cell by diffusion.

SO_2 inhibits microbial growth by interfering with intracellular processes. SO_2 is a highly reactive molecule and it binds to many metabolites and enzymes in the cell. The influx of SO_2 into an eukaryotic cell results in the immediate inhibition (mechanism unknown) of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a critical enzyme in the glycolysis

pathway (Hinze and Holzer 1986). This enzyme is responsible for the conversion of glyceraldehyde 3-phosphate to D-glycerate 1,3-bisphosphate in a two-step catalysis as seen in Figure 6. Casalone *et al.* (1992) showed that in *S. cerevisiae* S288C, GAPDH lost 100% of its activity after incubating in 2mM sulphite after 45 minutes and activity was not restored after 90 minutes. The inhibition of GAPDH results in the subsequent stalling of glycolysis and the reduction of ATP produced as well as NADH regeneration.

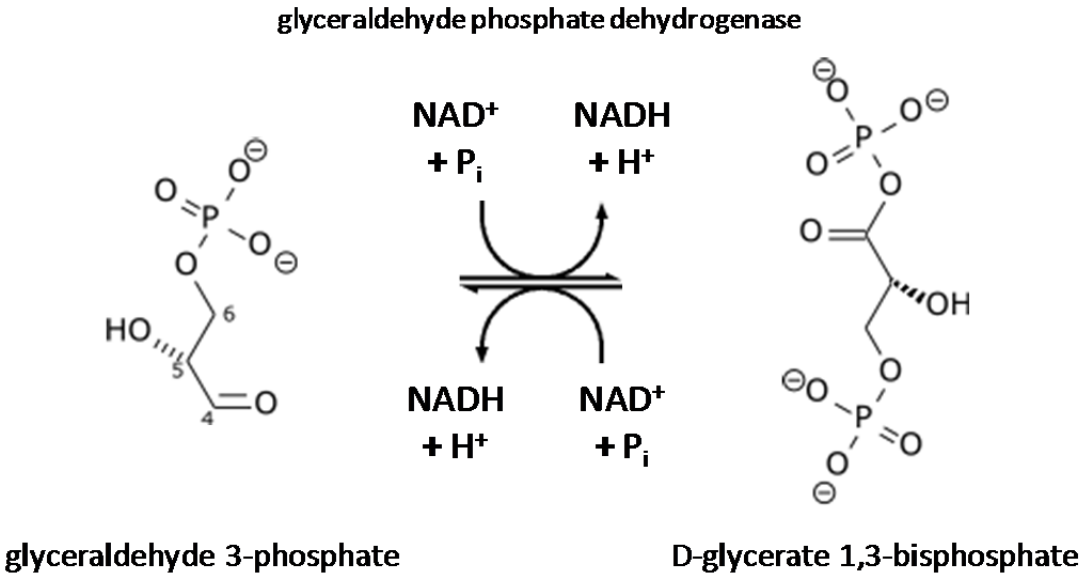

Figure 6
The two-step conversion of glyceraldehyde-3-phosphate to D-glycerate 1,3-bisphosphate (Glycolysis / Gluconeogenesis http://www.genome.jp/kegg-bin/show_pathway?map00010)

As reported by Maier *et al.* (1986), removal of sulphite from the cell by the addition of acetaldehyde to the medium resulted in ATP returning to 70% of its original intracellular level. Due to sulphite's high affinity for acetaldehyde, the formation of ethanol is inhibited and the subsequent NADH regeneration does not occur. Other enzymes such as ATPase, alcohol dehydrogenase, aldehyde dehydrogenase and NAD^+-glutamate dehydrogenase are inhibited by sulphite but it is not known if the inhibition is reversible (Maier *et al.* 1986).

Besides the inhibition of key metabolic enzymes, sulphite also binds to other proteins (destroying the disulphide bridge), co-enzymes (NAD^+ and FAD^+) and co-factors such as the vitamins thiamine and menadione (Carmack *et al.* 1950). It has also been shown that

sulphite can cause DNA point mutations by changing A/T to C/G (Meng and Zhang 1992; Mukai *et al.* 1970; Pagano and Zeiger 1987; Pagano *et al.* 1990).

As stated previously, sulphite has a major negative effect on the energy metabolism in eukaryotes. The increased stress placed on the cell can ultimately lead to cell death. In order to prevent this fatal outcome, yeasts have developed an array of defence mechanisms that will be described in the following sections.

2.2.5 Microbial SO_2 resistance mechanisms

S. cerevisiae has been used as a model organism to determine yeasts response to SO_2 (Park and Bakalinsky 2000). Bacteria, yeast and mammalian cells have been shown to have three cellular responses to the presence of SO_2 in their environment: 1) sulphur reduction (Kobayashi and Yoshimoto 1982 ; Yoshimoto and Sato 1968) 2) sulphur oxidation (I. Beck-Speier *et al.* 1985; Feng *et al.* 2007; C. G. Friedrich *et al.* 2005; Heimberg *et al.* 1953; U. Kappler and C. Dahl 2001) 3) acetaldehyde production (Stratford *et al.* 1987) and one molecular response, the active efflux of SO_2 by the sulphite transporter Ssu1p (Park and Bakalinsky 2000). Figure 7 is a summary of the above mentioned responses to SO_2 and it will be referred to in the following sections of this review.

2.2.6 Cellular responses in the presence of SO_2

Sulphur reduction

Sulphur is a crucial element in yeasts as it is used in the synthesis of sulphur-containing amino acids such as methionine, S-adenosylmethionine and cysteine (see Thomas and Surdin-Kerjan (1997) for a comprehensive review). The sulphur amino acid biosynthesis (SAAB) pathway plays a crucial role in the active transport of sulphate (SO_4^{2-}) into the cell by the membrane bound transporter protein *SUL1/SUL2*. The genes *MET3*, *MET14*, *MET16* and *MET5*/*MET10* code for the catalytic enzymes ATP sulfurylase, APS kinase, PAPS reductase and the two subunits of sulphite reductase respectively. The Met3p and Met14p each requires one ATP, Met16p one NADPH and Met5p/Met10p complex 3 NADPH

molecules for the catalysis of SO_4^{2-} to S^{2-} as illustrated in Figure 7. The available sulphide (S^{2-}) can be used in the synthesis of sulphur containing amino acids adenosine, methionine and cysteine as well as being excreted as H_2S.

As seen in Figure 7, HSO_3^- is an intermediary in the SAAB and can be viewed as a potential sink for excess HSO_3^- which has entered the cell. It can be speculated that SAAB may not play a crucial role in the removal of exogenous SO_2 from the cell due to the fact that sulphur-containing amino acids play a crucial role in cellular metabolism and preference would be given to their synthesis at a rate which does not exceed the demand for sulphur-containing amino acids. *MET16* is inhibited by sulphite and methionine down-regulates the enzymes of the sulphate assimilation pathway and therefore enhances toxicity of SO_2 due to decreased sulphite reductions. Adenine enhances SO_2 resistance and reverses the negative effect of methionine but the reason for this is only speculated at this time (Aranda *et al.* 2006).

Sulphur oxidation

The oxidation of SO_2 in eukaryotes is poorly researched (there is no known sulphite oxidase in *S. cerevisiae*) but it has been extensively researched in bacteria, especially thiobacilli (Charles and Suzuki 1966; Silver and Lundgren 1968; Suzuki and Silver 1966). Kurek (1985) characterised an enzymatic complex from *Rhodoturula* which possesses thiosulfate as well as sulphur oxidising activities and Heimberg *et al.* (1953) inferred sulphite oxidation from mammalian liver extracts. It was found that the optimum pH for these reactions is 7.8 and 9.3 respectively. The optimum pHs for known sulphite oxidase are relatively high and it can therefore be speculated that this enzyme complex plays only a minor role in the oxidation of sulphite due to its high optimum pH relative to the standard intracellular pH range [approximately pH 5.5 to 7 (Imai and Ohno 1995)] which is much higher than the intracellular pH as represented in Figure 7. Sulphite oxidation is therefore only a minor role player in the detoxification of sulphite and the future of the formed sulphate is unknown.

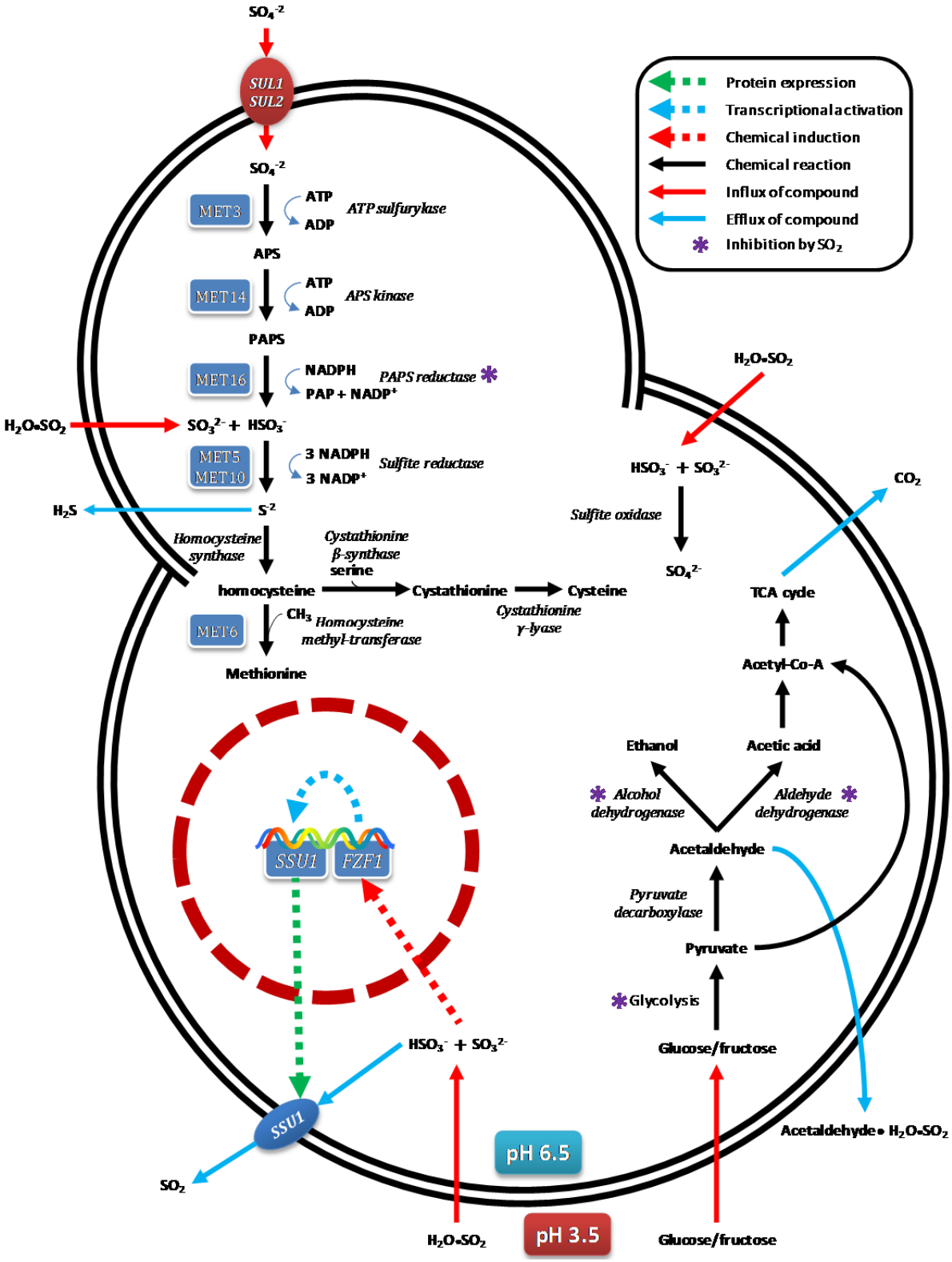

Figure 7
A summary of the sulphate assimilation pathway and the cellular and molecular responses of *S. cerevisiae* to the presence of SO_2. Aranda *et al.* (2006), Avram and Bakalinsky (1997), Goto-Yamamoto *et al.* (1998), Kobayashi and Yoshimoto (1982), Maier *et al.* (1986), Park and Bakalinsky (2000),Thomas and Surdin-Kerjan (1997).

Acetaldehyde production

The role of acetaldehyde in winemaking is very important as it can contribute both positively and negatively to the wine aroma profile, where at low levels it can add a fruity character, but at high levels imparts a pungent grassy and apple off-flavour. Acetaldehyde is a highly volatile and reactive compound and it binds to many compounds in wine such as amino acids, proteins and SO_2.

Acetaldehyde is an intermediate metabolite that is produced in numerous metabolic pathways in mammals, bacteria and yeast. In yeast, it is considered a leakage product and is most prevalent during the decarboxylation of pyruvate by pyruvate decarboxylase, during anaerobic fermentation with ethanol or acetic acid as the end products. It is also biologically toxic at high levels and can form covalent bonds with DNA and cause DNA point mutations (Cheng et al. 2003; Fang and Vaca 1997; Wang et al. 2000).

Acetaldehyde plays a negative role in oenology because of its green apple-like flavour and its strong affinity for unbound SO_2 where one mole of acetaldehyde binds one mole of SO_2 forming hydroxysulfonate, and thereby reducing the sulphite stress on any bacteria and yeast present during the fermentation. As mentioned in section 2.2.4 and as seen in Figure 7, SO_2 has direct inhibitory effects on many enzymes in energy metabolism pathways. A direct result of this is that the flux of intermediary metabolites changes drastically. As seen in Figure 8, increasing the level of SO_2 in the growth media resulted in the increased production and subsequent leakage of acetaldehyde by *S. cerevisiae* into the extracellular environment.

This increase, although minimal, in extracellular acetaldehyde will immediately bind to any free SO_2. The removal of this portion of free SO_2 from the extracellular environment will subsequently reduce the molecular SO_2 stress on the cell. Aranda *et al.* (2006) found that in the presence of 24 mg/L sulphite, the gene expression level of *ALD6* (the gene encoding for aldehyde dehydrogenase) progressively decreased with the concomitant increase of *SSU1* expression over a period of 50 hours. Maier *et al.* (1986) found that low levels of sulphite effectively inhibited the action of alcohol dehydrogenase, the enzyme responsible for the

conversion of acetaldehyde to ethanol. These gene expression changes will directly influence the cell by increasing acetaldehyde production by the decrease of acetaldehyde conversion to acetic acid or ethanol and increasing the rate of sulphite efflux.

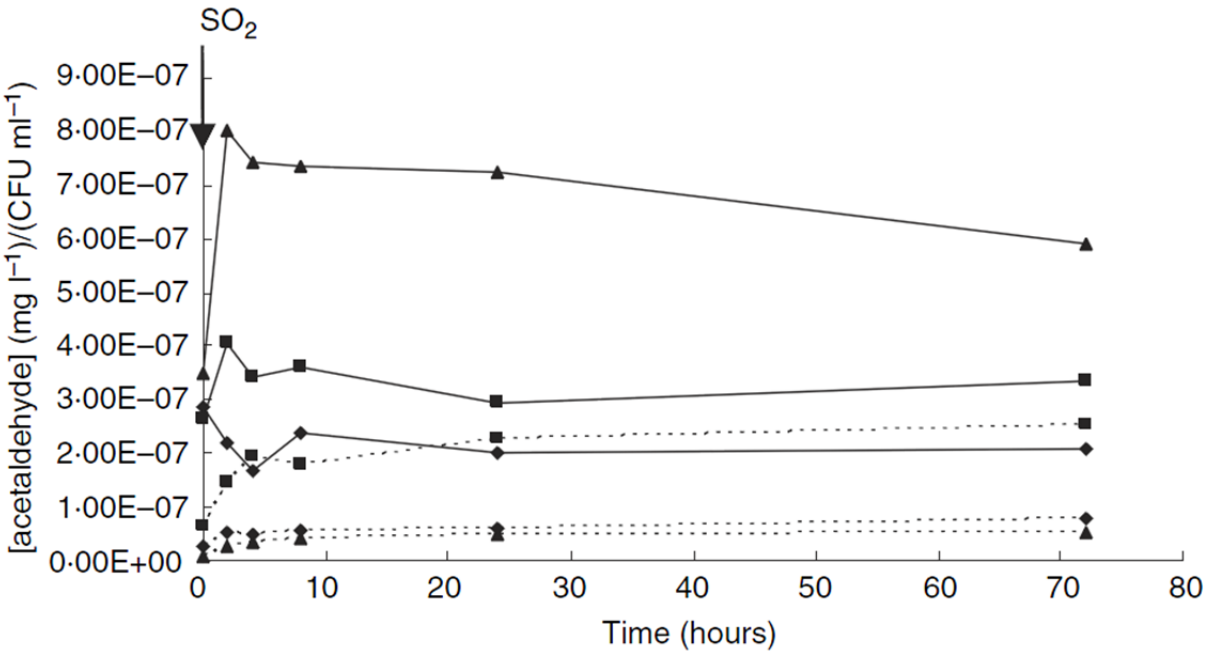

Figure 8
Acetaldehyde formation per cell during 72 h following different added concentrations ((◆) 0; (■) 10 and (▲) 50 mg/L) free SO_2) after 24 h cultivation for two strains of Saccharomyces cerevisiae: FOEB L0433 (solid lines) and Maurivin PDM (dashed lines) (Divol *et al.* 2006).

The increased level of acetaldehyde will bind any free SO_2 and together these two factors can increase the yeasts survival chances in the presence of high SO_2 levels. Whether this overproduction of acetaldehyde is a predetermined stress response or that it is only a side-effect of the enzymatic inhibition caused by SO_2 stress, is yet to be determined.

2.2.7 Molecular response

Active efflux of sulphite by Ssu1p

In *S. cerevisiae* the gene *SSU1* encodes a plasma membrane bound sulphite pump (Park and Bakalinsky 2000) belonging to the dicarboxylate transporter (TDT) family. The *SSU1* gene is positively regulated by a putative transcription factor *FZF1* (Avram *et al.* 1999). Fzf1p is a five zinc finger based transcription factor which binds directly to the promoter region of *SSU1*. Sulphite efflux pumps have also been shown to exist in *Apergillus fumigatus*

(AfuSSU1), Trichophyton rubrum (TruSSU1) and *Arthroderma benhamiae (AbeSSU1*) where the excreted sulphite is used to degrade keratin (Léchenne *et al.* 2007).

Sulphite accumulation and efflux assays were performed on *S. cerevisiae* strains 3090-9d carrying empty YEplac181 vector (referred to as wild-type), 3090-9d-T4-L1 (*ssu1*Δ), 3163-1b (*FZF1-4*) and in 3090-9d carrying multicopy YEplac181-*SSU1* (3090-9d-MC) by Park and Bakalinsky (2000) as seen in Figs. 9 and 10. It was shown that the *ssu1*Δ mutant could not efficiently transport sulphite out of the cell due to lack of Ssu1p expression while the multicopy *SSU1* strain showed minimal sulphite accumulation due to overexpression of Ssu1p. Therefore the Ssu1 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.

Sulphite resistance in yeast should not be stereotyped into singular mechanisms but rather work together directly and/or indirectly to detoxify the intracellular sulphite presence and maintain cell viability. The Ssu1p sulphite pump is the most important role player and the only direct cellular response to increase sulphite detoxification and without this pump, the yeast known to synthesise it will be unable to tolerate sulphite at moderate levels. The other sulphite resistance mechanisms assist in sulphite detoxification by indirect methods and their efficacy in this matter is yet to be determined. Most of the sulphite mechanisms have been studied in *S. cerevisiae* and their existence and effectiveness in other yeast species such as *B. bruxellensis* are yet to be investigated.

When these sulphite resistance mechanisms are unable to cope the cell must either adapt or die. As a result certain yeasts can enter the viable-but-non-culturable state to increase survival chances which will be discussed further in the next section.

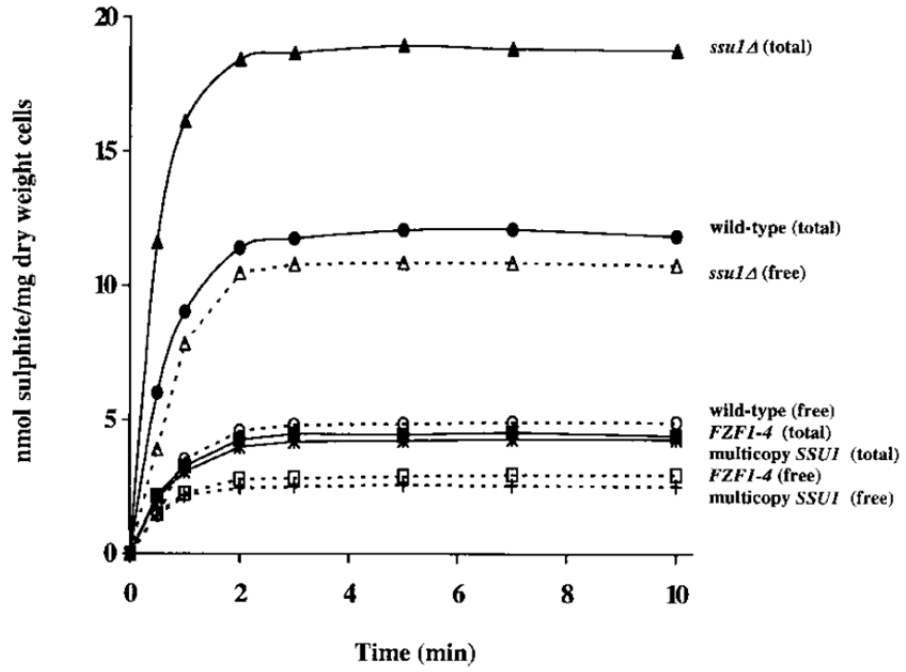

Figure 9
Sulphite accumulation in 3090-9d carrying empty YEplac181 (wild-type), 3090-9d-T4-L1 (*SSU1Δ*), 3163-1b (*FZF1-4*) and in 3090-9d carrying multicopy *SSU1* (3090-9d-MC)(Park and Bakalinsky 2000).

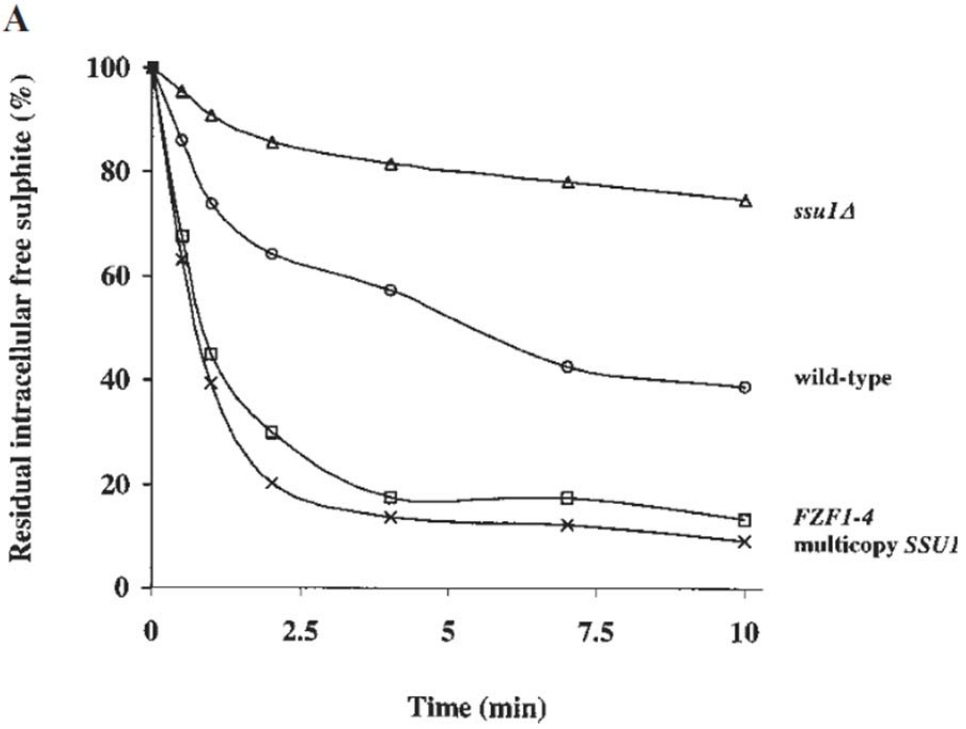

Figure 10
Efflux of free sulphite from 3090-9d (wildtype), 3090-9d-T4-L1 (*SSU1Δ*), 3163-1b (*FZF1-4*), and 3090-9d-MC (multicopy *SSU1*) (Park and Bakalinsky 2000).

2.3 The viable but non-culturable state in yeast

Fermenting grape must and wine presents challenging and stressful environments to any microorganisms trying to grow in them. To survive and eventually dominate in this environment, a microorganism must be able to either out-compete its rivals by reproducing faster or remain in a dormant and viable state so that it can flourish when the correct environmental conditions present themselves.

2.3.1 Definition

A microorganism is said to be in viable but not-culturable state when it is no longer culturable on routine culture media and metabolically active cells are still detected by comparing the enumeration obtained by culture-independent microbiological techniques such as flow cytometry and epifluorescence microscopy.

2.3.2 Occurrence in yeast and bacteria

The VBNC state was first identified and described in bacteria by Xu *et al*. (1982) in *Escherichia coli* and *Vibrio cholera.* The bacterial VBNC state is of particular importance as it is often correlated with pathogenic bacteria strains and their impact on human health (Peneau *et al.* 2007; Sardessai 2005). Since the ground breaking paper by Xu, approximately 60 bacterial species have been shown to demonstrate a VBNC response, of which approximately 50 are known as human pathogens (Oliver 2005). In wine, it is believed that the VBNC state plays a crucial role by allowing spoilage yeasts and bacteria to survive throughout the wine fermentation process and into the wine bottle (Millet and Lonvaud-Funel 2000).

In both bacteria and yeast, the VBNC state is characterised by a few key points: The complete loss of cellular division and reproduction on solid media (du Toit *et al.* 2005); a reduction in the metabolic rate (Quiros et al. 2009) to a basal level as a means to reduce energy expenditure to a minimum level; an increase in peptidoglycan cross-linking in Gram-positive bacteria (Signoretto *et al.* 2000); a reduction in cell size in bacteria and yeast

(Divol and Lonvaud Funel 2005); recovery from the VBNC state during favourable environmental conditions (Table 5) is proposed (du Toit *et al.* 2005).

It has been observed that viable bacteria passed through a 0.2-µm filter and *Brettanomyces* sp., *Pediococcus damnosus* and *Acetobacter aceti* cells were not recovered on a 0.45-µm filter membrane after wine filtration due to the reduction of cell size as a result of the VBNC state (Linder and Oliver 1989; MacDonell and Hood 1982; McDougald *et al.* 1998; Millet and Lonvaud-Funel 2000; Oliver 1993; Rahman *et al.* 1994; Serpaggi *et al.* 2011) (the average cell size of yeast and bacteria is (5–8 x 3–4 µm and 0.5–1 µm respectively). Serpaggi *et al* (2011) also observed, by means of scanning electron microscopy, a drastic change from smooth to rough and uneven in *Brettanomyces* cell surface characteristics of cells in the VBNC state.

Table 5
Characteristic effects of microorganisms in the VBNC state

Organism	VBNC effect	References
Acetobacter pasteurianus	Loss of reproduction on solid media	du Toit *et al.* (2005)
Lactobacillus hilgardii	Decreased metabolic rate	Quiros *et al.* (2009)
Enterococcus faecalis	Peptidoglycan cross-linking increase	Signoretto *et al.* (2000)
S. cerevisiae, Candida. stellata, Rhodotorula mucilaginosa, Zygosaccharomyces bailii	Cell size reduction; Loss of reproduction on solid media; Decreased metabolic rate	Divol and Lonvaud Funel (2005)
Brettanomyces bruxellensis, Acetobacter aceti, Pediococcus damnosus	Cell size reduction; Loss of reproduction on solid media	Millet and Lonvaud-Funel (2000)

This phenomenon has not been explained yet. The latter authors have also attempted to characterise the VBNC state in *B. bruxellensis* at a proteomic level. They showed that when the cells are in a VBNC state, they accumulate various enzymes directly or indirectly involved in the glycolytic pathway. These enzymes were shown to have a lower pI than native proteins, demonstrating that these enzymes are degraded according to the authors. This

accumulation of non-functional enzymes ultimately leads to a reduction of glycolytic flux. An induction on oxidative stress response was also noted, in particular, an increase in the amount of thioredoxin. It has previously been shown that thioredoxin peroxydases play a role in sulphite reductase activity (Lee *et al.* 2008) and peroxiredoxins are involved in signal transduction and DNA damage response (Morgan and Veal 2007).

Finally, a decrease in the amount of Dug1-like protein has been observed. This enzyme is involved in the degradation of glutathione. The latter peptide plays a role in DNA synthesis and repair and acts as a major antioxidant, neutralising reactive oxygen compounds that are formed after exposure to SO_2. All these observations seem consistent with SO_2 resistance.

2.3.3 Triggering of the entry into and exit from a VBNC state

The entry into and exit from a VBNC state has been well studied in bacteria (Nilsson *et al.* 1991; Oliver 1993, Oliver 2000) but very little research emphasis has been placed on yeast. In bacteria, environmental stressors such as change in temperature, osmotic, nutrient and chemical stresses have been well documented to induce a VBNC state. In yeast and particularly in wine conditions, the presence of chemical stress factors such as SO_2 has been shown to induce a VBNC state in *S. cerevisiae, Candida stellata, Rhodotorula mucilaginosa* and *Zygosaccharomyces bailii* (Divol and Lonvaud Funel 2005) and in *D. bruxellensis* (Agnolucci *et al.* 2010; Barata *et al.* 2008; du Toit *et al.* 2005). The specific molecular and genetic mode of action for the induction of the VBNC state has yet to be elucidated.

Very little is known on the mechanisms of exit from VBNC by yeasts under wine making conditions. As in bacteria, a favourable change in environmental conditions has been shown to resuscitate a cell in the VBNC state and the same response could occur with yeasts. Du Toit *et al.* (2005) found that *A. pasteurianus* recovered from the VBNC state when the wine it was growing in was aerated with O_2 and that low levels of O_2 can support the growth of *Brettanomyces* sp. in a VBNC state. It has been shown that the removal of SO_2 from the

environment by the increase in extracellular pH (and subsequent decrease in molecular SO_2) can trigger the exit from VBNC in *B. bruxellensis* (Serpaggi *et al.* 2011).

2.3.4 Detection of microorganisms in the VBNC state

The detection of microorganisms in a VBNC state presents itself with numerous challenges not usually encountered with viable and culturable cells. Standard microbiological techniques such as cell counting using optical microscopy, colony counting on growth media, culture optical density and standard PCR are unable to distinguish between culturable and non-culturable cells. As a result of this, detection techniques such as fluorescent *in situ* hybridisation (FISH) have been employed to distinguish between VBNC and viable and culturable microorganisms (Divol and Lonvaud Funel 2005; Millet and Lonvaud-Funel 2000; Roder *et al.* 2007).

Recently FISH, using species-specific fluorescent DNA probes, has been combined with flow cytometry (FCM-FISH) for the rapid detection and quantification of *Brettanomyces* in wine (Serpaggi et al. 2010) to a low level of 10^2 cells/mL. Andorrà *et al.* (2010) used the DNA binding dyes (DBD) ethidium monoazide bromide and propidium monoazide bromide in combination with qPCR (DBD-qPCR) to determine viable wine yeast. These DNA binding dyes block the PCR reaction by covalently binding to the DNA (after exposure to bright light) in cells with compromised cell wall and membranes (Nogva et al. 2003), hence allowing only the qPCR of viable cells (Nocker *et al.* 2006).

2.4 Conclusion

The environment that bacteria and yeast find themselves in fermenting grape must presents numerous metabolic stresses such as high osmotic potential, the dramatic increase in ethanol and loss of nutrients. This forces the microorganisms to adapt to the stresses or face being out-competed by better adapted microorganisms and ultimately, death. These stresses are further enhanced by the artificial addition of high levels of SO_2. Sulphiting the must allows only a narrow range of bacteria and yeast species to survive until the end of and beyond alcoholic fermentation. These species show some degree of resistance to the antimicrobial effect of SO_2. The antimicrobial effect of SO_2 is rapid and results in the loss of culturability within a day after sulphiting.

Four main mechanisms of resistance to SO_2 have been shown to exist, namely sulphur reduction, sulphur oxidation, acetaldehyde production and active sulphur efflux. Sulphur reduction is present in almost all eukaryotes as it is a vital part of the sulphur amino acid metabolism pathway where the amino acids methionine and cysteine are produced. Sulphur oxidation has been postulated as a possible means to remove intracellular SO_2 from the cell. There is significant literature available on bacterial sulphite oxidative pathways but very little literature is available on the presence of a sulphite oxidase in yeasts and no known sulphite oxidase is found in *S. cerevisiae.*

Acetaldehyde is a crucial intermediate metabolite in the pyruvate metabolism pathway, in particular during the anaerobic fermentation of glucose/fructose to ethanol. The enzymes in this pathway are particularly sensitive to the inhibitory effects of SO_2. As a result the metabolic flux through these pathways is greatly affected and a large increase in acetaldehyde leakage occurs. The acetaldehyde then automatically binds any free intra- or extracellular SO_2. The amount of acetaldehyde leakage needs to be at a sufficient concentration so that the sulphite stress on the cell is effectively reduced. It is not known if the increased acetaldehyde leakage is an evolutionary response to the presence of SO_2 or if

it occurs simply due to the inhibitory effect of SO_2 on the metabolic enzymes and the subsequent changes in metabolic flux.

In *S. cerevisiae,* a cell wall associated sulphite pump, Ssu1p, has been shown to actively efflux SO_2 from the cell. It is transcriptionally regulated by the transcription factor Fzf1p which itself is induced by elevated levels of SO_2. Ssu1p can effectively control the level of intracellular SO_2 so as to reduce the amount of cellular sulphite stress. Ssu1p has not only been found in *S. cerevisiae* but also in the fungal species *Aspergillus* sp. and therefore by extrapolation could be present in other yeast such as *B. bruxellensis*.

When the external environmental stress (such as the presence of high sulphite levels) increases to a level which a microorganism cannot tolerate, it usually results in death of the microorganism. However, certain bacteria and yeasts have been shown to shift their metabolism and cellular reproduction into a viable-but-not-culturable state. In this state, the microorganism is unable to grow on routine solid growth media yet it retains low level metabolic function. This survival mechanism is reversible and allows the microorganism to flourish once environmental conditions become favourable once again. This state is very important for wine production as spoilage microorganisms such as *A. aceti* and *B. bruxellensis* are capable of entering this state. *B. bruxellensis* is a notorious producer of high levels of volatile phenols in red wine which give the wine undesirable sweaty and medicinal characteristics.

Of the stresses placed on the wine microcosm, the presence of SO_2 is most probably the greatest driver behind forcing cells into a VBNC state. SO_2 has been shown to rapidly force sensitive cells into a VBNC state and this state is maintained until the sulphite stress is removed from the environment.

CHAPTER 3

Materials and methods

3 Materials and methods

3.1 Microbial strains, media and growth conditions

Yeast strains were selected from the Institute for Wine Biotechnology's culture collection. Yeast strains (see Table 6) were routinely cultured in yeast peptone dextrose (YPD) broth (Biolab Diagnostics, Wadeville, South Africa) at 30°C for with shaking and *Escherichia coli* DH5α was cultured in Luria-Bertani broth (Biolab Diagnostics, Wadeville, South Africa) at 37°C with shaking with ampicillin (100 mg/L, Sigma-Aldrich, St. Louis, Missouri, USA), X-Gal (100 mg/L, Roche, Basel, Switzerland), IPTG (100 mg/L, Sigma-Aldrich, St. Louis, Missouri, USA) (when appropriate). For solid media, 1.5% agar was added to the previously described media.

Yeast sporulation was induced by incubation on Yeast Extract-Malt Extract (YM) + vitamins agar (40 g/L YM agar, 0.2 mg/L biotin, 0.2 mg/L folic acid, 40 mg/L calcium pantothenate, 200 mg/L inositol, 40 mg/L niacin, 20 mg/L *p*-aminobenzoic acid, 40 mg/L pryidoxine hydrochloride, 20 mg/L riboflavin, 100 mg/L thiamin as well as potassium acetate (KAc) agar (20 g/L potassium acetate and 15 g/L agar) (Fugelsang and Edwards 2007).

50X Tris-Acetate-EDTA (TAE) buffer was made as a stock solution to be used as a running buffer for gel electrophoresis (242 g Tris base, 57.1 mL acetic acid, 100mL 0.5 M EDTA to 1 L with dH_2O and adjusted pH to 8.5 using KOH).

Other chemicals used in this study were obtained from Sigma-Aldrich (St. Louis, Missouri, USA).

Table 6
Yeast strains used

Collection	Strain number	Other name	Described genus	Described species	Source/description/Genotype	Reference
		VIN13	*Saccharomyces*	*cerevisiae*	Industrial wine strain	Anchor Yeast, South Africa
		BY4742	*Saccharomyces*	*cerevisiae*	Laboratory strain* *MATα his3Δ leu2Δ0 lys2Δ0 ura3Δ0*	Brachmann *et al.* (1998)
		BY4742 *ssu1*Δ	*Saccharomyces*	*cerevisiae*	BY4742; *Matα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; YPL092w:kanMX4*	EUROSCARF
ISA[a]	1649	CBS72[T], IGC-4179	*Dekkera*	*bruxellensis*	Isolated from lambic beer	
ISA	1653	IGC-5160	*Dekkera*	*anomala*	Isolated from spoiled beer	
ISA	1650	IGC-4801	*Brettanomyces*	*bruxellensis*	Tea fungus	
ISA	1721	CBS 6043, IGC-5163	*Brettanomyces*	*naardenensis*	Isolated from tonic water	
ISA	1791		*Dekkera*	sp.	Isolated from red wine	
FOEB[b]	CB61		*Brettanomyces*	*anomalus*	Isolated from red wine	
FOEB	CB63		*Brettanomyces*	*bruxellensis*	Isolated from red wine	
ARC[c]	Y0136		*Brettanomyces*	*lambicus*		
IWBT[d]	Y101	isolate 83	*Brettanomyces*	*bruxellensis*	Isolated from MLF, Cabernet Sauvignon in old barrel in 2004	Oelofse, 2008
IWBT	Y102	isolate 87	*Brettanomyces*	*bruxellensis*	Isolated from MLF, Cabernet Sauvignon in tank in 2004	Oelofse, 2008
IWBT	Y103	isolate S11	*Brettanomyces*	*bruxellensis*	Isolated from AF, Shiraz in barrel in 2004	Oelofse, 2008
IWBT	Y104	isolate 40a	*Brettanomyces*	*bruxellensis*	Cabernet Sauvignon, finished wine, barrel, 2005	Oelofse, 2008
IWBT	Y105	Isolate 8	*Brettanomyces*	*bruxellensis*		Oelofse, 2008
IWBT	Y111	7	*Brettanomyces*	*bruxellensis*	Rinsing water	Oelofse, 2008
IWBT	Y113	isolate WI14	*Brettanomyces*	*bruxellensis*		Oelofse, 2008
IWBT	Y117	59	*Brettanomyces*	*bruxellensis*	Shiraz, isolated from malolactic fermentation, 2004	Oelofse, 2008
IWBT	Y119	isolate 70	*Brettanomyces*	*bruxellensis*	MLF, old barrel, pinotage, 2004	Oelofse, 2008
IWBT	Y121	isolate 84	*Brettanomyces*	*bruxellensis*	MLF, Cabernet Sauvignon, old barrel, 2004	Oelofse, 2008
IWBT	Y130	isolate 39a	*Brettanomyces*	*bruxellensis*	Finished wine, Merlot, barrel, 2005	Oelofse, 2008
IWBT	Y131	39b	*Brettanomyces*	*bruxellensis*	Merlot, barrel, finished wine, 2005	Oelofse, 2008
IWBT	Y132	40b	*Brettanomyces*	*bruxellensis*	Cabernet Sauvignon, barrel, finished wine, 2006	Oelofse, 2008
IWBT	Y133	41	*Brettanomyces*	*bruxellensis*	Shiraz, barrel, finished wine, 2005	Oelofse, 2008
IWBT	Y134	OB1	*Brettanomyces*	*bruxellensis*	Old barrel shavings, 2005	Oelofse, 2008
IWBT	Y135	*Brettanomyces intermedius*	*Brettanomyces*	*bruxellensis*	2003	Oelofse, 2008
IWBT	Y136	W1	*Brettanomyces*	*bruxellensis*	2003	Oelofse, 2008

* S288C background. MLF= malolactic fermentation; AF = alcoholic fermentation; T = type strain; a = Instituto Superior de Agronomia, Lisbon, Portugal; b = Faculté d'Oenologie de Bordeaux, France; c = Agricultural Research Council, South Africa; d = Institute for Wine Biotechnology, Stellenbosch University, South Africa;

3.2 DNA extraction, PCR, RFLP analysis and agarose gel electrophoresis

DNA extraction

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

ITS PCR

An optimised colony PCR technique was used in place of genomic DNA extraction when individual yeast colonies required identification. 5 µL of 24-hour-old yeast culture in YPD was washed in dH_2O and resuspended in 10 µL mQ water (Millipore, Billerica, Massachusetts, USA). The PCR reaction consisted of 35.3 µL mQ water, 200 µM dNTP, 5 µL 10X ExTaq buffer (Takara, Otsu, Shiga, Japan), 500 µM ITS1 primer and 500 µM ITS4 primer (Table 7), 0.5 µL of the resuspended washed cells as a gDNA source and 0.25 µL ExTaq DNA-polymerase (Takara, Otsu, Shiga, Japan) in a final volume of 50 µL. The PCR reaction was conducted in an ABI 2720 Thermal Cycler (Applied Biosystems, Foster City, California, USA) using the following program: 95°C for 5 minutes, 40 cycles of 95°C for 1 minute, 58°C for 30 seconds and 72°C for 1 minute. A final extension step at 72°C for 7 minutes was used.

Table 7
Primers sequences

Primer	Sequence	Target organism	Target gene	Expected size (bp)	Reference
SSU1f-FW	5'-ATG GTT GCC AAT TGG GTA CT-3'	*S. cerevisiae*	*SSU1*	1376	This study
SSU1f-RV	5'-TTA TGC TAA ACG CGT AAA ATC TAG AG-3'				This study
SSU1_FW	5'-TGY TCN TAY MYY WTS TTT-3'	*S. cerevisiae*	Partial *SSU1*	~1000	This study
SSU1_RV	5'-CA DGY WCC YAD NGG RAA-3'				This study
ITS1	TCCGTAGGTGAACCTGCGG	Any yeast species	ITS-5.8S rDNA	450-1000	White *et al.*, 1990
ITS4	TCCTCCGCTTATTGATATGC				

SSU1 PCR

The PCR reaction consisted of 35 µL milliQ-H_2O, 200 µM dNTP, 5 µL 10X ExTaq buffer (Takara), 500 µM SSU1f-FW primer and 500 µM SSU1f-RV primer (Table 7), 0.5 µL gDNA and 0.5 µL ExTaq DNA-polymerase (Takara) in a final volume of 50 µL. The PCR reaction was carried out in an ABI 2720 Thermal Cycler (Applied Biosystems) using the following program: 94°C for 5 minutes, 45 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute and 30 seconds. A final extension step at 72°C for 7 minutes was used.

SSU1 degenerate primer PCR

The PCR reaction consisted of 18 µL milliQ-H_2O, 200 µM dNTP, 5 µL 10X ExTaq buffer (Takara), 500 µM SSU1-FW primer and 500 µM SSU1-RV primer (Table 7), 1 µL gDNA and 1 µL ExTaq DNA-polymerase (Takarain a final volume of 50 µL. The PCR reaction was carried out in an ABI 2720 Thermal Cycler (Applied Biosystems, Foster City, California, USA) using the following program: 95°C for 2 minutes, 45 cycles of 95°C for 30 seconds, 52°C for 60 seconds and 72°C for 50 seconds. A final extension step at 72°C for 7 minutes was used.

Restriction fragment length polymorphism analysis

RFLP analysis was carried out according to Esteve-Zarzoso *et al.* (1999). The restriction enzymes *Hinf*I and *Cfo*I (Roche, Basel, Switzerland) were used to digest ITS PCR products. The restriction enzyme reaction consisted of 17 µL mQ water, 0.5 µL restriction enzyme, 5 µL PCR reaction, 2.5 µL 10X appropriate buffer and the reaction was incubated at 37°C for 2 hours.

Vector based cloning and DNA sequencing

The vector system, pGEM®-T Easy (Promega, Madison, WI, USA), manufacturer's instructions were followed to transform *E. coli* DH5α. Both strands of the pGEM®-T Easy vector were sequenced in an ABI 3130XL Genetic Analyser at the Central Analytical Facility (Stellenbosch University, South Africa) using the SP6 and T7 primers (Promega, Madison, WI, USA).

Agarose gel electrophoresis conditions

DNA, be it genomic, PCR product, plasmid or from a restriction enzyme digest, was separated using agarose gel electrophoresis in pre-cast agarose gels stained with ethidium bromide. When the expected DNA fragment size was more than 500 bp a 1X TAE 0.8% agarose gel was used and when less than 500 bp a 1X TAE 1.5% agarose gel was used and agarose gels were run in 1X TAE buffer at a constant voltage of 7 $V.cm^{-1}$ for 45 minutes or 6 $V.cm^{-1}$ for 90 minutes respectively. The DNA was visualised using a GBOX ultraviolet illuminator imaging system (Syngene, Cambridge, England).

3.3 Sporulation of yeasts strains

Temperature stress and nutrient excess

Yeast strains were pre-cultured in 5 mL YPD broth overnight. 100 µL of fresh pre-culture was spread-plated onto triplicate Petri plates containing 20 mL YM + vitamins agar and sealed with Parafilm. Plates were then incubated at 30°C until visible colony growth had occurred. Each replicate was then incubated at either 4°C, 25°C or 45°C for one week. Sporulation was observed microscopically using a Nikon OptiPhot 2 upright light microscope.

Temperature stress and nutrient deficiency

Yeast strains were pre-cultured in 5 mL YPD broth overnight. 100 µL of fresh pre-culture was spread-plated onto YPD agar Petri plates and sealed with Parafilm. Plates were then incubated at 30°C until visible colony growth had occurred. Individual colonies from each plate was then picked and streaked, in 10 mm long lines, onto KAc agar. Each replicate was then incubated at either 4°C, 25°C or 37°C for two weeks. Sporulation was observed microscopically using a Nikon OptiPhot 2 upright light microscope.

3.4 Quantification of sulphur dioxide and assay optimisation

A method originally used to determine the concentration of SO_2 in beer (Association of Analytical Communities International, official method 963.11) was adapted for small scale use in the laboratory. The assay is based on a colorimetric reaction of SO_2 with *p*-rosaniline-

HCl and is quantified using a 96-well spectrophotometer at 550 nm and uses mercuric chloride to bind compounds that can bind sulphur. This assay is not limited to microtitre plate volumes and can be scaled up for quantifying SO_2 in larger volumes.

For the calibration curve, solution 1 contains 400 µL $HgCl_2$ (27.2 g/L $HgCl_2$ and 11.7 g/L NaCl in dH_2O), 40 µL (1 g/L) SO_2, and 1560 µL mQ water. The calibration standard range is made up in individual 1.5 mL micro-centrifuge tubes and consists of 50 µL of the chosen working medium, 0 to 100 µL of solution 1 (in 20 µL incremental steps), 750 to 650 µL mQ water (in 20 µL decremental steps), 100 µL *p*-rosaniline-HCl (0.4 g/L in 2.56% ($^{v}/_{v}$) HCL) and 100 µL 0.2 % ($^{v}/_{v}$) formaldehyde. After inverting the mixture 5 times, the colour is allowed to develop for 30 minutes at room temperature. 385 µL is then transferred to a 96 well flat-bottomed micro-titre plate and the absorbance determined at 550nm in a micro-titre plate reader. A scatterplot of absorbance versus the SO_2 concentration (mg/L) is plotted and the linear trend line equation of this plot is used to calculate the SO_2 concentration in a sample.

For total SO_2 sample quantification, 10 µL $HgCl_2$ (27.2 g/L $HgCl_2$ and 11.7 g/L NaCl in dH_2O), 25 µL 50 mM H_2SO_4, and 25 µL sample is pipetted into a 1.5 mL microcentrifuge tube. 75 µL fresh 0.1 M NaOH is then added, the mixture is mixed well and allowed to incubate at room temperature for 5 minutes. 50 µL 0.05 M H_2SO_4, 615 µL mQ water, 100 µL *p*-rosaniline-HCl (0.4 g/L in 2.56% ($^{v}/_{v}$) HCL) and 100 µL 0.2 % ($^{v}/_{v}$) formaldehyde is added. After inverting the mixture 5 times, the colour is allowed to develop for 30 minutes at room temperature. 385 µL is then transferred to a 96 well flat-bottomed micro-titre plate and the absorbance determined at 550nm in a micro-titre plate reader. For free SO_2 sample quantification, the 0.1 M NaOH and second 50 mM H_2SO_4 step is removed from the assay protocol and an additional 125 µL mQ water is added.

3.5 Sulphite accumulation

A method adapted from Park and Bakalinsky (2000) was used. Three *S. cerevisiae* strains (VIN13, BY4742 and BY4742 *ssu1Δ*) and three *B. bruxellensis* strains (ISA1649, CB63 and

Y121) were used in the experiment. Each strain was pre-cultured in YPD broth and inoculated into 400 mL YPD broth + 75 mM tartaric acid at pH 3.5 and grown overnight until late log phase. The cells were then washed with the same medium and transferred to a 250 mL side-ported Erlenmeyer flask and resuspended in 50 mL of the following buffer: 75 mM Tartaric acid, 2% glucose, pH 3.5. The flask was then filled with N_2 gas to create an anaerobic environment and sealed with a rubber stopper. A 1 mL sample was taken at T_0. SO_2 was added to the cell suspension at a final concentration of 100 mg/L. One mL samples were taken at the intervals 5, 10, 15, 30, 45, 60, 90, 120 and 150 s. Each sample was rapidly filtered through a new, sterile 0.45-µm syringe filter and the supernatant recovered into individual 1.5-ml centrifuge tubes. The SO_2 quantification assay described above was then used to determine the SO_2 in the recovered supernatant. The intracellular concentration was calculated indirectly by determining the decrease in SO_2 concentration in the cell-free supernatant at the various time points.

3.6 Sulphite efflux

A method adapted from Park and Bakalinsky (2000) was used. Three *S. cerevisiae* strains (VIN13, BY4742 and BY4742 ssu1Δ) and three *B. bruxellensis* strains (ISA1649, CB62 and Y121) were used in the experiment. Each strain was pre-cultured in YPD broth overnight and inoculated into 400 mL 75 mM Tartaric acid YPD broth at pH 3.5 and grown overnight until late log phase. The cells were then washed and resuspended in 200 mL of the following buffer: 75 mM Tartaric acid, 2% glucose, 100 mg/L SO_2, and pH 3.5. The cell culture suspension was then incubated for 10 minutes (Park and Bakalinsky, 2000) to allow the cells to accumulate the maximum amount of SO_2. The entire cell culture suspension was then rapidly vacuum filtered through a 0.45 µM cellulose membrane filter. The filter membrane (with adhered cells) was then placed cell-side up into a side-ported Erlenmeyer flask. The flask was filled with N_2 gas and sealed with a rubber stopper to create an anaerobic environment. While using a stirring magnetic stirrer bar, 50 mL of tartaric acid buffer was quickly added to the flask. Immediately 1-mL samples were taken anaerobically at time

intervals 0, 10, 20, 30, 40, 50, 60, 90, 120 and 150 s. Each sample was rapidly filtered through a new, sterile 0.45-µm syringe filter and the supernatant recovered into individual 1.5 mL centrifuge tubes. The SO_2 quantification assay described above was then used to determine the quantity of SO_2 effluxed into the supernatant at the different time points.

3.7 SO_2 and ethanol plate stress assay

80 mL YPD, 12 g/L agar, 75 mM Tartaric acid pH 3.5 plates were made by separately autoclaving pH 3.0 YPD broth and agar, allowed to cool and then mixed. Ethanol (0, 10 or 14%) was added to the cooling agar just before pouring. Defined SO_2 concentrations (0, 0.3, 0.4 or 0.8 mg/L molecular SO_2) were added onto the cooled agar surface and evenly spread and the plates were then incubated overnight at room temperature to allow the SO_2 to absorb into the agar. This method was used as SO_2 is volatile at pH 3.5 and adding it directly to the hot agar causes the SO_2 to evaporate which affects the final SO_2 concentration.

Yeast strains were pre-cultured in 5 mL YPD broth overnight. 100 µL pre-culture was then inoculated into 10 mL YPD broth and grown for 3 days until broth until late stationary phase. Each yeast strain was diluted in a series from 10^0 to 10^{-5} and 5 µL of each dilution was spotted on each respective SO_2 concentration plate and allowed to grow until all strains showed discernable colony growth. Scoring of the yeast growth was done by determining until which dilution (10^0 to 10^{-5}) the yeast could grow as shown in Table 8.

Table 8
Scoring guide for the SO_2 and ethanol plate stress assay

Dilution	Score	Description
10^{-5}	+++++	Very strong
10^{-4}	++++	Strong
10^{-3}	+++	Average
10^{-2}	++	Weak
10^{-1}	+	Poor
10^0	+/-	Very poor
	-	None

The lowest dilution at which the yeast grew at was scored as the highest score i.e. if visible colonies were seen at a dilution of 10^{-4} then a score of “++++” and “strong” was given.

3.8 Carbon energy metabolism over a 48 hour period

S. cerevisiae VIN13 and *B. bruxellensis* CB63 were used in the experiment. Both strains were pre-cultured in 5 mL YPD broth overnight and inoculated into individual 200 mL YPD + 75mM tartaric acid and grown until late log phase. Each culture was washed and then transferred to a 250 mL side-ported Erlenmeyer flask containing 200 mL minimal medium (75 mM Tartaric acid and 20 g/L D-glucose, pH 3.5) and the flasks were filled with CO_2 gas and sealed with a fermentation cap. 100 mg/L of SO_2 was added at T_0. Samples were taken at regular time points over a 24-hour period. Samples were divided into "cell-free supernatant" and "supernatant-free cells" and snap frozen in liquid N_2 and then stored at -80°C.

A KoneLab Arena 20XT (Thermo Scientific, Waltham, Massachusetts, USA) automated enzymatic kit robot was used to quantify three key cellular metabolites: acetic acid, ethanol and D-glucose (Thermo Scientific). A manual enzymatic kit to quantify acetaldehyde (R-Biopharm, Darmstadt, Germany) was optimised for use in a 96-well microtitre spectrophotometric plate reader. The "cell-free supernatant" was used as a representative matrix for the presence of these extracellular metabolites which had either been excreted or diffused across the yeasts cell membrane into the extracellular environment. The "supernatant-free cells" were mechanically ruptured and used as a representative matrix of the intracellular environment.

Additional 1 mL samples were taken at the 48 hour time point, centrifuged at maximum speed, the supernatant was removed and the wet cell mass was allowed to dry for one week at 60°C. The resulting dry cell mass was used in the normalisation of the data generated by the enzymatic metabolite quantification.

3.9 Carbon energy metabolism flux over a 5-week period

S. cerevisiae VIN13 and *B. bruxellensis* CB63 were used in the experiment. Each strain was pre-cultured in 5 mL YPD broth overnight and inoculated into individual 200 mL YPD + 75mM tartaric acid + 5% ethanol and grown until late log phase.

Each culture was washed and then transferred to a 250mL side-ported Erlenmeyer flask containing 200 mL synthetic wine media (SWM) and the flasks were filled with CO_2 gas and sealed with a fermentation cap. SWM consisted of the following: 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 5M NaOH. After autoclaving, it was supplemented with 10% (v/v) ethanol and *p*-coumaric (10 mg/L) and ferulic acid (10 mg/L).

SO_2 was added in the form of sodium metabisulphite on day 4 at three different molecular SO_2 concentrations: 0, 0.4 and 0.8 mg/L. 2mL samples were taken anaerobically every four days up to day 32. The samples were centrifuged at 13 200 rpm for 5 minutes and the supernatant was then transferred to 2 mL microcentrifuge tubes and stored at -80°C. The enzymatic analysis was conducted as described above with the exception that ethanol was removed from and D-fructose was added to the list of analysed compounds.

Additional 1 mL samples were taken at day 32, centrifuged at maximum speed, the supernatant was removed and the wet cell mass was allowed to dry for one week at 60°C. The resulting dry cell mass was used in the normalisation of the data generated by the enzymatic metabolite quantification.

3.10 Quantification of volatile phenols

4-Ethyl phenol, 4-Ethyl guaiacol, 4-vinyl phenol and 4-vinyl guaiacol were quantified using Gas Chromatography – Mass Spectrometry (GC-MS) with the approved volatile phenol method at the Central Analytical Facility, Stellenbosch University after a liquid-liquid extraction sample preparation procedure. This entailed the extraction of the volatile phenols with 2 mL diethyl ether after addition of the internal standard (2,6- dimethyl phenol, 50 µg/L in ethanol) during 30 minutes of sonication and shaking.

The organic phase was recovered and dried over anhydrous sodium sulphate. 2 µL of this extract was injected into an Agilent 5890 GC-MS. The capillary column used was a DB-FFAP (60m x 320.0 µm x 0.5 µm column) with helium as carrier gas at a flow of 1.2 mL/min. The injector (split/splitless) was operated at 260 ºC, in splitless mode, with a splitless time of 1 minute. The oven temperature was ramped from 40 ºC at a rate of 20 ºC/min up to 150 ºC and then at 5 ºC/min to 240 ºC, and held for 8 minutes. MS data acquisition was performed in selected ion monitoring (SIM) mode.

CHAPTER 4

Results and discussion

4 Results and discussion

4.1 Identification of yeast strains

According to Esteve-Zarzoso *et al.* (1999), partial amplification of the *B. bruxellensis* ITS-5.8S rDNA region yields a PCR product size of approximately 485 bp and the restriction-fragment-length-polymorphism analysis (RFLP) of this PCR product using *Hinf*I *yields* DNA fragments of 270 and 215 bp. *B. bruxellensis* and *B. anomalus* strains were obtained from the IWBT, ISA and CB culture collection and the method described above was used to confirm the identity of strains to be used in this study.

Figure 11 displays the PCR product and PCR product *Hinf*I restriction enzyme digest bands of the yeast strains used in this study and the numbers correspond to groups of yeasts that yield the same PCR-RFLP profile and that they are identified in Table 9. Almost all strains were positively identified according to their 5.8S-ITS PCR and RFLP profiles but certain freeze cultures were shown to be different as compared to the expected RFLP profiles according to Esteve-Zarzoso *et al.* (1999) and it was assumed that these freeze cultures were contaminated with other yeast species. Five groups of strains yielded the same results. One of the PCR products of each group was randomly selected as representative and sequenced and the sequences were BLASTed against online databases to identify the strains (Table 9).

DNA sequencing of the 5.8S-ITS PCR product and subsequent sequence alignment using BLAST (against available sequence databases), was used to confirm species identity of the identified *B. bruxellensis* and the contaminated freeze cultures.

B. bruxellensis was correctly identified but the contaminated free cultures were identified as *C. boidinii* and an unknown *Saccharomycete* sp. According to Esteve-Zarzoso *et al.* (1999), a *Hinf*I digest of the *B. anomalus* 5.8S-ITS rDNA-PCR product should yield 4 fragments of 360, 190, 160 and 80 bp. However in this study the *B. anomalus* yield only two bands of approximately 230 and 80 bp.

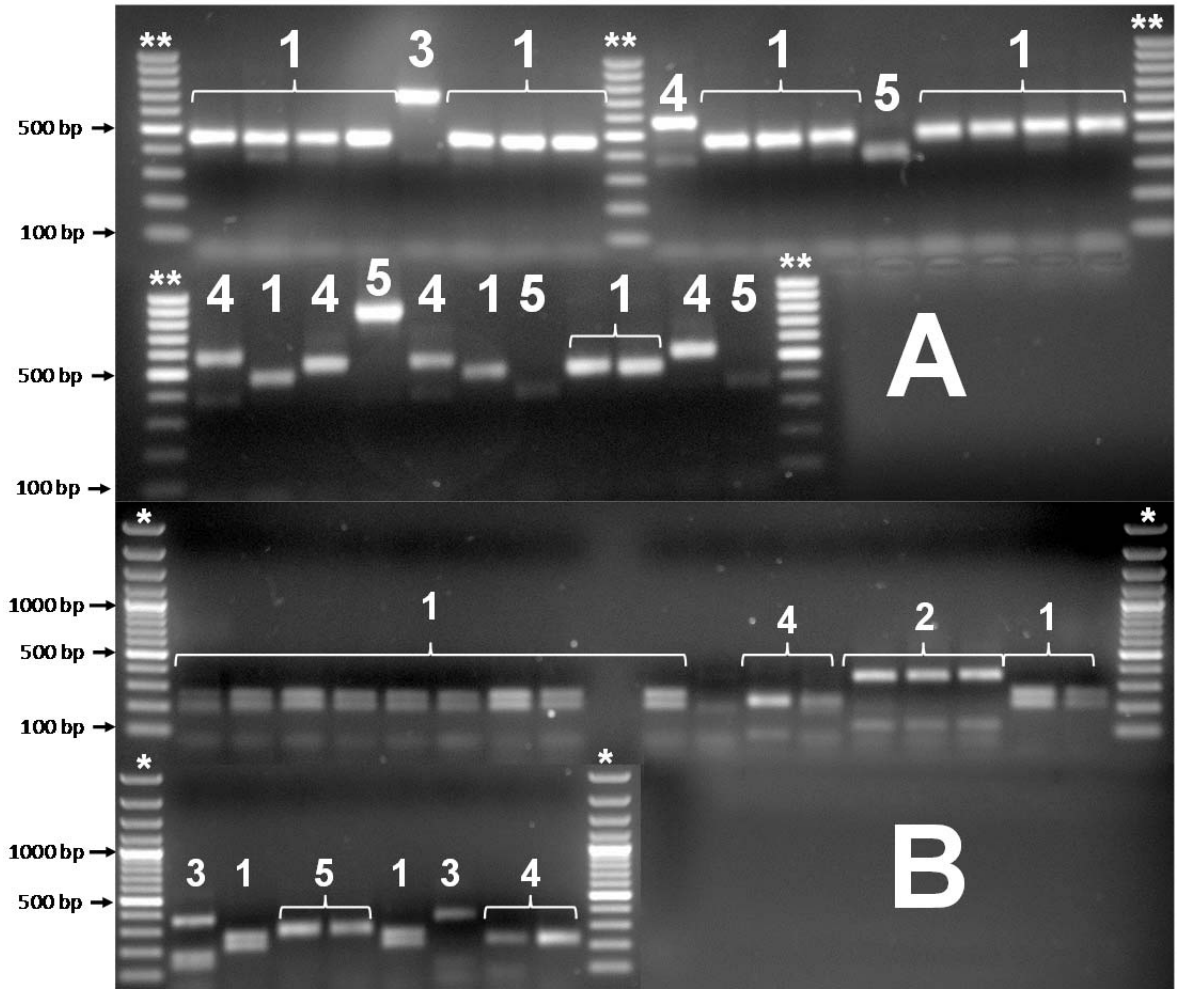

Figure 11
A: Gel electrophoresis of ITS PCR of strains used in this study **B:** Gel electrophoresis of *Hinf*I restriction enzyme digests of ITS PCR of strains used in this study. * = Fermentas GeneRuler 100 bp Ladder Plus; ** = Fermentas GeneRuler 100bp Ladder;

Table 9
Sequencing and identification results of strains used in this study

Group	Strain	Original species	Sequence species ID	Accession number	Sequence size (bp)	Gel band size (bp)[a]	*Hinf*I digest size (bp)[a]	QC (%)	Max. ID (%)
1	Y135	*B. bruxellensis*	*B. bruxellensis*	AM850055.1	470	485	270, 200	100	98
5	CB61[b]	*B. anomalus*	*Saccharomycete sp.*	EF121771.1	572	600	Unknown[c]	89	93
4	CB61[b]	*B. anomalus*	*Dekkera anomala*	AF043510.1	525	525	Unknown[c]	86	99
4	X1	Unknown[d]	*Dekkera anomala*	AF043510.1	526	525	Unknown[c]	86	98
4	ISA1721	*B. naardenensis*	*Dekkera anomala*	AF043510.1	525	525	Unknown[c]	86	99
3	Y105	*B. bruxellensis*	*Candida boidinii*	JF901806.1	702	750	380,180, 160	100	99
2	VIN13	*S. cerevisiae**	n/a	n/a	n/a	n/a	360, 150	n/a	n/a

a = estimated by comparison with DNA molecular weight marker
b = two colonies types were identified from a single freeze culture
c = incomplete *Hinf*I restriction enzyme digestion or lack of *Hinf*I cleavage site
d = unidentified yeast isolate
* = not sequenced
QC = query coverage; Max. ID = maximum identity;

The low QC value for group 4 and 5 (86 and 89% respectively) suggests that the contaminant yeast species is a species that has not been sequenced and annotated previously and this could be an explanation for the different *B. anomalus* RFLP profile. Sequencing of the D1-D2 26S rDNA region could provide more specificity from the BLAST result. The sequencing of the *B. bruxellensis* 5.8S-ITS PCR product yielded a size of exactly 470 bp, 15 bp less than Esteve-Zarzoso *et al.* (1999) obtained. The RFLP profile was also slightly different with fragment sizes of 270 and 200 bp compared to 270 and 215 bp obtained by Esteve-Zarzoso *et al.* (1999).

The discrepancy between the sequenced *B. bruxellensis* 5.8S-ITS PCR product size and that obtained by Esteve-Zarzoso *et al.* (1999) is most likely due to the fact that Esteve-Zarzoso *et al.* (1999) compared DNA ladder migration distance with the DNA band pattern obtained with agarose gel electrophoresis to estimate the size of the 5.8S-ITS PCR products obtained in their study. Although errors can occur during DNA sequencing, the obtained sequence length is always highly accurate and therefore we can assume that the 470 bp length obtained in this study for the *B. bruxellensis* 5.8S-ITS PCR product is correct.

4.2 Sporulation of yeasts

Sporulation by diploid yeasts is the less favourable manner of reproducing compared to simple asexual budding. Sporulation in *S. cerevisiae* occurs infrequently under normal growth conditions and it would normally sporulate due to stressful environment conditions where spore formation is the only possible survival strategy (Codon *et al.* 1995; Simchen *et al.* 1972). According to Van der Walt (1984) the teleomorphic forms of *B. bruxellensis and B. anomalus, D.* bruxellensis and *D. anomala,* should form ascospores on YM agar after 14 days.

Twenty four wine-related yeast strains were tested for the ability to sporulate under different environmental growing conditions and nutrient availability (Table 10). Yeast were spread plated onto nutrient rich YM + vitamins agar and incubated at 4°C, 25°C or 45°C and

sporulation was observed microscopically after two weeks of incubation. Yeast were also spread plated on YPD agar and grown at 30°C until visible colonies had formed. Colonies were selected randomly and then picked sterilely and streaked in 10 mm lines onto nutrient deficient KAc agar. These plates were incubated at 4°C, 25°C or 37°C and sporulation was observed microscopically after two weeks of incubation. According to (Codon *et al.* 1995), the transfer of actively growing *S. cerevisiae* from a nutrient rich growth medium to the nutrient deficient KAc agar medium should induce sporulation overnight.

The *D. bruxellensis* strain ISA1649 is the species type strain and it was used a positive control for sporulation, as confirmed by the curator of the CBS culture collection (personal communication). *S. cerevisiae* was used as a second positive control for sporulation but the diploid strain VIN13 unexpectedly failed to sporulate. Only *D. anomala* X1 and *C. boidinii* were shown to sporulate after 14 days on YM agar and KAc agar and it can therefore be deduced that the sporulation media used were capable of inducing sporulation. The reason for the lack of sporulation by *S. cerevisiae* VIN13 is unknown as this species should sporulate within a few days after exposure to stressful environmental conditions (Codon *et al.* 1995).

Without the positive sporulation control of *S. cerevisiae* it cannot be said for certain that the lack of sporulation by *B. bruxellensis* is a confirmation that the strains are indeed *Brettanomyces* and not *Dekkera*.

Table 10
Sporulation of yeasts (or not) used in this study on YM or KAc agar incubated at 4, 25, 37 or 45°C.

		Sporulation on:					
		YM + Vitamins			KAc		
Species	**Strain**	**4°C**	**25°C**	**45°C**	**4°C**	**25°C**	**37°C**
B. anomalus	CB61	-	-	-	-	-	-
B. bruxellensis	CB63	-	-	-	-	-	-
D. bruxellensis	ISA1649	-	-	-	-	-	-
B. bruxellensis	Y0136	-	-	-	-	-	-
B. bruxellensis	Y101	-	-	-	-	-	-
B. bruxellensis	Y102	-	-	-	-	-	-
B. bruxellensis	Y104	-	-	-	-	-	-
B. bruxellensis	Y111	-	-	-	-	-	-
B. bruxellensis	Y117	-	-	-	-	-	-
B. bruxellensis	Y119	-	-	-	-	-	-
B. bruxellensis	Y121	-	-	-	-	-	-
B. bruxellensis	Y131	-	-	-	-	-	-
B. bruxellensis	Y132	-	-	-	-	-	-
B. bruxellensis	Y133	-	-	-	-	-	-
B. bruxellensis	Y136	-	-	-	-	-	-
B. bruxellensis	Y135	-	-	-	-	-	-
B. nanus	ISA1985	-	-	-	-	-	-
D. bruxellensis	ISA1650		-		-	-	
D. anomala	X1[a]	-	+	-	-	+	-
Dekkera sp.	ISA1791	-	-	-	-	-	-
C. boidinii	Y105[b]	-	+	-	-	+	-
S. cerevisiae	VIN13	-	-	-	-	-	-
S. cerevisiae	BY4742	-	-	-	-	-	-
S. cerevisiae	BY4742 *ssu1*Δ	-	-	-	-	-	-

a – Strain X1 was previously unidentified and was temporarily assigned as X1 until further identification steps have been taken
b– Strain Y105 was previously designated as *B. bruxellensis* and was reassigned as *C. boidinii* after sequence identification

4.3 SO_2 assay optimisation

The quantification of free and total SO_2 in wine is most commonly determined by using the Ripper titration technique (Iland *et al.* 1993; Ripper 1892). The sensitivity and repeatability of this technique were considered insufficient for the needs of this study and therefore other means of SO_2 quantification was sought.

A method previously described to quantify SO_2 in beer (AOAC International, official method 963.11) was scaled down for use in the laboratory. The linearity of the SO_2 quantification assay was tested using three different media YPD+TA, TA+2% D-glucose and SWM. As seen in Figure 12 the calibration curves for each media were linear and reproducible (R^2 values of 0.9971, 0.9988 and 0.9909 for YPD+TA, TA+2% D-glucose and SWM, respectively). Differences are seen in the level of absorption between the three media with SWM consistently showing a lower absorption range and because of this unique assay calibrations were performed with each medium for each experiment.

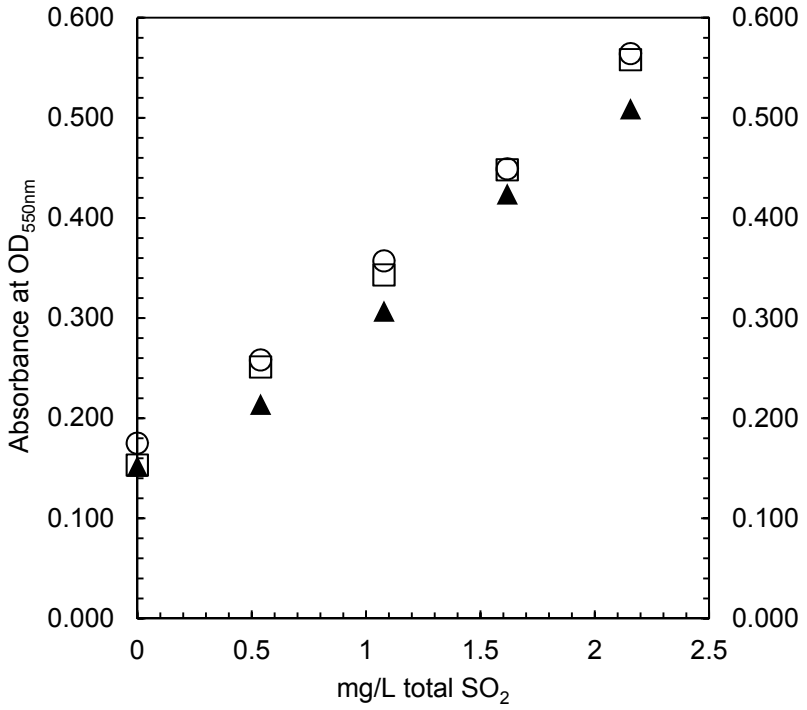

Figure 12
Linear calibration curve of optical density at 550nm versus total mg/L SO_2 for the following media: ○: YPD + 75 mM tartaric acid, pH 3.5 (y = 0.1797x + 0.1668 R^2 = 0.9971); □: 75 mM tartaric acid + 2% D-glucose, pH 3.5 (y = 0.1868x + 0.1492 R^2 = 0.9988); ▲: SWM, pH 3.5 (y = 0.1714x + 0.1364 R^2 = 0.9909); Standard deviations are less than 10% for all points.

The detection range of the assay is very low (0.5 – 2.5 mg/L SO_2) and is therefore suited to detecting small changes in SO_2 levels. Above the 2.5 mg/L SO_2 level, absorbance saturation occurs due to the increasingly dark purple colouration, resulting in inaccurate SO_2 concentration determination (data not shown).

4.4 The binding power of various microbiological media

It is essential to obtain highly accurate free and total SO_2 data when a specific theoretical molecular SO_2 concentration is required in a given medium. Due to the fact that the SO_2 quantification technique used is influenced heavily by the spectrophotometric absorbance characteristics of the medium wherein SO_2 is to be quantified, it is vital to use a calibration curve equation unique to a specific medium.

Many compounds are known to bind to SO_2 and significantly reduce the amount of free SO_2 and by extension molecular SO_2. As seen in Figure 13, three different media were tested for their ability to bind SO_2: YPD + TA (50 g/L yeast peptone dextrose, 75 mM tartaric acid, pH 3.5), TA (75 mM tartaric acid, 2% ($^{m}/_{v}$) glucose, pH 3.5) and SWM, pH 3.5.

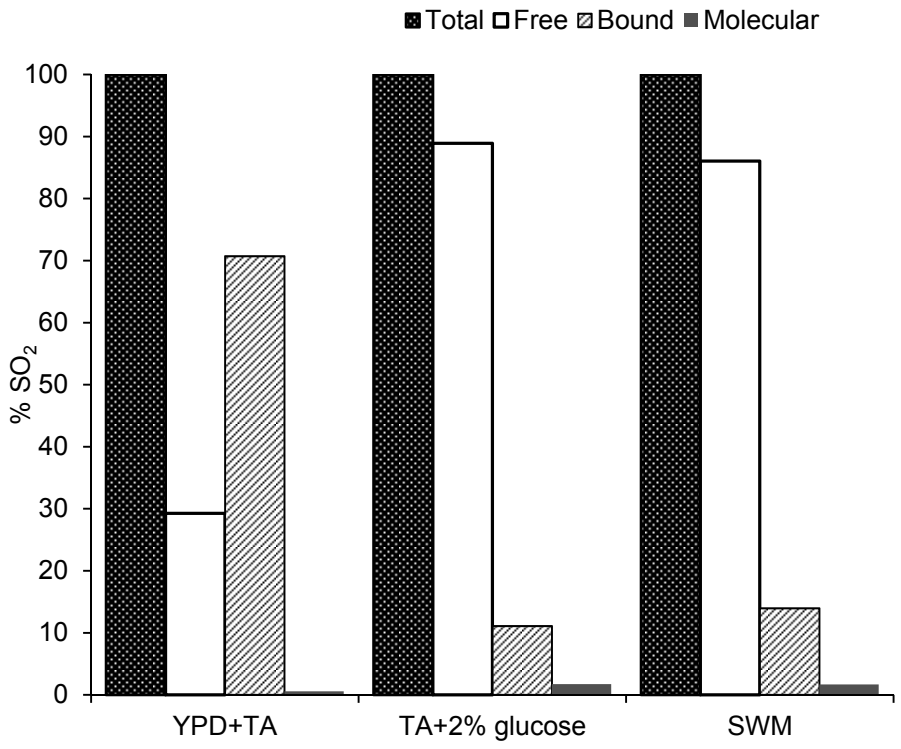

Figure 13
The approximate ratios of the three states of SO_2 in YPD+TA, TA+2% D-glucose and SWM media at pH 3.5.

Each medium showed greatly different degrees of SO_2 binding capability. YPD+TA showed the highest percentage of bound SO_2 with approximately 70.7% of total SO_2 being in a bound state compared to 11.1 and 14.0% bound SO_2 for TA + 2% glucose and SWM, respectively.

The TL50 value of a given medium is the amount of total SO_2 concentration required to obtain a free SO_2 concentration of 50 mg/L (Barbe *et al.* 2002). It is a useful value to quickly compare various media SO_2 binding power. The TL50 for the three tested media are as follows: YPD + TA: 171.1 mg/L; TA + 2% glucose: 55.6 mg/L; SWM: 58.055 mg/L. These TL50 values confirm what is seen with Figure 13 that YPD + TA requires more than three times more total SO_2 than TA + 2% or SWM to achieve the same amount of free SO_2 and by extrapolation molecular SO_2.

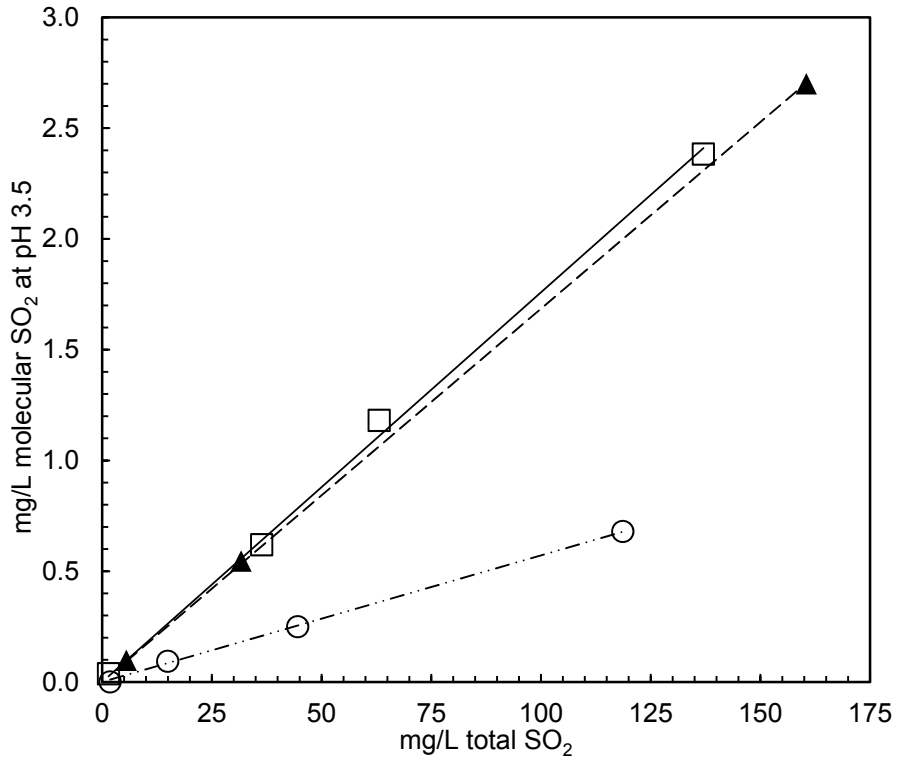

Figure 14
mg/L molecular SO_2 versus total mg/L SO_2 for the following media: ○: YPD + 75mM tartaric acid, pH 3.5 (y = 0.0057x R^2 = 0.9993); □: 75mM tartaric acid + 2% D-glucose, pH 3.5 (y = 0.0168x R^2 = 0.9999); ▲: SWM, pH 3.5 (y = 0.0176x R^2 = 0.9979); Standard deviations are less than 10% for all points.

This is most likely due to the complex and undefined nature of the yeast extract component and high sugar levels in YPD which can potentially bind free SO_2. In Figure 14, molecular SO_2 concentration is plotted against total SO_2 concentrations for each media which was tested. To obtain 0.5 mg/L molecular SO_2 at pH 3.5, SWM and TA both require approximately 30 mg/L total SO_2 whereas YPD + TA requires approximately 90 mg/L total SO_2. YPD + TA therefore requires approximately three times more total SO_2 to achieve a

similar molecular SO_2 concentration and as such this result was very important for all future experiments using YPD + TA (or the other two media) requiring a specific molecular SO_2 concentration.

The exact reason for the high degree of SO_2-binding that occurs with YPD (relative to the other two media) is unclear. According to Jarvis and Lea (2000), glucose accounts for only 0.1% of bound SO_2 whereas acetaldehyde, pyruvate and α-keto glutarate account for 99.8, 83 and 58% of bound SO_2, respectively. However, the latter three compounds are however usually only found in a given medium during or after growth of a microorganism (unless the compounds are present in the medium).

YPD consists of yeast extract (1% $^m/_v$), peptone (2% $^m/_v$) and glucose (2% $^m/_v$) (Sherman 1991). Yeast extract is created from an extraction of autolysed or hydrolysed yeast and peptone usually originates from the proteolytic digestion of animal proteins. Yeast extract is therefore a highly undefined medium as it contains unknown ratios of carbon, nitrogen, vitamins and minerals and it is speculated that this undefined portion contains compounds with a high degree of SO_2-binding capability. The degree of peptide binding from the peptone portion is also speculated to contribute to the bound SO_2 portion. Very little literature is available for these compounds however there is extensive coverage of the major juice and fermentation compounds (Burroughs and Sparks 1964; Burroughs and Sparks 1973; Jarvis and Lea 2000; Würdig 1989).

4.5 Tolerance of yeasts to molecular SO_2 and/or ethanol

One of the primary uses of SO_2 during winemaking is to prevent the growth of unwanted microorganisms. Certain wine-related yeast species show high resistance to the antimicrobial properties of SO_2 and therefore it is crucial to understand how these yeasts deal with the presence of potentially toxic levels of SO_2 in their environment. The maintenance of molecular SO_2 at a 0.8 mg/L concentration has been shown to prevent growth of almost all unwanted microorganisms in a wine fermentation (du Toit *et al.* 2005).

Park and Bakalinsky (2000) showed the existence of a membrane bound protein sulphite pump, Ssu1p, in *S. cerevisiae.* Strains lacking the sulphite pump showed increased sensitivity to SO_2 and strains with an overexpressed Ssu1p showed increased resistance to SO_2. A similar approach was taken in this study with the incorporation of yeasts found in the wine environment, *S. cerevisiae* VIN13 and various *B. bruxellensis* strains.

Twenty one different yeast strains were grown on pH 3.5 YPD agar supplemented with three levels of molecular SO_2, 0, 0.3 or 0.4 and 0.8 mg/L and/or 10% ($^{v}/_{v}$) ethanol (Table 11) in order to determine their sensitivity to molecular SO_2 on solid growth media.

Table 11
Tolerance of various *S. cerevisiae, Dekkera* and *Brettanomyces* strains to varying molecular SO_2 levels and/or ethanol. Scores are average of duplicate repeats.

		SO_2 tolerance at				
		mol. SO_2 (mg/L)			mol. SO_2 (mg/L) + ethanol (v/v)%	
Species	Strain	0	0.4	0.8	0.3, 10%	0.8, 14%
S. cerevisiae	VIN13	+++++	+++++	+++++	+++++	+++
S. cerevisiae	BY4742	+++++	+++++	+++++	++++	-
S. cerevisiae	BY4742 *ssu1*Δ	+++++	+++++	-	+++	-
B. anomalus	FOEB CB61	+++++	+++++	++++	+	-
B. bruxellensis	FOEB CB63	+++++	++++	-	++++	-
B. bruxellensis	ISA1649	+++++	++	+	+	-
Dekkera sp.	ISA1791	+++++	+++	-	+	-
D. anomala	IWBT X1	+++++	+++	-	-	-
B. bruxellensis	ARC Y0136	+++++	+++++	+++	++	-
B. bruxellensis	IWBT Y101	+++++	+++++	-	++	-
B. bruxellensis	IWBT Y102	+++++	+++++	-	+++++	-
B. bruxellensis	IWBT Y104	+++++	+++++	-	+++	-
B. bruxellensis	IWBT Y111	+++++	-	-	-	-
B. bruxellensis	IWBT Y117	+++++	+	-	+++	-
B. bruxellensis	IWBT Y119	+++++	+++++	+++++	-	-
B. bruxellensis	IWBT Y121	+++++	+++++	-	+++	-
B. bruxellensis	IWBT Y131	+++++	+++++	-	-	-
B. bruxellensis	IWBT Y132	+++++	-	-	-	-
B. bruxellensis	IWBT Y133	+++++	+++++	-	++	-
B. bruxellensis	IWBT Y135	+++++	++	-	++++	-
B. bruxellensis	IWBT Y136	+++++	++	-	+++	-

Growth	
+++++	Very strong
++++	Strong
+++	Average
++	Weak
+	Poor
+/-	Very poor
-	None

S. cerevisiae VIN13 is said to be tolerant to 50 mg/L free SO_2 and was used as positive control for SO_2 and and/or ethanol tolerance (Anchor 2011). *S. cerevisiae* VIN13 showed the greatest resistance to SO_2 and it grew very well in the presence of 0.8 mg/L and 14% ethanol. The laboratory strain *S. cerevisiae* BY4742 tolerated SO_2 well but it could not tolerate the additional presence of ethanol while the sulphite sensitive strain *S. cerevisiae* BY4742 *ssu1*Δ grew poorly in the presence of SO_2 and/or ethanol confirming the results obtained by Park and Bakalinsky (2000).

The *B. bruxellensis* strains that were used in this experiment come from a variety of sources such as beer fermentations (low SO_2 levels), wine cellar equipment (intermittent exposure and moderate to high SO_2 levels) and wine samples (long exposure and moderate to high SO_2 levels). Since *B. bruxellensis* manages to survive throughout fermentation and into the wine bottle, in the presence of SO_2, it was expected that most strains would show similar levels of tolerance to molecular SO_2. However, strain-dependent behaviour is seen regarding the *B. bruxellensis* SO_2 tolerance. Comparison of the SO_2 tolerance results obtained in this experiment with the strain source in Table 6 does not provide any insight with regards to the influence of the source of the strain on SO_2 tolerance. Certain strains (IWBT Y111 and IWBT Y132) are incapable of growing at 0.4 mg/L molecular SO_2 or 0.3 mg/L molecular SO_2 + 10% ethanol and others (Y0136 and IWBT Y119) capable of growing at 0.8 mg/L molecular SO_2. The presence of ethanol greatly enhanced the toxicity of SO_2 for *B. bruxellensis* as most strains grew poorly at 0.3 mg/L molecular SO_2 + 10% ethanol and none grew at 0.8 mg/L and 14% ethanol. This strain dependent behaviour was observed by Barata *et al* (2008) in wine with 12% ethanol and 9 out of 19 *D. bruxellensis* strains inoculated into this wine did not grow after the addition of 100 mg/L PMB.

The inclusion of only two *B. anomalus* strains in this experiment does not give any insight into a possible strain dependent behaviour as shown for *B. bruxellensis.* However the fact that IWBT X1 could not grow in the presence of 0.3 mg/L molecular SO_2 and 10% ethanol

while FOEB CB61 could grow under these conditions, suggests that similar strain dependent behaviour will be observed when testing a larger strain set.

4.6 Yeast sulphite accumulation and efflux

The ability of a yeast to cope with the presence of toxic levels of SO_2 in its environment will determine its survival chances and if it can out-compete other microorganisms for nutrients. To test intracellular accumulation of SO_2, yeast cells were resuspended in a TA buffer containing 100 mg/L SO_2. The SO_2 concentration was measured in the supernatant at regular time intervals and the intracellular SO_2 concentration was extrapolated as described in the materials and methods section.

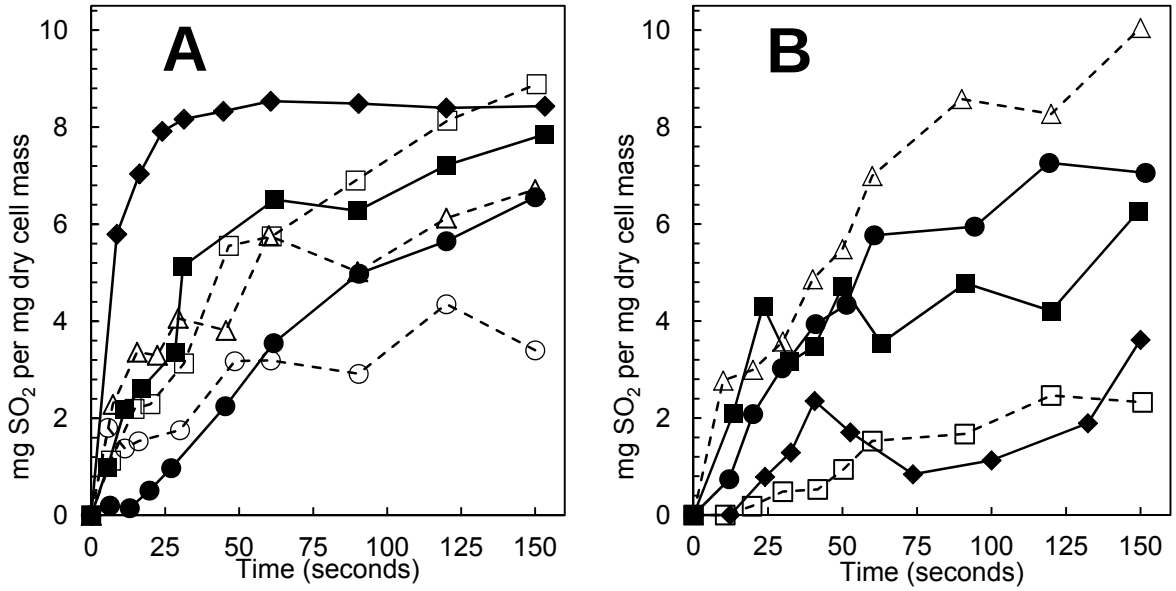

Figure 15
A: Sulphite accumulation **B**: Sulphite efflux. -◆-: *S. cerevisiae* VIN13; -●-: *S. cerevisiae* BY4742; -■-: *S. cerevisiae* BY4742 *ssu1Δ;* □: *B. bruxellensis* Y121; - -○- -: *B. bruxellensis* CB63; - -△- -: *B. bruxellensis* ISA1649; Standard deviations are less than 15% for all points.

Figure 15A shows the intracellular accumulation of SO_2 by the yeasts *S. cerevisiae* (strains VIN13, BY4742 and BY4742 *ssu1Δ)* and *B. bruxellensis* (strains ISA1649, CB63 and Y121). The intracellular accumulation of SO_2 occurred rapidly within the first minute after which the intracellular SO_2 concentration began to saturate, although saturation did not occur at the same time for all strains. *S. cerevisiae* VIN13 accumulated SO_2 at the highest rate, the deletion mutant BY4742 *ssu1Δ* accumulated SO_2 at a faster rate compared to its wild-type

parental strain BY4742. *B. bruxellensis* intracellular accumulation of SO_2 was highly strain dependent and followed a similar trend to that of the *S. cerevisiae* strains.

Figure 15B shows the extracellular efflux of SO_2 by the yeasts *S. cerevisiae* (strains VIN13, BY4742 and BY4742 *ssu1*Δ*)* and *B. bruxellensis* (strains ISA1649 and Y121). The yeast strains were loaded with maximal SO_2 and resuspended in a sulphite free medium. The increase in SO_2 in the supernatant was determined at regular time intervals. A rapid and almost linear efflux of SO_2 occurred in all strains, albeit at different rates. As seen in Figure 15B, *S. cerevisiae* VIN13 effluxed SO_2 at the slowest rate. The deletion mutant BY4742 *ssu1*Δ effluxed SO_2 at a slower rate compared to its wild-type parental strain BY4742. Both *Brettanomyces* strains effluxed SO_2 at varying rates. *B. bruxellensis* Y121 effluxed at rate which was very similar to *S. cerevisiae* VIN13 while *B. bruxellensis* ISA1649 effluxed at a rate closer to that of *S. cerevisiae* BY4742.

Comparison of the yeasts SO_2 tolerance data (Table 11) with the SO_2 intracellular efflux data (Figure 15) yielded unexpected results. It was expected that yeast showing a high tolerance to SO_2 should correlate with the cells ability to remove intracellular SO_2 rapidly and efficiently via the membrane bound Ssu1p SO_2 transporter. *B. bruxellensis* Y121 and *S. cerevisiae* VIN13 both show strong growth at high molecular SO_2 concentrations yet their SO_2 efflux rates are amongst the lowest tested. The opposite is seen with *B. bruxellensis* ISA1649 as it has the highest efflux rate yet it grows poorly at high molecular SO_2 concentrations. The only yeast strains where tolerance to SO_2 correlated with the cells ability to remove intracellular SO_2 is *S. cerevisiae* BY4742 and the deletion mutant *S. cerevisiae* BY4742 *ssu1*Δ.

The difference between the growth of yeasts on solid media and in liquid cultures could provide an explanation for the differences seen between the two methods. Čáp *et al.* (2009) compared the stress response of *S. cerevisiae* BY4742 grown in liquid media or on solid media. It was shown that cell survival in liquid media is dependent on its ability to scavenge reactive oxygen species (ROS) whereas growth on solid media, colonies develop a complex

environment where central cells provide nutrients for cells on the colony margin which ensures the expansion and growth of the colony.

An alternative to testing the SO_2 tolerance of yeast on solid media is presented by Uzuka *et al.* (1985) where yeast's growth is monitored spectrophotometrically over time in a defined liquid medium. The logarithmic growth profile of a specific yeast strain in the presence/absence of SO_2 and/or ethanol in this liquid medium can then be compared with other strains as well as compared with the strains SO_2 tolerance on solid media.

Therefore, it can be concluded from this study that yeast SO_2 stress tolerance on solid media in comparison to a yeasts SO_2 efflux rates are not a sufficient indicator of yeasts sensitivity to SO_2 and that other SO_2 tolerance tests, such as liquid growth assays, must be included in an experiment of this nature to provide conclusive evidence .

4.7 The effect of molecular SO_2 on carbon energy-metabolism flux

At the normal wine pH range of pH 3 to 4, the molecular form ($H_2O.SO_2$) of SO_2 crosses the cell membrane by passive diffusion and dissociates into the anionic forms (HSO_3^- and SO_3^{2-}) due to the higher intracellular pH (6-7). SO_2, in the anionic states, can be severely disruptive to a cells metabolism at the correct concentration due to its inhibitory effects on intracellular enzymes. It has been shown that SO_2 inhibits key enzymes in glycolysis and enzymes involved in anaerobic fermentation (Figure 7).

Maintenance of an oenologically relevant SO_2 level during wine fermentation is therefore crucial to prevent growth of unwanted yeast and bacteria. It was shown by du Toit *et al.* (2005) and Coulon *et al.* (2011) that a molecular SO_2 concentration of 0.8 mg/L is sufficient to inhibit growth of *B. bruxellensis* (although there are exceptions to this general rule, see Table 7 for a full list as tested in this study). The yeast metabolic response to molecular SO_2 has not been studied in detail with previous studies covering only individual metabolic response aspects such as cell viability (Agnolucci *et al.* 2010; Barata *et al.* 2008;

du Toit *et al.* 2005; Jensen *et al.* 2009), acetaldehyde production (Frivik and Ebeler 2003; Liu and Pilone 2000) and volatile phenolic production (Benito *et al.* 2009).

4.7.1 Short term response to molecular SO_2 in TA medium

S. cerevisiae VIN13 and *B. bruxellensis* CB63 were evaluated for their short term (48 hour) metabolic response to the presence of three molecular SO_2 concentrations in a medium containing only D-glucose and tartaric acid. Each yeast was inoculated into individual Erlenmeyer flasks containing 200 mL TA medium (75 mM tartaric acid and 20 g/L D-glucose). Acetic acid, acetaldehyde, ethanol and D-glucose concentrations were monitored in this experiment over a period of 48 hours following the addition of SO_2 (after 30 minutes of incubation) to the medium at 0.53 and 1.0 mg/L molecular SO_2. The results were compared to those obtained in the absence of SO_2.

These four compounds were chosen as they are central to the energy metabolism of the cell and SO_2 has a direct negative effect on the enzymes involved in their respective metabolic pathways. As seen in Figure 16, immediate differences are apparent between how *S. cerevisiae* VIN13 and *B. bruxellensis* CB63 responded to incubation in a minimal medium. *S. cerevisiae* VIN13 characteristically fermented D-glucose very rapidly to dryness producing mostly ethanol as a fermentation product irrespective of the level of molecular SO_2 present in the medium. *B. bruxellensis* CB63 fermented D-glucose very slowly and could not consume all the available sugar within 48 hours but it produced slightly more ethanol per mg dry cell mass than *S. cerevisiae* VIN13 could.

Molecular SO_2 had a direct impact on the rate that *B. bruxellensis* CB63 fermented D-glucose and an increased molecular SO_2 level had a direct correlation to a decrease in the fermentation rate. In the presence of 1.0 mg/L molecular SO_2 *B. bruxellensis* CB63 fermented slower than in the presence of 0.53 mg/L molecular SO_2.

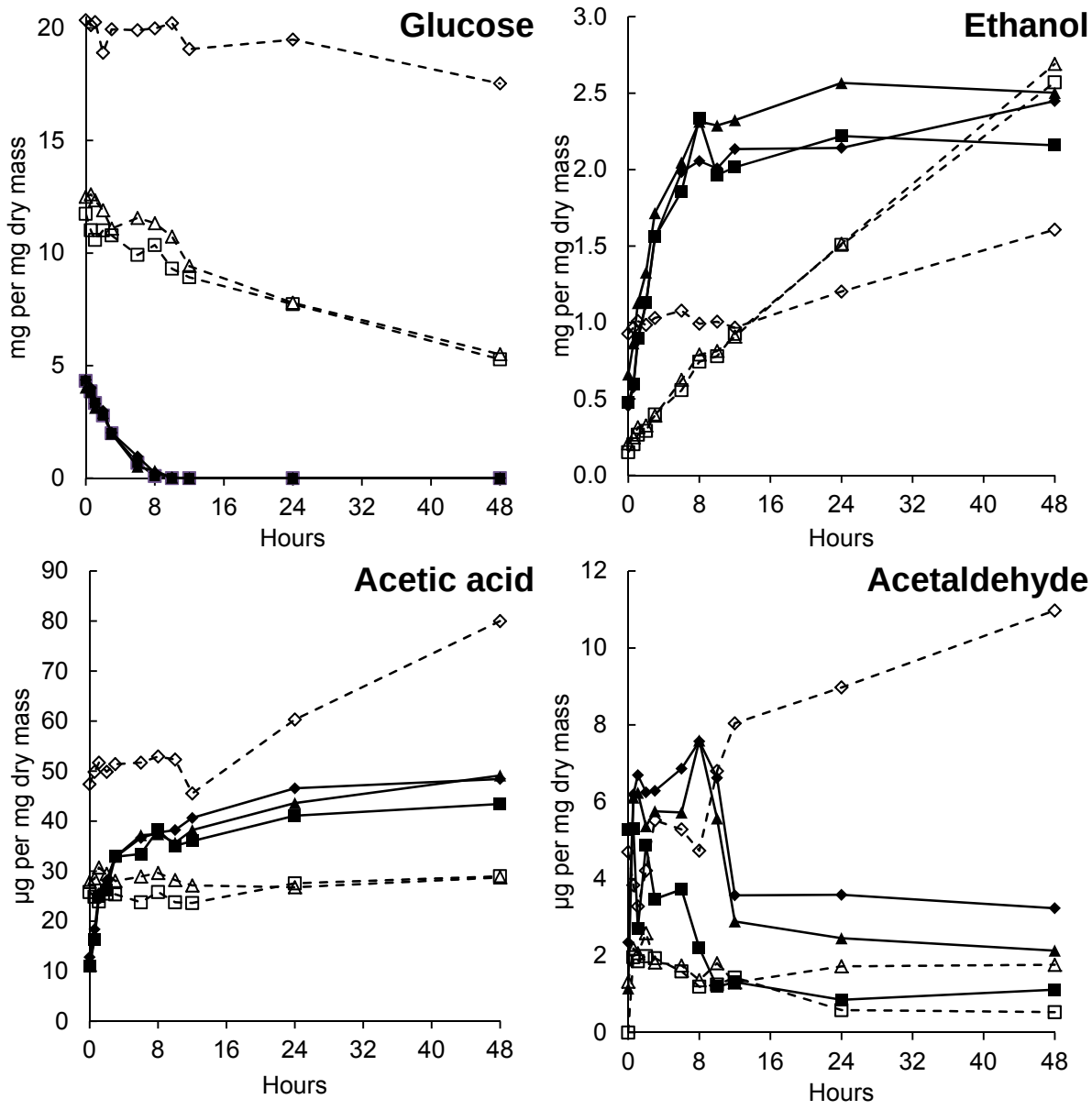

Figure 16
The effect of molecular SO_2 on key primary metabolites over a 48 hour period in TA + 2% D-glucose. **SO_2 was added at 30 minutes**. Values are normalised against dry mass per 1 mL culture. Standard deviations for all points are less than 10%. - -□- -: *B. bruxellensis* CB63 0 mg/L molecular SO_2; - -△- -: *B. bruxellensis* CB63 0.53 mg/L molecular SO_2; - -◇- -: *B. bruxellensis* CB63 1.0 mg/L molecular SO_2; -■-: *S. cerevisiae* VIN13 0 mg/L molecular SO_2; -▲-: *S. cerevisiae* VIN13 0.53 mg/L molecular SO_2; -◆-: *S. cerevisiae* VIN13 1.0 mg/L molecular SO_2.

One mg/L molecular SO_2 had the greatest impact on *B. bruxellensis* metabolism. The inhibitory effect of SO_2 on anaerobic fermentation could be seen by the complete stalling of D-glucose consumption and ethanol production for approximately 8 hours for samples treated with 1 mg/L molecular SO_2. Even though glycolysis had stalled, acetaldehyde production rapidly increased for approximately 3 hours. Acetic acid production did not

increase for approximately 8 hours but thereafter it increased along with a decreased ethanol concentration. The increase of acetaldehyde after 8 hours did correlate with a slight decrease in D-glucose concentration and an increase in ethanol. This suggests that the acetaldehyde bound to sufficient molecular SO_2 to decrease the degree of enzymatic inhibition caused by the molecular SO_2 and allowed D-glucose consumption and ethanol production to progress, albeit at a slow rate.

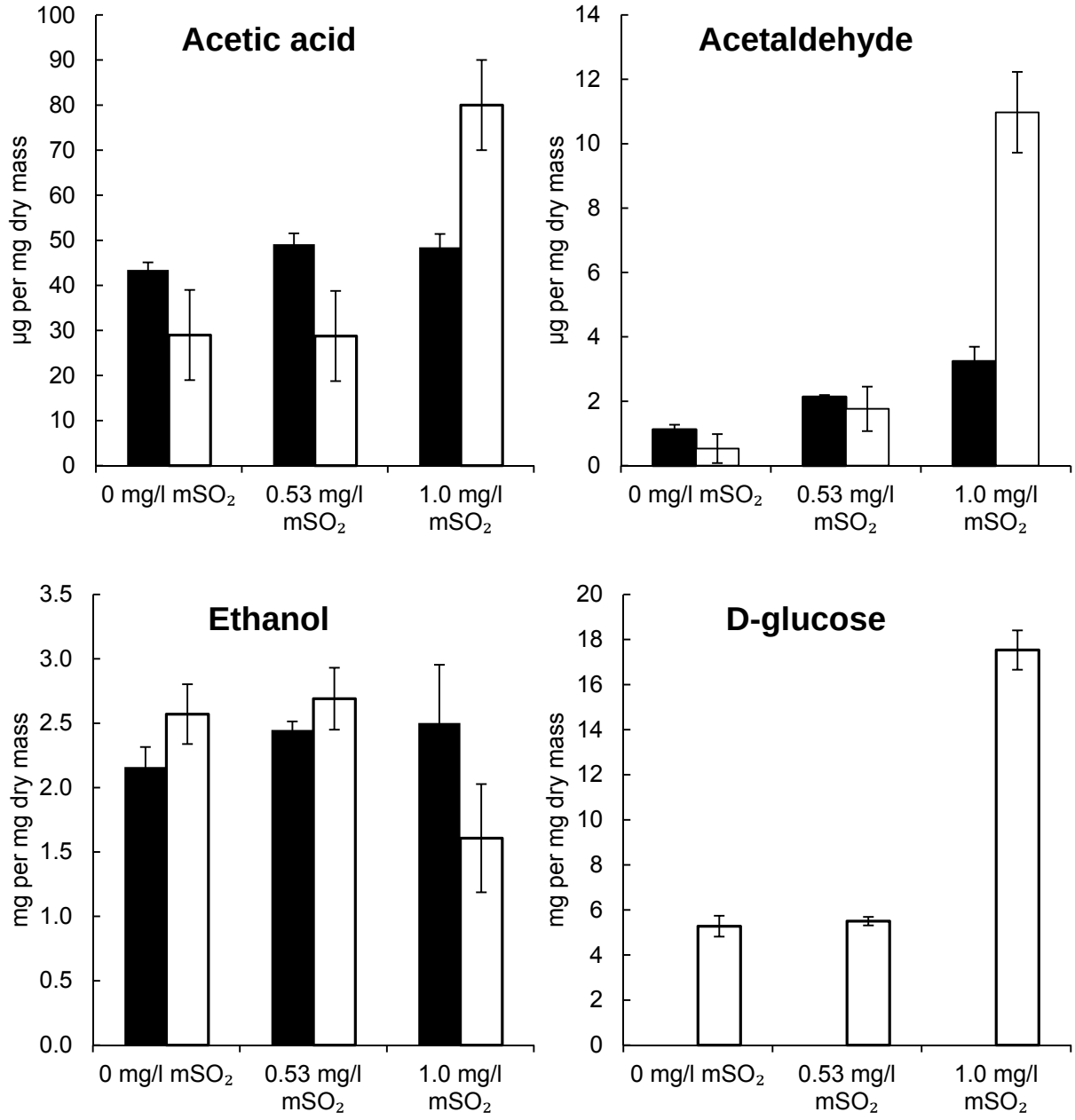

Figure 17
The effect of molecular SO_2 on the key primary metabolites: final concentrations at the 48 hour time point in TA media. Values are normalised against dry mass per 1 mL culture. *S. cerevisiae* VIN13 (■) *B. bruxellensis* CB63 (□).

At the 8 hour time point, *S. cerevisiae* VIN13 had completely consumed all available sugar and ethanol production peaked. However acetaldehyde levels decreased dramatically after this and remained at a constant level for the next 40 hours while acetic acid levels increased slowly. This suggests that the acetaldehyde was consumed by the yeast and was directed towards acetic acid production. A similar acetaldehyde trend was observed by Frivik and Ebeler (2003) in white grape juice fermentations with 50 mg/L SO_2 and also by Osborne *et al.* (2000) in a tartaric acid buffer with 2 g/L D-glucose. Acetaldehyde levels were also proportionate to the amount of molecular SO_2 present in the medium which suggest that acetaldehyde plays an indirect protective SO_2 detoxification role in the cell (Liu and Pilone 2000).

Figure 17 illustrates the final metabolite levels per mg dry cell mass at the 48 hour time point for this experiment. Acetic acid and acetaldehyde productions were stimulated by the presence of 1.0 mg/L molecular SO_2 for *B. bruxellensis* CB63 whereas in *S. cerevisiae* VIN13, molecular SO_2 had a low impact on acetaldehyde and no effect on acetic acid production. The consumption of D-glucose and production of ethanol by *B. bruxellensis* CB63 was negatively affected by the presence of 1.0 mg/L molecular SO_2 compared to *B. bruxellensis* CB63 at 0 or 0.53 mg/L molecular SO_2 whereas for *S. cerevisiae*, molecular SO_2 had no impact on the final D-glucose and ethanol concentrations.

The Custer effect (Carrascosa *et al.* 1981; Scheffers and Wiken 1969), or negative Pasteur effect, is the inhibition of alcoholic fermentation in an anaerobic environment along with the production of acetic acid from glucose and the reduction of NAD^+. This effect could explain the slow glucose consumption of *B. bruxellensis* CB63 relative to *S. cerevisiae* VIN13 as this effect is not present in *S. cerevisiae*, unlike in *B. bruxellensis* (Scheffers and Wiken 1969). The metabolic inhibition caused by 1 mg/L molecular SO_2 most likely negatively influenced the redox balance in the cell and the exaggerated increase of acetic acid and decrease in ethanol could be as a result of *B. bruxellensis* facing a shortage of NAD^+. The production of NADH by aldehyde dehydrogenase in the conversion of acetaldehyde to acetate could assist

in restoring the cells redox balance by decreasing the NAD^+/NADH ratio. *S. cerevisiae* on the other hand most likely produced more higher-alcohols or glycerol rather than acetic acid and quantification of these compounds could verify this hypothesis.

A less robust *S. cerevisiae* strain, with a lower fermentative capacity and a higher sensitivity to SO_2 compared to *S. cerevisiae* VIN13, would have presented more informative results with regards to the effect of molecular SO_2 on D-glucose consumption and ethanol production.

4.7.2 Response to molecular SO_2 in wine-like conditions over a 32-day period

SWM medium was used to mimic the stressful growing/survival conditions of an ageing wine where little sugar was available and high amounts of ethanol was present. SO_2 was added after 4 days of incubation to the medium at 0.4 and 0.8 mg/L molecular SO_2 and the results were compared to those obtained in the absence of SO_2.

S. cerevisiae VIN13 and *B. bruxellensis* CB63 were inoculated into individual Erlenmeyer flasks containing 200 mL SWM media. Acetic acid, acetaldehyde, D-fructose and D-glucose concentrations were monitored in this experiment over a period of 32 days. These four compounds were chosen as they are central to the energy metabolism of the cell and SO_2 has a direct negative effect on the enzymes involved in their respective metabolic pathways. Ethanol was not quantifiable due to technical difficulties with available quantification method.

As shown in Figure 18, differences are apparent between how *S. cerevisiae* VIN13 and *B. bruxellensis* CB63 survive in SWM although the addition of SO_2 on day 4 markedly affects the metabolism of both yeasts. Almost all available D-glucose had been consumed for all samples of *S. cerevisiae* VIN13 by day 4. D-fructose consumption by *S. cerevisiae* VIN13 however was not complete by day 4 and its continued consumption rate decreased markedly after the addition of SO_2 up until it was entirely consumed on day 12. Acetic acid was continuously produced throughout the 32-day period by both yeast species. Differences are seen between *S. cerevisiae* VIN13 and *B. bruxellensis* CB63 acetic acid production response to the presence of SO_2.

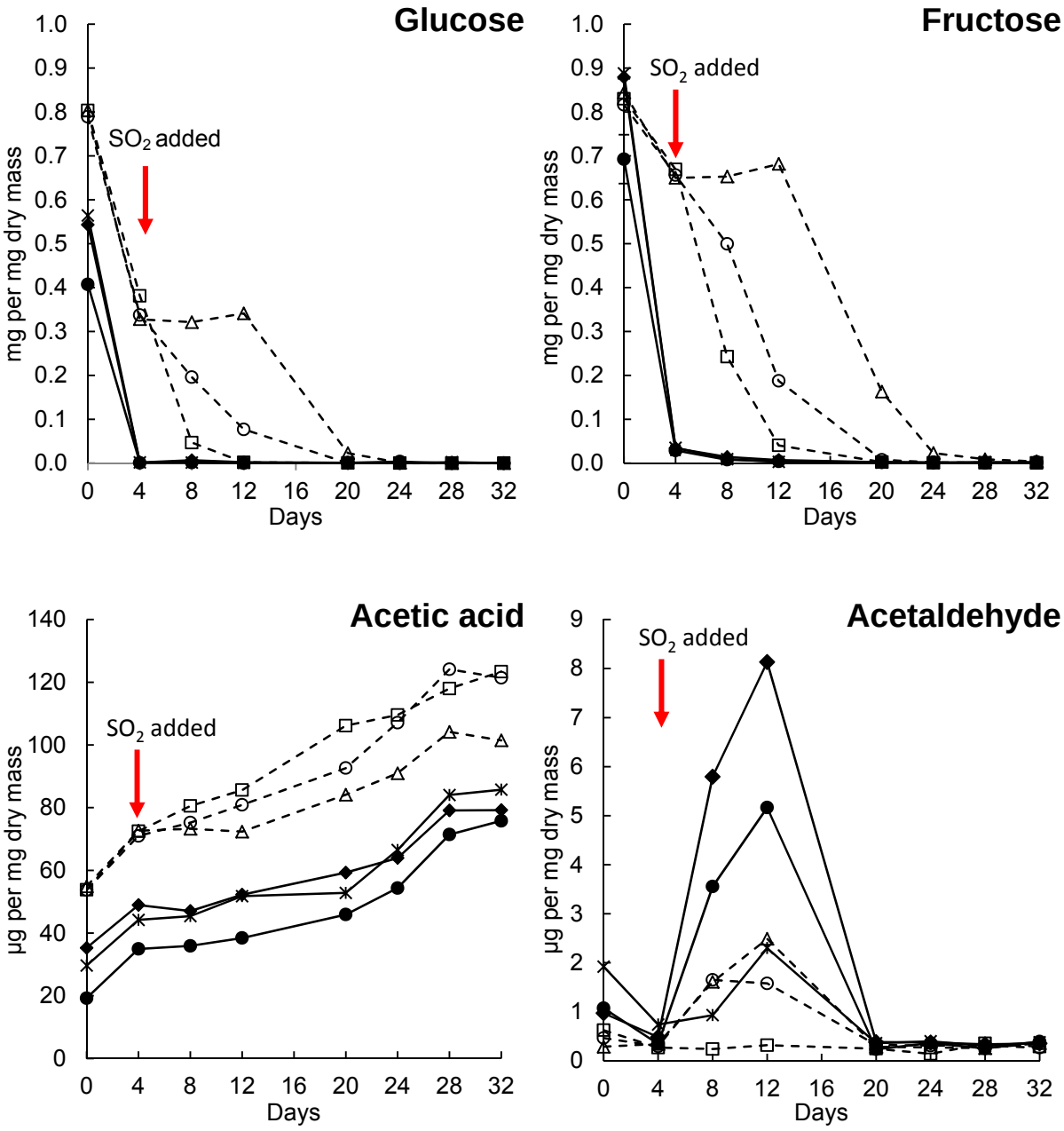

Figure 18
The effect of molecular SO_2 on key primary metabolites over a 32 day period in SWM. **SO_2 was added on day 4.** Standard deviations for all points are less than 12%. Values are normalised against dry mass per 1 mL culture. - -□- -: *B. bruxellensis* CB63 0 mg/L molecular SO_2; - -○- -: *B. bruxellensis* CB63 0.4 mg/L mol. SO_2; - -△- -: *B. bruxellensis* CB63 0.8 mg/L molecular SO_2; -✳-: *S. cerevisiae* VIN13 0 mg/L SO_2 ; -●-: *S. cerevisiae* VIN13 0.4 molecular mg/L SO_2; -◆-: *S. cerevisiae* VIN13 0.8 mg/L molecular SO_2.

B. bruxellensis CB63 acetic acid production was very similar for all samples on day 4. However after SO_2 addition, divergence occurred between the samples with more SO_2 reducing the rate and overall amount of acetic acid produced. *S. cerevisiae* VIN13 acetic acid production responded less to SO_2 and but it followed a similar trend as *B. bruxellensis* CB63.

Even after total sugar depletion, both yeasts continued to produce acetic acid. The Custer effect could be involved here but without knowing the production of ethanol the occurrence of this effect is only speculative. Acetaldehyde production and consumption by both yeast species was affected most by the presence of molecular SO_2.

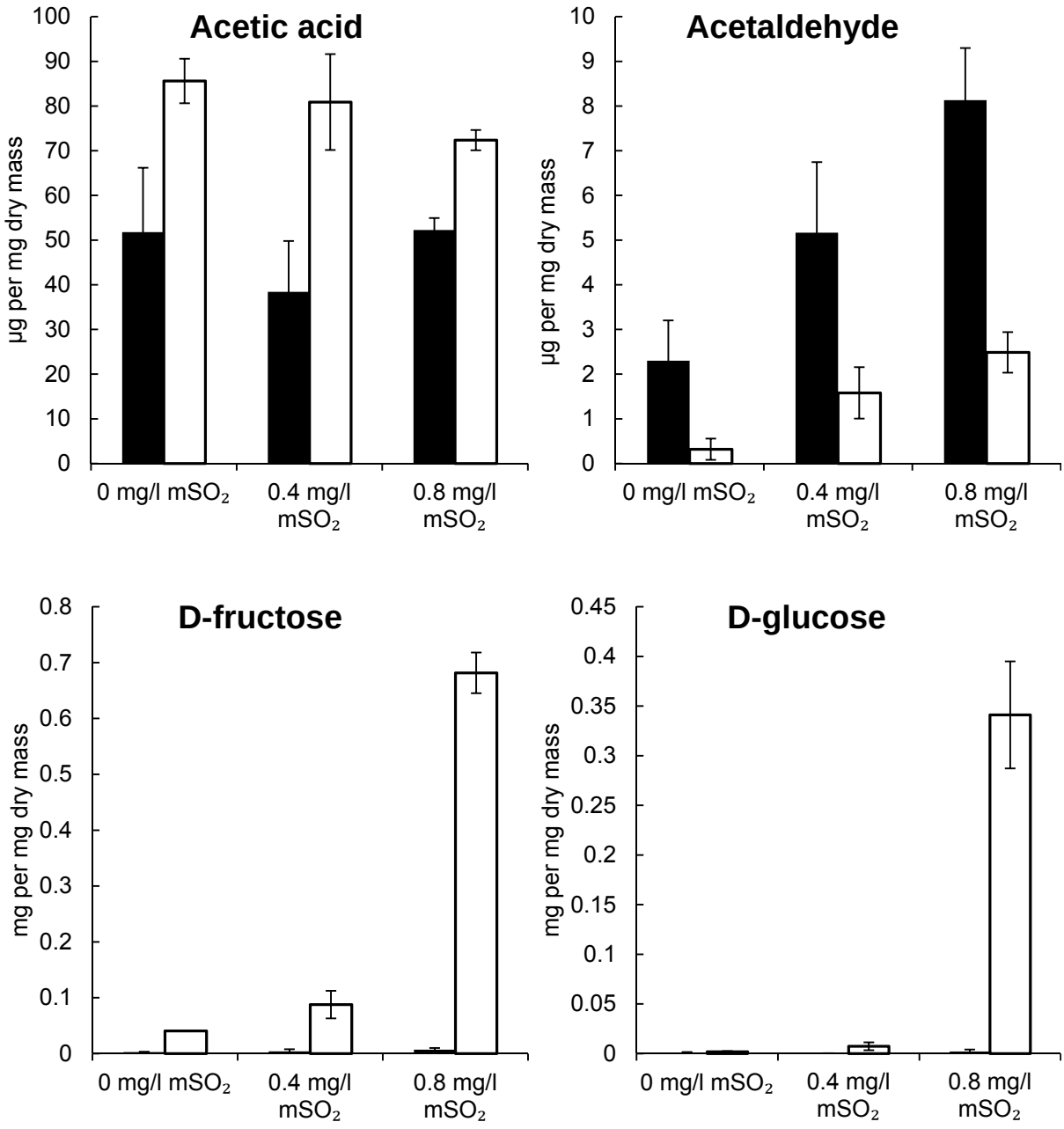

Figure 19
The effect of mSO_2 on the key primary metabolites: final concentrations at the day 12 in SWM media. Values are normalised against dry mass per 1 mL culture. *S. cerevisiae* VIN13 and (■) *B. bruxellensis* CB63 (□).

Immediately after SO_2 addition, acetaldehyde production increases significantly and as seen in Figure 18, it peaked at day 12 after which it decreased to almost 0 µg per mg dry cell mass. This event also correlated with the restarting of sugar consumption for *B. bruxellensis* CB63 (0.8 mg/L molecular SO_2) and could be a result of acetaldehyde binding to sufficient molecular SO_2 to remove the SO_2 inhibition on glycolytic enzymes.

Volatile phenolic content was determined by means of GC-MS on *B. bruxellensis* CB63 samples taken at day 32 of the experiment (Figure 20). The enzymes phenolic acid decarboxylase (PAD) and vinyl phenol reductase (VPR) are responsible for their production from the phenolic precursors in the SWM media, *p*-coumaric acid (10 mg/L) and ferulic acid (10 mg/L) respectively, with the regeneration of one mole NADH per mole of substrate. As seen in Figure 20, the presence of 0.4 and 0.8 mg/L molecular SO_2 resulted in an approximate 58% increase of 4-ethyl guaiacol concentration compared to the samples containing 0 mg/L SO_2.

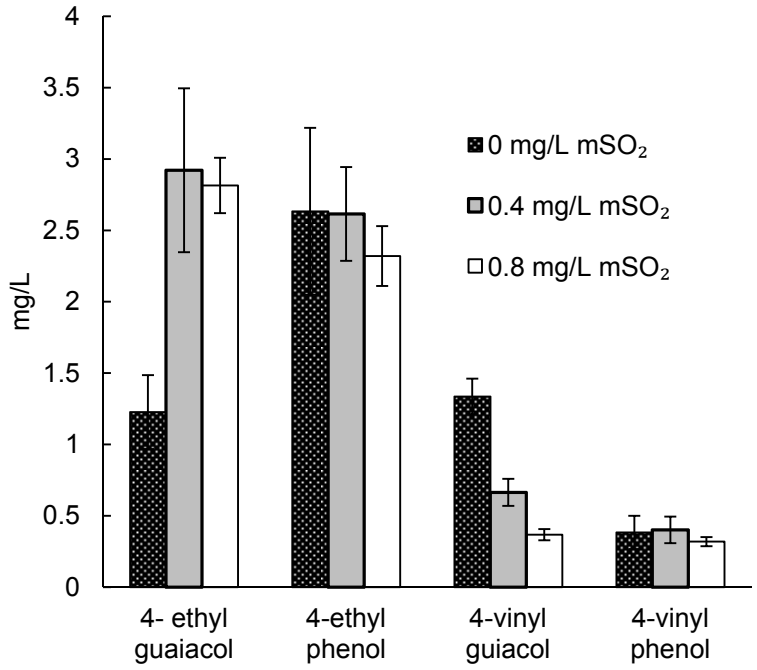

Figure 20
Volatile phenol production by *B. bruxellensis* after incubation with either 0, 0.4 or 0.8 mg/L molecular SO_2 after 32 days.

0.8 mg/L molecular SO_2 had a slight negative effect on the level of 4-ethyl phenol with an approximate 12% decrease compared to 0 and 0.4 mg/L molecular SO_2. The volatile phenol intermediate metabolites, 4-vinyl guaiacol and 4-vinyl phenol, followed the opposite trend to 4-ethyl guaiacol and 4-ethyl phenol.

According to Tchobanov *et al.* (2008), the activity of VPR is 30% higher when using 4-vinyl guaiacol instead of 4-vinyl phenol as a substrate, which could explain the results obtained in this study. However, Harris *et al.* (2009) found strain dependent behaviour with regards to VPR substrate specificity in *D. anomala* CBS77*, D. bruxellensis* CBS 1499 and *D. bruxellensis* CBS 2336. Tchobanov *et al.* (2008) results could be due to the fact that they did not use a pure form of 4-vinyl phenol and/or as a result of strain dependent behaviour (Agnolucci *et al.* 2010; Harris *et al.* 2009; Oelofse *et al.* 2009). The results obtained in this study show that in the absence of SO_2, 4-ethyl guaiacol concentration was approximately 50% that of 4-ethyl phenol. It is therefore necessary to investigate the effect of molecular SO_2 on volatile phenol using more *B. bruxellensis* strains to be able to draw definitive evidence from such an experiment. The increased production of 4-ethyl guaiacol in the presence of SO_2 suggests that this process could be contributing to the maintenance of the cellular redox balance through the increased oxidation of NADH/NADPH to NAD^{+}/$NADP^{+}$.

It has been shown previously by Agnolucci *et al* (2010) and du Toit *et al* (2005) that an increase in molecular SO_2 from 0 to 0.4 and 0.8 mg/L is sufficient to inhibit the growth of *B. bruxellensis* on YPD agar. On day 32, fresh samples were taken and serially diluted with 100 µL of each dilution plated onto YPD agar. Once visible colonies had grown, the yeast colonies on the YPD agar plates were manually counted and plotted (Figure 21). Unexpectedly, both yeast species survived remarkably well over the 32 day period irrespective of the concentration of molecular SO_2 present in the flasks. *S. cerevisiae* VIN13 population size remained relatively constant at all molecular SO_2 levels and *B. bruxellensis* CB63 decreased 0.5% when comparing the 0.8 mg/L molecular SO_2 to the 0 mg/L molecular SO_2 control.

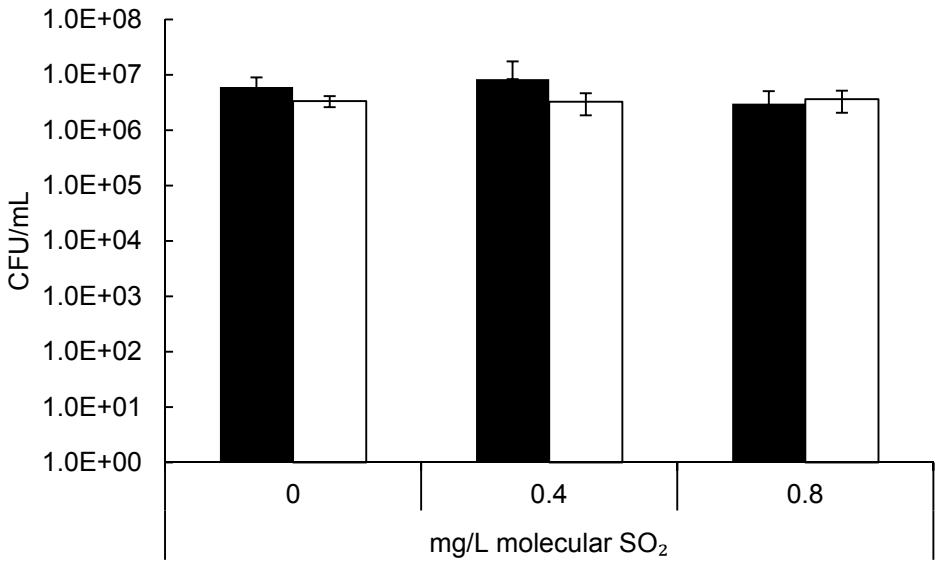

Figure 21
Plate counts (CFU/mL) at day 32. ■: *B. bruxellensis* CB63; □: *S. cerevisiae* VIN13

The reason for the survival of both yeast species is unclear but it can be speculated that sufficient acetaldehyde was produced by both species over the 32 day period to sufficiently reduce the SO_2 stress on the cells and prevent cell death.

The impact of molecular SO_2 on a carbon metabolic flux followed similar trends in both TA and SWM. The enzymatic inhibition caused by SO_2 affected all the compounds tested. However a direct comparison is not possible due to the different duration of each experiment, (i.e. 48 hours versus 32 days). A characteristic difference between the two media is the time taken for the level of acetaldehyde to peak. For the 48-hour experiment, acetaldehyde peaked in only 8 hours whereas for the 32 day experiment acetaldehyde took 12 days to peak. This is most likely due to the difference in glucose levels, 20 g/L for TA vs. 5 g/L for SWM, and the presence of 10% ethanol for SWM, which certainly affected the physiological state of the cultures.

The initial rate of acetic acid production in TA remained steady for *B. bruxellensis* CB63 at 1.0 mg/L molecular SO_2 for approximately 12 hours where after its production increased

dramatically. This coincided with a peak in acetaldehyde at 10 hours. For SWM, the rate of acetic acid production increased at a similar rate for all samples until SO_2 was added, thereafter the rate of production decreased and this is most likely due to enzymatic inhibition on aldehyde dehydrogenase. Even though TA had a higher initial sugar concentration of 20 g/L (favouring the production of ethanol), less acetic acid (on average for all samples) was produced per mg cell mass for TA compared to SWM where the initial sugar concentration was 5 g/L.

The ethanol in SWM significantly reduced the rate of fermentation for both yeast species, *S. cerevisiae* VIN13 took 4 days and *B. bruxellensis* CB63 took 12 – 24 days to completely ferment 5 g/L of sugars (glucose and fructose) in SWM whereas in TA, *S. cerevisiae* VIN13 fermented 20 g/L of glucose in only 8 hours and *B. bruxellensis* did not ferment to dryness. Extending the 48 hours to 72 hours for the TA experiment would provide more insight into whether *B. bruxellensis* CB63 can ferment to dryness in the presence of 1.0 mg/L molecular SO_2 and how acetaldehyde production is affected and if it will decrease as seen in the SWM experiment.

CHAPTER 5

General conclusion & Future prospects

5 General conclusion and future prospects

The increased use of SO_2 in the wine fermentation process over the years has resulted in the increased prevalence of SO_2 tolerant yeast species (Godden and Gishen 2005). Certain species such as *S. cerevisiae* VIN13 have been bred specifically to tolerate high levels of SO_2 so as to outcompete any unwanted microorganisms for available nutrients. Even though *S. cerevisiae* tends to dominate most commercial wine fermentations, certain yeasts such as those from the *Dekkera*/*Brettanomyces* species have the ability to persist at relatively low cell numbers throughout the wine fermentation.

Brettanomyces spp. are easily introduced into a wine fermentation due to poor cellar hygiene standards and previously contaminated equipment such as tanks and oak barrels. *B. bruxellensis* is a notorious red wine spoilage yeast due to its ability to produce high levels of volatile phenols (Chatonnet *et al.* 1992), acetic acid (Carrascosa *et al.* 1981) and other organic acid off-flavours (Romano *et al.* 2009) as well as its capability to enter a VBNC state (Agnolucci *et al.* 2010; Divol and Lonvaud Funel 2005; Millet and Lonvaud-Funel 2000) and persist throughout fermentation and into the wine bottle (Agnolucci *et al.* 2009; Andorrà *et al.* 2010; Barata *et al.* 2008; Chatonnet *et al.* 1992; Cocolin *et al.* 2004; Conterno *et al.* 2006; Coulon *et al.* 2010; Couto *et al.* 2005; Curtin *et al.* 2007; Delaherche *et al.* 2004; Hierro *et al.* 2006; Millet and Lonvaud-Funel 2000; Mitrakul *et al.* 1999; Oelofse 2008; Oelofse *et al.* 2009; Phister and Mills 2003; Puig *et al.* 2010; Romano *et al.* 2008; Serpaggi *et al.* 2010).

This study aimed to investigate how wine-related yeast, and specifically *B. bruxellensis,* response to SO_2 on a molecular and cellular level. A highly accurate SO_2 quantification technique was optimised for use in the laboratory (the technique originated as a means to quantify SO_2 concentration in beer on a large scale). This technique was shown to quantify free and total SO_2 concentrations accurately from 0.5 mg/L to 2.5 mg/L.

Numerous *B. bruxellensis* strains were obtained from the IWBT culture collection and positively identified *via* 5.8S rDNA-ITS RFLP analysis (Esteve-Zarzoso *et al.* 1999) and DNA

sequencing. These same strains were also tested for their ability to sporulate to confirm whether they were a *Dekkera* (able to sporulate) or *Brettanomyces* (unable to sporulate) species. *D. anomala* did sporulate and *B. bruxellensis* strains did not sporulate. However the yeast used as a positive sporulation control, the diploid strain *S. cerevisiae* VIN13, unexpectedly did not sporulate and therefore it cannot be said for certain that the *Brettanomyces* sporulation results are correct.

The ability of *S. cerevisiae* and *B. bruxellensis* to tolerate molecular SO_2 was evaluated by growing these yeast on YPD agar in the presence of SO_2 and ethanol. Highly strain dependent results were obtained with *B. bruxellensis* strains ranging from poor to very high tolerance to SO_2 and ethanol. As expected, *S. cerevisiae* showed a higher overall tolerance compared with *B. bruxellensis,* especially in the presence of ethanol.

Park and Bakalinsky (2000) showed the existence of a membrane bound sulphite protein transporter, Ssu1p, in *S. cerevisiae* which actively effluxes SO_2 out of the cell. It was also shown that increased/decreased SO_2 tolerance was related to the over or under-expression of Ssu1p. This knowledge was applied to *S. cerevisiae* strains and *B. bruxellensis strains*. It was shown in experiments with these yeasts that their rate of SO_2 accumulation and efflux is highly strain dependent. The fact that *B.* bruxellensis could efflux SO_2 at rates similar to that of *S. cerevisiae* suggests that *B. bruxellensis* does possess a membrane bound SO_2 transporter similar to Ssu1p found in *S. cerevisiae.*

The SO_2 efflux results contradicted the SO_2 tolerance results where a yeast with a high SO_2 efflux rate was less tolerant to SO_2 and vice versa [except for *S. cerevisiae* strains BY4742 and BY4742 *ssu1Δ* where the expected results confirmed those observed by Park and Bakalinsky (2000)] whereas the results obtained by Park and Bakalinsky (2000) showed an increase in SO_2 tolerance associated with a higher SO_2 efflux rate. The *S. cerevisiae* strains used in their experiments were purposely over or under-expressing Ssu1p and this could explain the results in this study as all the yeast used in this study (with exception of BY4742

and BY4742 *ssu1Δ*) where yeasts originating from wine or beer environments and therefore are not as predictable as strains with a fully understood geno- and phenotype.

To further understand the effect of SO_2 on yeast energy metabolism, *S. cerevisiae* strains VIN13 and *B. bruxellensis* CB63 were subjected to different molecular SO_2 concentrations over a 48-hour period, in a minimal buffer, and over a 32-day period in a SWM replicating wine ageing conditions, respectively. Molecular SO_2 had an immediate effect on the yeasts metabolism and both species reacted in a similar manner. *S. cerevisiae* VIN13 is a commercial yeast strain and it is naturally highly tolerant to both SO_2 and ethanol and because of this the impact of molecular SO_2 on its metabolism was not as great as that of *B. bruxellensis.* In TA + 2% glucose and SWM, the Custer effect was seen with an increase in molecular SO_2 exaggerating the Custer effect and reducing the rate of glucose (and fructose in SWM) consumption and ethanol production by *B. bruxellensis* along with a steady increase in acetic acid levels. Acetaldehyde production showed the characteristic response to SO_2 for both *S. cerevisiae* and *B. bruxellensis* where its levels peaked after the addition of SO_2 followed by a steady decline. Along with this occurrence, *B. bruxellensis* restarted its consumption of glucose/fructose and ethanol production, albeit at a rate slower than that prior to SO_2 addition. This suggests that sufficient acetaldehyde was produced to bind to any free SO_2, thereby reducing the inhibitory effects of SO_2 on the enzymes involved in glycolysis and anaerobic fermentation. Whether this production of acetaldehyde is a characteristic response to SO_2 by *B. bruxellensis* and *S. cerevisiae* or if it is only as a result of the inhibitory effect on glycolysis and anaerobic fermentation associated enzymes, is yet to be determined.

The production of 4-ethyl phenol and 4-ethyl guaiacol occurs with the oxidation of one NADH/NADPH (Godoy *et al.* 2008; Tchobanov *et al.* 2008). It was shown in the SWM experiment that an increased molecular SO_2 level resulted in an increase in the level of 4-ethyl-guaiacol but not 4-ethyl-phenol. It can therefore be suggested that the increased 4-EG level occurred due to an increase demand for NAD^+/$NADP^+$ in order to maintain a healthy intracellular redox balance. Plate counts were performed for samples on day 32 of the SWM

experiment to ascertain if either yeast species entered a VBNC state (with the characteristic inability to grow on routine solid growth media). Unexpectedly, both yeast species survived exceptionally well and molecular SO_2 did not have an impact on final cell numbers and therefore it would have been advantageous for the interpretation of the results to have monitored the yeasts growth at regular intervals. Monitoring of the free SO_2 and dissolved O_2 content could also provide more insight into the metabolic response of the yeast to the medium they are inoculated into. Repeating these experiments in finished red wine under various environmental conditions (for example differing O_2, SO_2 and temperature) will elucidate further *B. bruxellensis* response to stress in an oenological environment.

A direct comparison between *S. cerevisiae* and *B. bruxellensis* response to SO_2 shows that similar mechanisms exist to cope with SO_2 stress. The greatest factor in this comparison is the high degree of strain-dependent characteristics shown for *Brettanomyces/Dekkera*. Although no direct proof is given for the existence of these mechanisms in *Brettanomyces/Dekkera,* it can be extrapolated from the SO_2 efflux results that *B. bruxellensis* must have a Ssu1p-like transporter in order to efflux SO_2 at rates similar to that of *S. cerevisiae*. The influence of SO_2 on both yeast species metabolic energy flux shows similar trends indicating that *B. bruxellensis* possesses similar metabolic pathways to *S. cerevisiae* in this regard and that the respective enzymes of these pathways are inhibited by SO_2 in a similar manner. Where *B. bruxellensis* differs compared to *S. cerevisiae* in this regard is the presence of the Custer effect that directly impacts *B. bruxellensis* ability to ferment sugars to ethanol in an anaerobic environment.

Further understanding of the specific effects of sulphite stress on the *S. cerevisiae* eukaryotic cell model as well as in other non-*Saccharomyces* yeasts such as *B. bruxellensis* should be examined. In particular, focus should be placed on the enzymes involved in energy metabolism (alcohol dehydrogenase, aldehyde dehydrogenase, pyruvate decarboxylase and GAPDH to name a few) to determine to what degree the enzyme kinetics are affected by the presence of SO_2. Attention should also be given to ascertain if the three speculated sulphite

stress reduction mechanisms (sulphur reduction, sulphur oxidation and acetaldehyde production) decrease the intracellular sulphite stress to a level which influences the cells metabolic processes positively and which of these mechanisms are found in other yeasts besides *S. cerevisiae* such as *B. bruxellensis.*

The VBNC state has been shown to exist in bacteria and yeast yet very little knowledge is available on the molecular and cellular aspects of the state. When the full genome sequence is available for *B. bruxellensis,* it is recommended that a RNA microarray analysis is performed on strains which show high levels of VBNC. With this experiment, it would be crucial to extract RNA in the various metabolic states involved with VBNC i.e. before induction of VBNC, during induction of VBNC, during actual VBNC, during removal of the VBNC inducer and lastly after resuscitation of the cells from VBNC. It would be necessary to experiment with potential VBNC inducers such as temperature, ethanol, osmotic and chemical stress and special attention must be given to SO_2. Selectively reducing the expression of the genes which are directly involved in the sulphite stress reduction mechanisms mentioned above could provide insight as well. It will be vital to examine the global shifts in gene expression so as to ascertain what exactly is occurring during each VBNC stage.

It has been shown in this study that SO_2 has a far reaching negative impact on yeast metabolism and that not all yeast strains are created equal with regards to their ability to cope with the stress presented by molecular SO_2 at high concentrations. Further knowledge of the genetic potential and of the transcriptomic, proteomic and metabolomic responses of yeast to SO_2 is required to fully understand their response and ultimately the control of spoilage yeast with a high tolerance to SO_2.

CHAPTER 6

Addendum **A**

6 Addendum A

The discovery of the genes *AfuSSU1* in *Apergillus fumigatus*, *AbeSSU1* in *Arthroderma benhamiae* and *TruSSU1* in *Trichophyton rubrum* (Léchenne *et al.* 2007), that are orthologous to *SSU1* in *S. cerevisiae*; prompted further investigation to elucidate if an orthologous gene existed in *B. bruxellensis*.

These fungal gene sequences were aligned with the *S. cerevisiae SSU1* gene. Regions were found with high homology in the alignment and these regions were used to create a degenerate PCR primer pair (data not shown). The primer pair SSU1-FW and SSU1-RV was used to amplify DNA from *S. cerevisiae* (strains VIN13, BY4742 and BY4742 *ssu1*Δ) and *B. bruxellensis* (strains ISA1649 and CB63). Due to the highly degenerate nature of the primers used, multiple PCR products were produced (Figure 22) using this method, further complicating selection of a possible *SSU1* candidate for sequencing.

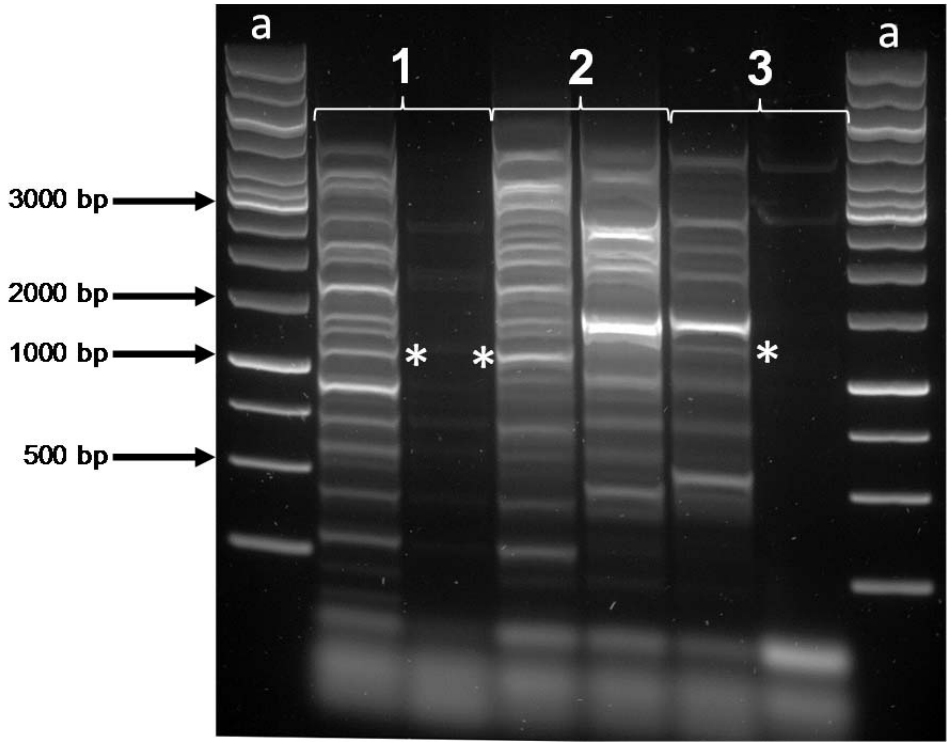

Figure 22
Agarose gel electrophoresis band pattern of *SSU1* degenerate primer PCR. 1 = *S. cerevisiae* VIN13, 2 = *B. bruxellensis* CB63 and 3 = *B. bruxellensis* ISA1649. * = possible SSU1 candidates; a = 1kb ladder (Fermentas, St. Leon-Rot, Germany);

Numerous attempts were made at cloning and sequencing the correct *SSU1* candidates but none were successful as it was very difficult to isolate a unique band from the agarose gel due to the large amount of closely spaced PCR product bands. Those bands that were successfully cloned and sequenced were identified and found to be unrelated DNA fragments with no association with *SSU1*.

S. cerevisiae VIN13 *SSU1* gene sequence was used to design specific DNA primers to amplify *SSU1* from *B. bruxellensis* using PCR. Only *S. cerevisiae* gave a positive result of 1376bp for *SSU1* amplification as seen in Figure 23. This PCR sample was cloned into pGEM®-T Easy and transformed into *E. coli* DH5α. Mini-prep plasmid extraction was then performed on positive *E. coli* DH5α clones and the plasmid was sent for sequencing. The gene sequence was compared against public databases using the BLAST nucleotide algorithm and a positive result was returned for *S. cerevisiae SSU1* with 100% query coverage and identification.

These results suggest that *B. bruxellensis* either does not possess a gene orthologous to the *S. cerevisiae SSU1* gene (which is highly unlikely considering the fact the *B. bruxellensis* is capable of actively effluxing SO_2 as previously shown in this study) or that a potential *B. bruxellensis SSU1* gene orthologue sequence is too distantly related from the known *SSU1* genes present in *S. cerevisiae, A. fumigatus*, A*. benhamiae* and *T. rubrum.*

A potential solution to this problem would be to search for a potential *B. bruxellensis SSU1* orthologue by constructing a genomic or cDNA library or by whole genome sequencing.

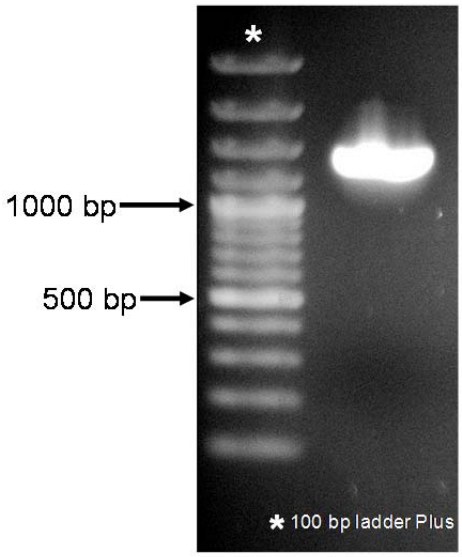

Figure 23
Agarose gel electrophoresis band pattern of *SSU1* PCR.

CHAPTER 7

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7 Bibliography

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