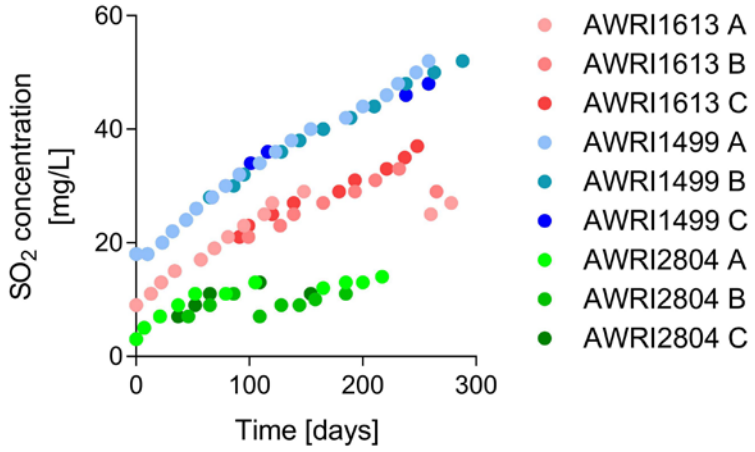
Australian Government

Australian Grape and Wine Authority

The Australian Wine Research Institute

Ensuring the continued efficacy of *Brettanomyces* control strategies for avoidance of spoilage



Sulfite tolerance of *Brettanomyces* populations undergoing evolution in the laboratory

FINAL REPORT to

AUSTRALIAN GRAPE AND WINE AUTHORITY

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1. Abstract:

Spoilage of wine by *Brettanomyces* yeasts remains one of the foremost microbiological problems faced by winemakers. Current strategies to minimise risk of spoilage do not eliminate *Brettanomyces* yeasts from wineries and are heavily reliant upon use of the preservative sulfite to stabilise wine against *Brettanomyces* growth. This project demonstrated that existing *Brettanomyces* wine strains have potential to evolve greater tolerance to sulfite and found evidence for this occurring in industry. The importance of developing new research tools for these yeasts was underscored by observed variation in efficacy of an emerging sulfite alternative, chitosan, which was ineffective against some strains.

2. Executive summary:

Spoilage of wine by *Brettanomyces* yeasts remains one of the foremost microbiological problems faced by winemakers. The risk of 'Brett' spoilage is currently managed through application of a multi-faceted strategy previously developed by the AWRI in collaboration with Australian winemakers, which facilitated industry-wide decreases in levels of spoilage compounds in finished wines. This strategy does not, however, eliminate *Brettanomyces* yeasts from wineries, and is heavily reliant upon use of the preservative sulfite to stabilise wine against *Brettanomyces* growth. To ensure Australian winemakers' continued ability to manage 'Brett' in a cost-effective manner, this project examined the interactions of sulfite with *Brettanomyces* biology at the population, strain and molecular levels.

Contrary to work done elsewhere, this project did not find evidence that *Brettanomyces* cells enter a viable but non-culturable (VBNC) state in response to sulfite. Exhaustive, long-term experiments instead suggest that resumption of population growth is dependent upon low numbers of viable, culturable cells, that may fall below limits of detection for standard microbiological and rapid tests.

For the first time, laboratory experiments revealed potential for common Australian winery strains of *Brettanomyces bruxellensis* to evolve greater tolerance to sulfite. Strikingly, when new industry isolates were obtained and compared to strains isolated over the past two decades it was evident that selection for sulfite-tolerance is already occurring. Attempts to characterise the mechanisms by which *B. bruxellensis* gains tolerance to sulfite did not reveal a common pattern, though parallel work showed that variations in amino acid sequence of the sulfite efflux pump Ssu1p can partly explain strain variation.

Efforts to identify genetic determinants of sulfite tolerance were hampered by the lack of molecular genetics tools for *Brettanomyces* yeasts. In response to this limitation, new tools were developed for the genetic transformation of *B. bruxellensis*, which were applied to study competitiveness of different strains under sulfite stress. The importance of developing tools for further study of *Brettanomyces* biology was emphasised by observed strain-level variation in efficacy of an increasingly popular alternative control agent, chitosan, which was shown to be ineffective against a key Australian wine strain.

Key outcomes of this research project are described in greater detail below.

Outcome 1: Knowledge of *B. bruxellensis* population genetics/genomics

While all strains of *B. bruxellensis* represent the same species, there is substantial variation within this species with respect to a number of wine-relevant traits, also known as phenotypes (e.g. growth rate, sulfite tolerance, nitrogen source utilisation). Previous studies have compared the genomes of a small number of *Brettanomyces* strains, revealing significant intra-specific genomic variation including evidence for possible inter-specific hybridisation.

To gain improved insight into the *B. bruxellensis* species complex, the genomes of an additional 40 strains, isolated from a variety of environmental sources (majority from wine), were sequenced. Comparative genomics was used to produce a unified phylogeny for all of the *B. bruxellensis* strain sequences to date, while also estimating the genome copy number (ploidy level) of each isolate. A complex phylogenetic relationship emerged from this work, with several instances of independent intra- and possibly inter-specific hybridisation occurring such that diploid, triploid and potentially tetraploid strains were encountered in the analysis. Polyploidisation may therefore represent a significant driving force in the development of phenotypic variation in *B. bruxellensis*, which is significant as these normally rare events can produce 'large-effect' phenotypic changes that then provide significant selective advantage when exposed to anthropomorphic environments such as wine fermentation.

Outcome 2: Evidence that *Brettanomyces* wine strains can evolve greater tolerance to sulfite than encountered in commonly observed strains

The judicious use of sulfite is the major means by which spoilage by *Brettanomyces* is controlled in the winery. However, reliance on a single point of control greatly heightens the risk that the target organisms will evolve strategies to circumvent the measures that are levelled against them. This process can be further exacerbated if sub-lethal doses of the agent are commonly used, such as when high pH, low-sulfite wines are produced.

In order to determine if *Brettanomyces* was genetically capable of developing increased sulfite tolerance, a directed evolution approach was instigated in order to provide conditions that would favour (and accelerate) the development of sulfite tolerance in the laboratory.

Three strains of *Brettanomyces* (AWRI1499, AWRI1613 and AWRI2804) that represent high-, mid- and low tolerance to sulfite were subjected to increasing concentrations of sulfite for up to 100 generations. These evolved populations were able to tolerate more than twice the concentration of sulfite than their respective parental strains, and individual colonies isolated from the populations were found to stably retain this property.

Having observed potential for evolution of sulfite tolerance in the laboratory, project staff obtained new industry isolates and compared them to strains isolated during previous studies. While the results are preliminary, and require further isolate numbers for statistical rigour, this snapshot suggests that sulfite tolerance of *Brettanomyces* in Australian wineries may be increasing. More extensive sampling would be required to confirm the generality of this preliminary finding.

Genome sequencing was used in an attempt to identify genetic changes that accompanied enhanced sulfite tolerance, but to date has not revealed any consistent patterns across replicated experiments or new industry isolates.

Outcome 3: Knowledge of the role that sulfite efflux pump Ssu1p plays in sulfite tolerance

One of the key proteins involved in the tolerance in other yeast species is the sulfite efflux pump Ssu1p (encoded by the gene *SSU1*), which provides a detoxifying role by directly pumping sulfite out of the cell. Variation in *SSU1* function therefore represents a likely candidate for differences that have been observed in sulfite tolerance between strains of *B. bruxellensis*. Whole genome sequence analysis has shown that there are several different versions of the Ssu1 protein in the population and often two different versions in the same strain.

In order to determine if differences in the Ssu1 protein composition affect the ability of this protein to remove sulfite from the cell, distinct *SSU1* gene sequences that were present in AWRI1499, AWRI1613 and AWRI2804 were tested for their ability to impart sulfite tolerance when expressed in a strain of *S. cerevisiae* in which the native *Saccharomyces SSU1* gene had been replaced with the *Brettanomyces* sequence. This complementation approach has been used in other studies to evaluate functionality of *SSU1* from diverse fungal species.

In total, four slightly different *SSU1* genes were identified that were predicted to encode proteins that differed slightly in length (438 - 450 amino acids) and exact sequence (8 sites with amino acid variations). The results showed that the four different *B. bruxellensis SSU1* protein variants conferred different sulfite tolerance when expressed in single copy in *S. cerevisiae*, providing a potential mechanism by which differences in sulfite tolerance may be established in *B. bruxellensis*.

Outcome 4: Knowledge of whether *B. bruxellensis* enters a VBNC-state in response to sulfite

Microbial species respond to stress in various ways, including entering a physiological state where cells remain viable (intact and metabolically competent) but stop replicating. This state is known as ‘viable but non-culturable’ (VBNC) because it is most commonly detected through failure to grow on laboratory media. There are many caveats associated with observing this phenomenon, and the burden of proof necessary to demonstrate loss of culturability and subsequent recovery upon removal of stress conditions has been met for a limited number of species and conditions.

Five long-term survivability experiments were established (three of which for took place over more than one year) for *B. bruxellensis* strains exposed to sulfite stress in model wine under anaerobic conditions. Population size and viability were monitored using various methods. In each experiment it was evident that at high concentrations of molecular sulfite (>1 mg/L) recovery was never observed, even when artificially reducing the amount of molecular sulfite by raising the pH of the model wine after treatment. At moderate concentrations of mSO_2 (0.2-0.5 mg/L) recovery of culturability was observed in some but not all treatment replicates. In each case the observed growth rate did not support a conclusion that

this was recovery of culturability from VBNC status – it appeared plausible that small numbers of culturable cells (below the analytical detection limit) survived mSO_2 treatment and were sufficient to enable development of observed growth.

During these experiments continual refinement of flow cytometry methodology revealed that an overlooked analytical artefact was most likely responsible for prior research showing a constant level of viable cells when nothing could be cultured. Flow cytometry reliant upon a commonly used DNA molecular probe (propidium iodide) requires a certain volume of sample to be analysed in order to yield signal. In our hands this created an artificial signal approaching 10^4cells/mL. Additional physiological probes were trialled to improve this analytical baseline; however, none of the alternatives improved this baseline noise sufficiently.

A recent report indicated the a VBNC state was possible with *S. cerevisiae* treated with sulfite. Two experiments were undertaken to investigate to this potential phenotype; however, no classical VBNC response was found, despite accurately repeating the published methodology. It was concluded that under the conditions examined, a VBNC response does not explain the observations previously reported for both *Brettanomyces* or *Saccharomyces*.

Outcome 5: Alternative control strategies

Chitosan was investigated as a possible alternative control strategy for *Brettanomyces*. To test the efficacy of chitosan, two commercially available products were examined using the manufacturer's recommendations. Both commercial preparations were successful in reducing active *Brettanoymces* populations; however, a strain-dependent variability was evident. This indicates that chitosan may not be effective against all *Brettanomyces* genotypes present in Australian wine.

A number of detection methods based on quantitative polymerase chain reaction (QPCR) have been reported and are currently used in the Australian wine industry. The potential of a common QPCR method to detect pre-determined cell numbers spiked into wine and model wine was investigated. An important consideration of the QPCR approach is the differentiation of live versus dead cells. It was determined that dead cells (heat-killed) can contribute significantly to a detectable QPCR signal. This could lead to false positive determinations of *Brettanomyces* populations as viable or further contribute to controversy surrounding the reported VBNC phenotype.

Outcome 6: Development of a genetic transformation toolkit for *B. bruxellensis*

Genetic transformation is a foundational technology that allows for a host of molecular biology tools to be applied to the study of a species (gene overexpression, gene deletion, incorporation of marker genes for competition experiments etc). Transformation has been available in *S. cerevisiae* for well over 30 years, with much of the knowledge that has been generated for this species being due to this early development. In the past, there were no reliable transformation protocols for *Brettanomyces*; however, a recent publication was able to demonstrate very low efficiency transformation of *B. bruxellensis* using a single drug-resistant selectable marker, although this protocol was only able to randomly integrate foreign DNA into the genome and therefore is unsuitable for gene deletion strategies.

To address these limitations, work in this project sought to improve the efficiency of the *Brettanomyces* transformation protocol, while providing an increased number of selectable markers and the ability to produce targeted gene deletions.

Transformation efficiency was increased by an order of magnitude relative to the original published protocol by incorporating highly expressed *Brettanomyces* promoters (as identified through genome sequencing and RNAseq performed previously at the AWRI), while also providing two additional drug selection markers (ClonNAT and hygromycin). The high efficiency protocol, combined with the presence of multiple markers has provided the means to 'tag' strains for performing pooled competition experiments to investigate *Brettanomyces* fitness.

In this project it was possible to demonstrate low-efficiency targeted gene deletion of one *B. bruxellensis* gene (KU80) through the use of long-flanking homology. When orthologues of *KU80* have been deleted in other fungal systems, this has often resulted in strains which are then highly efficient at homologous recombination, thereby improving the ability to perform gene deletions. However, this is not always the case, and the *B. bruxellensis KU80* strain was not shown to provide an increased frequency of homologous recombination. Indeed, it was not possible to delete any additional genes (including those of wine relevance such as *SSU1*) in this genetic background. Furthermore, additional attempts to delete

wine-relevant genes in a wild type background using the same low-efficiency technique used for *KU80* were unsuccessful. Further work will be required to assess the applicability of new molecular tools, such as CRISPR-Cas9, to allow for routine targeted genomic alterations to be made in *B. bruxellensis*.

3. Background:

Genotype analysis of more than 300 Australian *Brettanomyces* isolates obtained from 2000 to 2005 revealed that one strain increased in frequency of isolation from ~60 to ~95%(Curtin et al. 2007). This strain was subsequently found to tolerate higher concentrations of sulfite (Curtin et al. 2012a). When considered in light of changing industry practices to minimise risk of 'Brett' taint in red wines, which includes management of pH and sulfite, these observations could be seen as analogous to the problem of antibiotic resistant human pathogenic bacteria. More effective use of sulfite, resulting in a higher proportion of free and molecular forms in wine, means that residual *Brettanomyces* populations exposed to these conditions have been placed under more selective conditions. This represents an evolutionary bottleneck, which population ecology theory suggests will lead to emergence of adaptations that render some individuals in the population more fit than others, and these individuals will propagate and become dominant. This process is known as a selective sweep.

Sulfite tolerance in other fungal species, particularly *Saccharomyces cerevisiae*, has been linked to membrane protein Ssu1p that functions as a sulfite-efflux pump, reducing the intracellular concentration of sulfite (Park and Bakalinsky, 2000). It is unknown whether the *B. bruxellensis SSU1* gene encoding this protein performs this function, or whether alternative mechanisms mediate sulfite tolerance in *Brettanomyces* yeasts. Knowledge of mechanisms that confer enhanced sulfite resistance in some *B. bruxellensis* strains may facilitate design of rapid methods to test for emergence of new, potentially problematic, strains.

Indeed, a weak point in many 'Brett' control strategy implementations is a lack of well-validated rapid monitoring tools. Plating methods are laborious and slow, and while rapid methods are of interest, data interpretation can be difficult. Some rapid methods infer the existence of VBNC populations, that under certain conditions may resume growth and spoil the wine. If these putative VBNC yeast are in fact dead, risk averse winemakers may conduct costly and unnecessary processing. If VBNC *Brettanomyces* do exist, then plating methods may underestimate spoilage risk. VBNC status has been studied extensively in bacteria, and for species to be described as having the capacity to enter VBNC status, several criteria must be met, including proof of 'recovery'.

The concept of VBNC *Brettanomyces* has been described in studies where 'culturable' cell numbers differ from 'viable' population estimates made using dyes (Serpaggi et al. 2012). Quantitative real-time PCR methods have also inferred VBNC populations (Willenburg and Divol 2012). However, these interpretations have been proven misleading (Andorrà et al. 2010) and most have suffered from inadequate experimental design, whereby sampling frequency does not exclude the possibility that re-growth of the population can be attributed to culturable cells present at levels below method detection limits (Serpaggi et al. 2012). Indeed, it is clear that the key criteria that define VBNC status (Oliver 2005) have not been met for *Brettanomyces*.

4. Project aims and performance targets

The objectives of this project were to:

1. Develop knowledge of mechanisms that enable *Brettanomyces* to adapt to environmental conditions, particularly in response to sulfite stress
2. Develop molecular diagnostics and apply them to new industry isolates as an early warning system for emergence of sulfite-tolerant strains
3. Determine whether *Brettanomyces* enters a VBNC state, through rigorous experimentation that meets criteria previously described for VBNC bacteria
4. If VBNC demonstrated, generate knowledge of what wine-relevant conditions trigger this state, and under what conditions *Brettanomyces* exits its VBNC state and resumes growth
5. Develop new, or improve existing, rapid tests (QPCR or flow cytometry) that enable accurate assessment of spoilage risk
6. Investigate alternatives to sulfite for control of *Brettanomyces* during wine maturation
7. Provide Australian winemakers with an updated *Brettanomyces* control strategy.

5. Methods:

5.1. Comparative genomics of *B. bruxellensis* isolates

Genomic DNA was prepared using a standard zymolyase and phenol-chloroform extraction from cultures grown in YPD at 30°C. DNA sequencing was performed using 2 x 300 bp paired-end chemistry on the Illumina Miseq (Ramaciotti Centre, Sydney Australia). Short reads were mapped to the AWRI2804 reference genome using Novoalign. Single nucleotide polymorphisms (SNPs) were identified from the alignments using the *pileup2snp* functionality of Varscan v2.3 (default parameters; -min-coverage 10 (Koboldt et al. 2012) combined with custom Python scripts.

Sequencing coverage was extracted from alignments in .bam format using *mpileup* from the samtools v0.1.18 package (Li et al. 2009) with actual coverage values converted to changed ploidy levels in sliding windows using custom Python scripts.

Phylogenies were constructed using PhyML v3.0 (GTR model; default parameters) and visualised using Seaview v4.0 (Gouy et al. 2010).

5.2. Sulfite tolerance screening

Sulfite tolerance assays were performed in both minimal media and synthetic wine, with each isolate replicated four times. Synthetic wine was prepared as follows: chemically defined grape juice (CDGJ) containing 80 g/L each of glucose and fructose, pH 3.5 was fermented to dryness with *S. cerevisiae* AWRI 1631. For this 4 mL of YPD were inoculated from a plate culture and incubated overnight at 28ºC. A volume of 3 mL of YPD culture were inoculated into 300 mL of 50% CDGJ and incubated overnight at 28ºC with shaking. This culture was then used to inoculate 8 L of CDGJ at an initial OD_{600} 0.1 and incubated at 22ºC with agitation until fermentation was completed. The resulting wine was cold settled at 4ºC, racked and filtered through at 0.45-0.2 μm filter. Carbon sources and vitamins were then added to the synthetic wine at the following final concentrations: glucose 2.5 g/L, fructose 2.5 g/L, myo-inositol 25 mg/L, pyridoxine 1 mg/L, nicotinic acid 10 mg/L, thiamine 1 mg/L and biotin 30 μg/L. After adjusting pH to 3.5, the wine was filter sterilised (0.22 μm) and stored at 4ºC until required.

For sulfite tolerance assays in minimal media, a 200 µL starter culture was made in YPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) medium and incubated at 28ºC for two days. A 5 µL sample was then subcultured into 195 µL of minimal medium, and grown for two days at 28ºC. For the tolerance assay 5 µL of starter culture were inoculated in a 96-well microplate containing 195 µL of YNB supplemented with different concentrations of sulfite. Plates were incubated at 28ºC and OD_{600} measured after day 5 using a Tecan infinite M200 plate reader.

For sulfite tolerance assays in synthetic wine, a 200 µL starter culture was made in YPD medium and incubated at 28ºC for two days, 5 µL was then subcultured into 195 µL of YPD containing 6% ethanol. After three days of incubation at 28ºC, 5 µL was subcultured into 195 µL of synthetic wine and incubated for three days at 28 ºC. For the tolerance assay 5 µL of starter culture was inoculated in a 96-well microplate containing 195 µL of synthetic wine supplemented with different concentrations of sulfite. Plates were incubated at 28ºC and OD_{600} measured after day 5 using a Tecan infinite M200 plate reader.

5.3. Sulfite tolerance of industry isolates

To investigate if sulfite tolerance has increased in *B. bruxellensis* natural populations, industry isolates from different time periods were assessed for sulfite tolerance. Thus, 38 isolates collected from 2002-2004, 41 isolates collected from 2010-2014, and 97 isolates collected in 2016-2017 were screened (for details of isolates see Appendix 5). *Brettanomyces* cultures were maintained on MYPG (3 g/L malt extract, 3 g/L yeast extract, 5 g/L peptone, 10 g/L glucose, 50 g/L calcium carbonate, 20 g/L agar) plates and stored at 4ºC. Sulfite screening was performed in 96-well plates, sealed with Breathe-Easy membranes as indicated in 5.2. Each isolate was screened in quadruplicate.

5.4. Evolution of sulfite tolerance in laboratory experiments

5.4.1. Development of adaptive evolution methodology for *B. bruxellensis*

Two different experimental approaches commonly used in adaptive evolution studies were evaluated: continuous and batch culture. For continuous culture, AWRI1613 and AWRI1499 were inoculated separately into 700 mL minimal medium (MM), which consisted of 5 g/L glucose and 6.7 g/L yeast nitrogen base with amino acids (pH 3.5), in a Biostat 1L bioreactor with agitation (120 rpm) maintained at 28 °C. These cultures were allowed to reach stationary phase and then fresh media was pumped in to dilute the media at a rate equivalent to the maximum measured growth rate during batch culture (0.05 h^{-1}), in this way establishing a 'steady-state' where the population could grow continuously. Once the cultures were in steady-state, SO_2 was added to the feed media at increasing concentration. In parallel, batch cultures were performed in 250 mL flasks containing 50 mL MM (pH 3.5). Cultures were incubated at 22°C with shaking (140 rpm). When cultures reached an OD_{600} of 1.5 to 2.0 after approximately 7 generations, 1 mL was subcultured into 50 mL of fresh MM containing a higher concentration of sulfite than used in the previous flask. Samples from the bioreactor and triplicate batch culture flasks were periodically taken and plated on YPD for 3 days at 28°C. Individual colonies were picked for sulfite tolerance screening.

5.4.2. Batch-culture SO_2 'morbidostat'

B. bruxellensis founding strains, AWRI1613, AWRI1499 and AWRI2804 were subjected to adaptive evolution 'morbidostat' experiments, alongside an experiment with a mixture of 50 *Brettanomyces* strains. Triplicate 250 mL flasks containing 50 mL MM (pH 3.5) were inoculated, and cultures were incubated at 22°C with shaking (140 rpm). When cultures reached an OD_{600} of 1.5 to 2.0 after approximately 7 generations, 1 mL was subcultured into 50 mL of fresh minimal media containing an increasing concentration of sulfite. Depending on the founding strain, sulfite concentration was increased 1-2 mg/L with every population subculture. Triplicate control populations that were not exposed to sulfite stress were established in parallel for each founding strain, and subcultured for an equivalent number of generations. In addition, triplicate populations of AWRI1499 were subjected to increasing sulfite concentration for 50 generations, followed by 50 generations without any sulfite in the medium. Population samples were taken at every subculture and stored at -80°C as freeze cultures. Screening of individual isolates for every population was performed at 50 generations for all *B. bruxellensis* strains and at 100 generations for AWRI1613 and AWRI1499. For this, an aliquot of freeze culture was plated in YPD and grown at 28°C for 3 days. All biomass was then recovered, resuspended in sterile saline medium, diluted and plated in YPD to isolate single colonies. From these plated isolates, 15 to 17 colonies per individual population were screened for sulfite tolerance according to the method described in 5.2.

5.5. Expression of *Brettanomyces SSU1* genes in *S. cerevisiae*

Three types of gene modifications were performed: (i) gene deletions, where the open reading frame (ORF) of the target gene was deleted; (ii) *SSU1* replacement, where the ORF for *SSU1* from the *B. bruxellensis* strain was inserted in the chromosome replacing the native *S. cerevisiae SSU1* coding sequence; and (iii) multicopy expression of the *B. bruxellensis SSU1* genes, where *SSU1* was cloned in a plasmid under the control of a strong constitutive yeast promoter and introduced into *S. cerevisiae*. All yeast transformations were carried out using the lithium acetate/polyethylene glycol method.

Deletions were carried out by replacing targeted ORFs with either a natMX cassette encoding clonNAT resistance or a hygroB cassette encoding hygromycin resistance. Cassettes were PCR amplified using primers containing 50 base pair (bp) flanking regions corresponding to up- and down-stream regions outside the ORF. The natMX and hydroB cassettes were amplified from plasmids pAG25 and pAG32 (EUROSCARF collection), respectively.

DNA sequences for all *B. bruxellensis SSU1* genes, two haplotypes from AWRI1499, one from AWRI1613 and one from AWRI2804, as well as modified sequences resulting in amino acids substitutions in Ssu1p, were synthesised by Invitrogen GeneArt (ThermoFisher Scientific, Massachusetts, USA). Sequences were codon optimised for their expression in *S. cerevisiae.*

Synthesised fragments were cloned into pCV2_BB and pCV3. In both plasmids, the *SSU1* gene was flanked by the strong yeast promoter *FBA1p* and the *PGK1* terminator. To replace the native *S. cerevisiae SSU1* coding sequence, constructs containing the *B. bruxellensis SSU1* gene and the kanMX cassette (encoding for geneticin resistance) but not the *FBA1* promoter were amplified by PCR from pCV2_BB and introduced in *S. cerevisiae* as indicated above. To evaluate the multicopy expression of the *B. bruxellensis SSU1* genes, pCV3 derived plasmids were introduced in *S. cerevisiae.*

To assess sulfite tolerance, *S. cerevisiae* starter cultures were made in 2 mL YPD medium (yeast extract 10 g/L; peptone 20 g/L; glucose 20 g/L) and incubated overnight at 28ºC with shaking (120 rpm). Cultures were then inoculated into minimal medium containing 20 g/L glucose and 6.7 g/L yeast nitrogen base with amino acids (YNB, Difco, USA) pH 3.5 (MM20) and grown overnight at 28ºC with shaking (120 rpm). These cultures were then used for assessing SO_2 tolerance as follows: 5 µL of starter culture were inoculated in a 96-well microplate containing 195 µL of MM20 supplemented with different concentrations of a sterile potassium metabisulfite (PMS) solution (20 g/L). Microplates were sealed with a Breathe-Easy® sealing membranes (Diversified Biotech, USA) and incubated at 28ºC. Optical density at 600 nm (OD_{600}) was measured twice daily using a Tecan infinite M200 plate reader (Tecan, Austria) Each strain was replicated at least three times per plate and experiments were repeated twice. Free SO_2 was measured in minimal media supplemented with freshly prepared PMS using the aspiration/titration method; these values were then used to estimate the concentration of molecular SO_2.

5.6. Analysis of potential VBNC in *B. bruxellensis* and *S. cerevisiae*

A summary of the strains and conditions for the VBNC experiments conducted is presented in Table 5.6.1. *Brettanomyces bruxellensis* strains (AWRI 1499 and/or AWRI 1613) were streaked out onto YPD agar plates from glycerol storage and grown at 28ºC for five to seven days. Several colonies from these plates were inoculated into YPD liquid medium and grown to late log/stationary phase over one to two days. Cultures inoculated at $5x10^6$ viable cells/mL as determined by flow activated cell sorting (FACS, see below) were grown/adapted overnight in YPD supplemented by the addition of 6% (v/v) ethanol and grown aerobically overnight with shaking at 28ºC prior to inoculation into a model Wine Medium (WM, Harris et al. 2008).

Adapted cultures were inoculated into WM in the anaerobic hood at $5x10^6$ viable cells/mL. The anaerobic hood was maintained at room temperature and 0 ppm O_2 with H_2 (used as a scavenger for O_2) kept between 1.5 and 4% (v/v) in an otherwise N_2 atmosphere.

For Experiments #1 (AWRI1499) and #2 (AWRI1613), inoculations were made directly into 200 mL media per bottle for all replicates.

Table 5.6.1 Summary of VBNC experiments

VBNC	Yeast	Conditions
Experiment 1	*B.b* AWRI1499	pH 3.5. No SO_2, Low SO_2, Mid SO_2 and High SO_2
Experiment 2	*B.b* AWRI1613	pH 3.75. No SO_2, Low SO_2, Mid SO_2 and High SO_2
Experiment 3	*B.b* AWRI1613	pH 3.5. No SO_2, Low SO_2, Mid SO_2 and High SO_2
Experiment 4	*B.b* AWRI1613	pH 3.5. No SO_2, Low SO_2, Mid SO_2 and High SO_2
Experiment 5	*B.b* AWRI1613	pH 3.5-4.0. No SO_2, Low SO_2, Mid SO_2 and High SO_2
Experiment i	*S.c* AWRI1631	pH 3.5-4.0. No fSO_2, Low fSO_2, Mid fSO_2 and High fSO_2
Experiment ii	*S.c* AWRI1631	pH 3.5-4.0. No mSO_2, Low mSO_2, Mid mSO_2 and High mSO_2

For Experiments #3, #4 and #5 (all AWRI1613) 5-litre volume initial cultures were inoculated and grown to approximately 10^7 CFU/mL (determined by FACS and culturability plating). Cultures were split into one-litre batches and SO_2 added, to concentrations required for experimental conditions.

The experiments exploring the VBNC phenomenon in *S. cerevisiae* are described in Table 5.6.1. These experiments were performed at room temperature in 250 mL SCHOTT bottles with the same lids as used in the *B. bruxellensis* VBNC experiments. The addition of 2 M NaOH was used to adjust the pH from 3.5 to 4.0 (pH shifting), thus removing the described VBNC stimuli. Volumes required were determined empirically and added to 50 mL aliquots of cultures removed from the 'parent vessel'. The number and size of shifted fractions overwhelmed available bottles, 50 mL aliquots were decanted in the anaerobic hood (*B. bruxellensis*) or laminar flow hood (*S. cerevisiae*). NaOH additions were performed either in the anaerobic hood (*B. bruxellensis*) or laminar flow (*S. cerevisiae*) with mixing on an orbital mixer.

Difficulties inherent in working in an anaerobic hood required the development of a suitable lid for all culture vessels (50 mL pH-shifted aliquots excepted) consisting of a BC-422N GL-45 bottle cap (Vaplock) with four ports. Two ports were blocked with ¼-28 UNF Hex headed plugs (White Nylon, Valueplas). Ports and fittings were assembled and autoclaved. Taps and sterile 1 mL syringes were added in a laminar flow hood. Bottles had 25 x 6 mm stir bars for mixing immediately before sampling or aliquoting by magnetic stir platform. Samples of 1 mL volume were collected at regular intervals.

FACS analysis was performed using three different instruments. Propidium iodide (PI) exclusion was used in all cases. PI from a 1 mg/mL stock was used at 10 µg/mL final concentration in phosphate buffered saline (PBS). Different equipment required different methods for total cell enumeration as outlined below:

- **FACSCalibur** (BD Biosciences). FlowCount beads (25 µL Beckman Coulter) were added in equal volume to cells harvested from cultures, in a final volume of 200 µL PBS with BSA added at 125 µg/mL to prevent bead adhesion, in 5 mL Falcon FACS, tubes. Calibration factors associated with the beads were used to determine volumes analysed and absolute counts.
- **Accuri C6** (BD Biosciences). Beads were not required as the unit calculates absolute counts based on aspiration volume. Samples were analysed in 5 mL Falcon FACS tubes or 1.5 mL Axygen MCT-150C tubes.
- **Guava Eascyte 12HT** (Merck Millipore) Beads were not required, as the unit calculates absolute counts based on aspiration volume. Samples were analysed in Costar Corning 3799 round bottom 96 well plates.

Analysis of flow cytometry data was performed using Flowing Software v2.5.1, FCS Express v5/6, Accuri C6 Software v1.0.264.21 or Guavasoft InCyte v3.1. Other fluorophores investigated included CFDA, BOX-1 and Rh123.

Colony forming unit (CFU) counts (culturability) were determined by spiral plating onto YPD agar plates (WASP-Don Whitley Scientific). 50 µL of cell suspension (neat/diluted or concentrated as appropriate) was dispensed onto agar plates. Agar plates were poured using a plate pourer (PS20

PetriSwiss BIOTOOL with PetriRack stacks) with 20 mL of YPD media dispensed into each plate. CFU count determination was performed using a plate imager (ProtoCOL 3, Synbiosis). Images were acquired and spiral count determination was made with correction for dilution/concentration where required.

5.7. Investigation of alternative control methods in *B. bruxellensis*

5.7.1. Chitosan for control of *B. bruxellensis*

All experiments involving chitosan (except the pilot experiment), were conducted in a red wine model (red-WM) - the same medium (WM) as used for the VBNC *B. bruxellensis* experiments but with Grapex extract (Tarac Ltd) added at 50 mL/L to the medium. All adjustments of the red-WM were as per that used for the VBNC experiments other than the Grapex addition. Strains used in this experiment were AWRI 1499 and 1613, plus their sulfite-tolerant evolved counterparts AWRI 1499 38C and AWRI 1613 13A

Chitosan was sourced from:

- OENOBrett (Laffort Oenologie). Used at recommended 10 g per hL
- NoBrettInside (Lallemand Australia). Used at both recommended 4 g and 8 g per hL

All *B. bruxellensis* strains were grown as per VBNC experiments in WM (post-adaptation) at room temperature in the anaerobic hood. Cell suspensions were prepared at 2x the desired final concentration (i.e. $2x10^1$, $2x\ 10^2$, $2x\ 10^3$, $2x10^4$ and $2x10^5$ cfu/mL for the pilot experiment) in fresh red-WM.

Chitosan suspensions were prepared at 2x concentration, with OENOBrett at 20g/hL in fresh WM and NoBrettInside at 8 g/hL and 16 g/hL in fresh WM. For the experimental setup, 5 mL of each of cell suspension was mixed with 5 mL of chitosan preparation (or 5 mL of Red-WM for the positive control). All samples were prepared in triplicate for each point, volumes were combined in 15 mL screwcap Corex tubes and mixed immediately on an orbital mixer. Four experiments were conducted in two pairs, parent and evolved strain together, with tubes were arranged in racks, 48 samples per strain for two strains, therefore 96 in total. For all sampling points, 1 mL of resuspended medium was removed from the tube and transferred to a 96-well deep well plate. Dilutions or concentrations (spin down entire plate and transfer of residual after aspiration of supernatant by vacuum) was carried out into a new plate; a 96-well flat-bottomed microplate (Costar Corning Cat:3596). All data points from chitosan testing were determined by CFU/mL using spiral plating as per the VBNC experiments.

5.7.2.QPCR for detection of live vs dead *B. bruxellensis*

For QPCR investigations primers were prepared as per Phister and Mills (2003) (DBRUX-F/R, Figure 5.7.1). Two extra oligonucleotide primers (shorter in length, DBRUXShort-F/R) were designed to allow the use of Taqman probes (www.thermofisher.com). This was to improve both specificity and sensitivity of the original (Phister and Mills 2003).

The original primer set (DBRUX F/R) and shorter Taqman oligonucleotide pairs (DBRUXShort F/R) were tested for specificity using SYBR green and Taqman chemistry. Taqman probe/primer sets were used with TaqPath ProAmp Master Mix (Thermofisher Scientific) as per dilutions recommended in Phister and Mills (2003). A 2 µL volume of template was used in all QPCR reactions with final reaction volume of 10 µL, using the following oligo concentrations: forward (DBRUX-F 900nM), reverse (DBRUX-R 300nM) and probe (FAM-TMR) at 250 nM. Cycling was conducted on a BioRad CFX96, with the following cycling conditions: denaturing at 95ºC, 300 sec, thermocycler profile 95ºC/60 sec, 66ºC/45 sec, 72ºC /7 sec) x 60 cycles. Fluorescence data acquisition occurred at the end of extension.

Production of dead cell suspensions was performed by heat killing AWRI 1613 resuspended in PBS at 65ºC for 20 mins in a water bath. Cell counts (live/dead) were determined by FACS (Guava) and suspensions once made were reassessed by FACS and spiral plating prior to DNA extraction. The DNA from samples was extracted using DNeasy PowerSoil Kit (Qiagen). The manufacturer's protocol was followed and DNA was eluted in 100 µL volume.

5.8. Transformation of *B. bruxellensis*

To increase the transformation efficiency of *B. bruxellensis TDH1* promoter generating plasmids pMK-T-TDH1pr-KanMX, pMK-T-TDH1pr-NatMX and pMK-T-TDH1pr-HygMX, respectively, the *TDH1* promoter fragment was synthesised by Invitrogen GeneArt (ThermoFisher Scientific, Massachusetts, USA). Transformation cassettes were amplified from their corresponding plasmids, purified using the Wizard® SV Gel and PCR clean-up system (Promega, Madison, USA) and used for transformation. *B. bruxellensis* strains were transformed by electroporation following the protocol suggested by Miklenič et al. (2015). Briefly, strains were grown overnight in liquid YPD medium, then 1 mL of culture was used to inoculate 200 mL of fresh medium and incubated on an orbital shaker (180 rpm, 28°C) until a concentration of $5x10^7$ cells/mL was achieved. Cells were then centrifuged (3000 rpm, 4 min, room temperature), supernatant was carefully decanted and cells were washed three times with 50 mL deionised water. Cells were resuspended in 20 mL of 35 mM of dithiothreitol and 100 mM lithium acetate and incubated for 45 minutes at 28°C with gentle shaking (140 rpm). Cells were then isolated by centrifugation (3000 rpm, 4 minutes, 4°C), with the resulting pellet washed twice with 20 mL of ice-cold sterile deionised water and then twice with 20 mL of ice-cold 1M sorbitol solution. Finally, cells were re-suspended in 1M sorbitol (ice-cold), to a final volume of 500 µL, with 50 µL aliquots used for each transformation reaction. A volume of 1 µL containing DNA for transformation was then introduced into the samples, mixed thoroughly and incubated on ice for five minutes. Samples were then placed in separate 0.2 cm-gap electroporation cuvettes (Bio-Rad) previously kept on ice, and pulsed (1.8 kV, 5 ms) with Bio-Rad Gene Pulser Electroporator (600 Ω, 25 µF). Immediately after the pulse, 1 mL of 1M sorbitol:YPD (1:1) ice-cold solution was added, and the samples were incubated at room temperature for 20 minutes without shaking. After the incubation step, the cells were placed in 10 mL sterile tubes and 1 mL of YPD was added. The samples were incubated overnight at 28°C with shaking (140 rpm) and then spread on plates containing the appropriate antibiotic. Plates were then incubated at 30°C and transformants were visible after five to ten days.

Targeted deletion of *B. bruxellensis* coding regions was attempted following successful strategies implemented in other fungal species where non-homologous end joining (NHEJ) DNA repair is dominant. Briefly, in order to improve efficiency of targeted DNA modifications NHEJ must be impaired by deletion of genes encoding proteins homologous to Ku70 or Ku80. We targeted the *B. bruxellensis KU80* gene flanking regions (up- and down-stream to the ORF) with long homology (approximately 1.2 kb each) that were synthesised by Invitrogen GeneArt (ThermoFisher Scientific, Massachusetts, USA) and cloned up- and down-stream to the NatMX cassette in pMK-T-TDH1pr-NatMX. Thus, a cassette containing the upstream region to *KU80*, the NatMX cassette and the downstream region to *KU80* was amplified from the plasmid pMK-T-KU80up_TDH1pr-NatMX_KU80down, purified and used to transform AWRI2804. A similar strategy was used to obtain a transformation cassette for the deletion of *SSU1*. The *SSU1* deletion cassette was used to transform AWRI2804 and AWRI2804 lacking *KU80* to evaluate the impact of *KU80* deletion upon efficiency of NHEJ.



Figure 5.7.1. QPCR primers. DBRUX F/R from Phister and Mills (2003), with shorter primers DBRUXShort F/R and Taqman probe sequence (in red).

5.9. Competition experiments using antibiotic resistance 'tagged' *B. bruxellensis* strains

Antibiotic resistance cassettes were used to 'tag' different *B. bruxellensis* strains and perform competition experiments. Three strains, which represent the three major *B. bruxellensis* genetic groups, AWRI1499 (SO_2-tolerant), AWRI1608 (SO_2-sensitive) and AWRI1626 (SO_2-sensitive) were transformed with the KanMX and NatMX cassettes as described in section 5.6. As transformed DNA inserts in a non-targeted fashion in *B. bruxellensis*, multiple transformants were compared to the corresponding parental strains to determine the phenotypic neutrality of the transformation event. Only phenotypically neutral transformants were used for the subsequent competition experiments.

To begin the competition experiments, competing pairs (each strain containing a different antibiotic marker) were grown separately in YNB liquid medium until stationary phase and then inoculated together in equivalent proportions in synthetic media containing YNB, 6.7 g/L, glucose, 2.5 g/L, fructose, 2.5 g/L and ethanol 5%, at pH 3.5. Competing pairs were tested under different molecular SO_2 concentrations including 0, 0.2, 0.4, and 0.6 mg/L.

Competitive growth was then allowed to proceed in 50 mL centrifuge tubes (Grenier Bio-One) containing 25 mL of medium at 28 °C and with shaking (140 rpm). Cultures were grown until an approximate OD_{600} of 1.5-2. At this point, samples were taken and spread on YPD plates containing antibiotics for colony enumeration; simultaneously cells were re-inoculated in fresh medium at OD_{600} 0.1. These transfers were repeated until 20 generations for the total population were attained. To assess the possible impact of the introduced DNA cassette, strains containing either antibiotic marker were evaluated. Additionally, to avoid the potential bias introduced by using strains resulting from random insertion, different transformants (different transformed clones) were used as 'biological triplicates'. Finally, to test the repeatability of the competition experiments the same AWRI1499 transformants were used in both competition sets, AWRI1499 vs AWRI1608 and AWRI1499 vs AWRI1626.

6. Results/Discussion:

6.1. Knowledge of *B. bruxellensis* population genetics/genomics

Previous data had shown that *Brettanomyces* isolates were genetically diverse, and were often present with genetic complements that would be consistent with either inter-specific hybridisation or intra-specific hybridisation between very diverse strains (Borneman et al. 2014, Curtin et al. 2012b). In order to better understand the population genomics of *B. bruxellensis*, whole-genome sequence data was obtained for an additional 40 strains from a variety of sources (Table 6.2.1).

The pattern of single-nucleotide (SNP) variation across the genome of each isolate was used to estimate a species phylogeny (Figure 6.1.1), which could be sub-divided into six main clades. By further analysis of SNP ratios within each genome, it was also possible to estimate the ploidy, or genome copy number, of each strain. There were clear differences in ploidy observed between clades, with exclusively diploid (2n) and triploid (3n) clades observed, although in three clades, individual strains existed that differed in their ploidy relative to the majority of that group, indicating that additional hybridisation events had occurred. In addition, haploid (1n) strains were also found to be present within one clade. This represents the first instance that haploid strains of *B. bruxellensis* have been documented.

When viewed holistically, these genomic data indicate that at least nine different genomic configurations exist across the six phylogenetic clades, with three examples of possible inter-specific hybridisation and an additional two examples of intra-specific hybridisation/genome duplication (Figure 6.1.2).

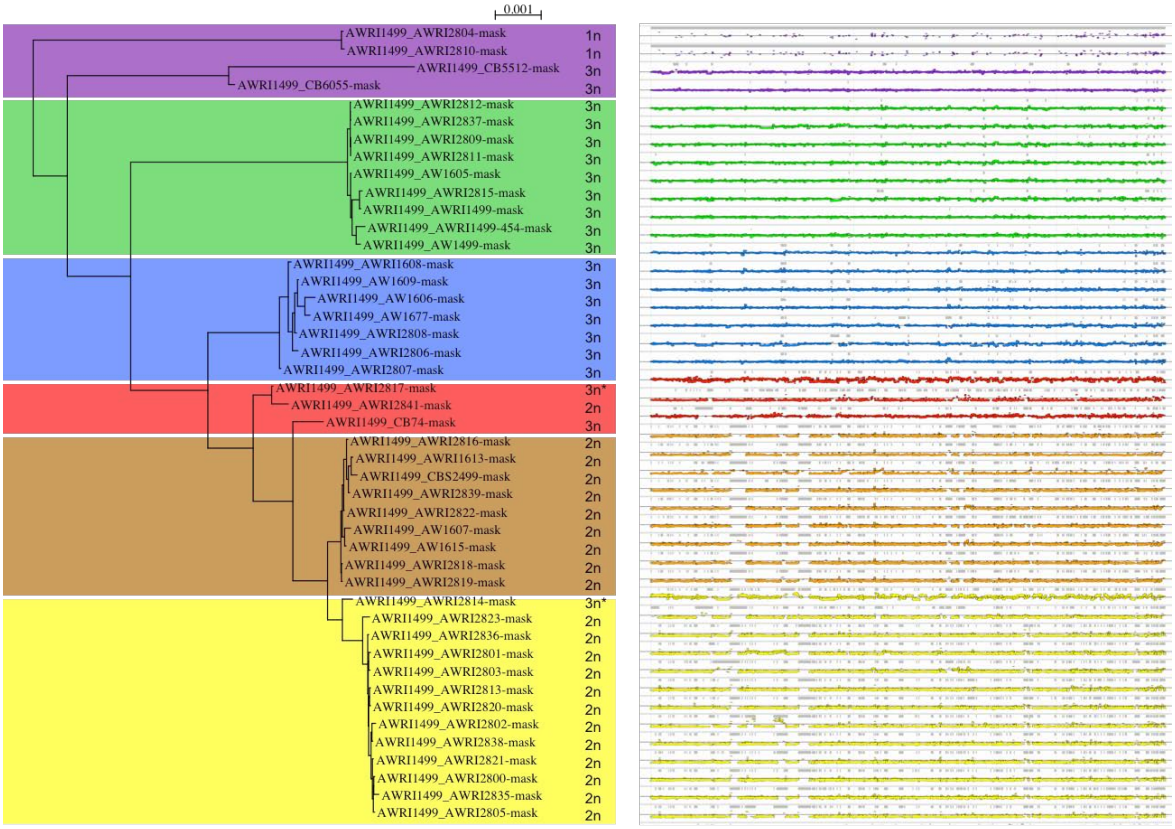


Figure 6.1.1. Phylogenetic analysis of *Brettanomyces* isolates. Isolates were sourced from several industrial and geographical sources (See Appendix) and subjected to whole genome sequencing. A genomic phylogeny (left) was produced from SNP variation across strains, with six main strain clades identified (colour coded). SNP allele proportions (right) were used to estimate strain ploidy as either haploid (1n), diploid (2n) or triploid (3n).

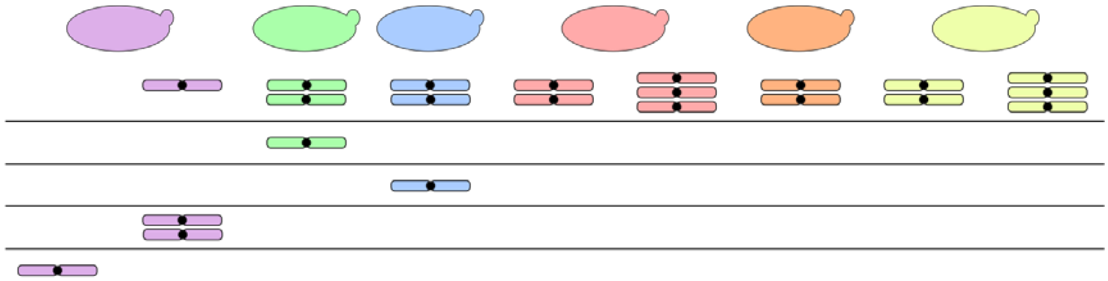


Figure 6.1.2 Genomic organisation of *B. bruxellensis*. Clades are shaded as in Figure 6.1.1. Average chromosomal ploidy is indicated by the number of chromosomes (2 = diploid), with independent instances of hybridisation indicated by chromosomes separated by lines.

6.2. Evolution of sulfite resistance in laboratory experiments

6.2.1. Establishing adaptive evolution systems

Laboratory evolution experiments are ideally performed under conditions that most closely mimic the environment being simulated, with tight control such that only the variable under study can contribute to evolutionary change in the target organism. Continuous culture 'chemostats' are one commonly used system, where environmental parameters are maintained as a constant and therefore the physiological state of the microbial population is in 'steady-state'. Evolution experiments can then be performed by introducing a single variable, such as increased sulfite concentration, and establishing a new steady-state that can be maintained for hundreds of generations. A chemostat was established for AWRI1613 and AWRI1499 and over a period of 30 days the dosage of sulfite being pumped into the growth medium was increased. Upon screening of single colony isolates from this experiment no significant differences were found in sulfite tolerance compared to controls, despite the apparent increase in tolerance for the population. Further investigation revealed that there was no detectable free SO_2 in the culture medium, despite there being 45 mg/L total SO_2. While it is possible this reflects an adaptation by the population, whereby it gained tolerance by producing metabolites that bind free SO_2, experimental artefacts could not be excluded and this approach was therefore abandoned.

Another, relatively simple experimental setup commonly used in adaptive evolution studies is long-term repeat batch culture. This more closely mimics the way microbes grow in nature (or in wineries) but does not allow the same degree of control over environmental parameters. Batch cultures were established in both minimal laboratory medium and a synthetic wine medium and increased concentrations of sulfite were introduced with each batch subculture. It quickly became apparent that in synthetic wine the duration of batch cultures under sulfite stress was too long to allow the attainment of the target number of yeast division generations necessary to study adaptive evolution (100+).

6.2.2. Long-term batch culture evolution experiment

Long-term batch cultures for AWRI1613, AWRI1499 and AWRI2804 were run for nearly 12 months in minimal laboratory media. All populations were established in triplicate to observe the reproducibility associated with selection. It was observed that all of the *B. bruxellensis* experimental evolution populations showed increased tolerance to sulfite over time, although both the speed of adaptation and the ultimate level of tolerance was highly dependent on the parental strain. The highest sulfite concentrations at which evolved populations were successfully sub-cultured were: 37 mg/L for AWRI1613, 54 mg/L for AWRI1499 and 15 mg/L for AWRI2804. For each founding strain, individual colonies from each of the evolving populations (A, B and C) and from control populations (D, E and F) isolated at 50 and 100 generations were assessed for sulfite tolerance (Figure 6.2.1).

For AWRI1613 at 50 generations there was a significant increase in tolerance for all individual isolates irrespective of the presence of sulfite in the media (Figure 6.2.1A). However, at 100

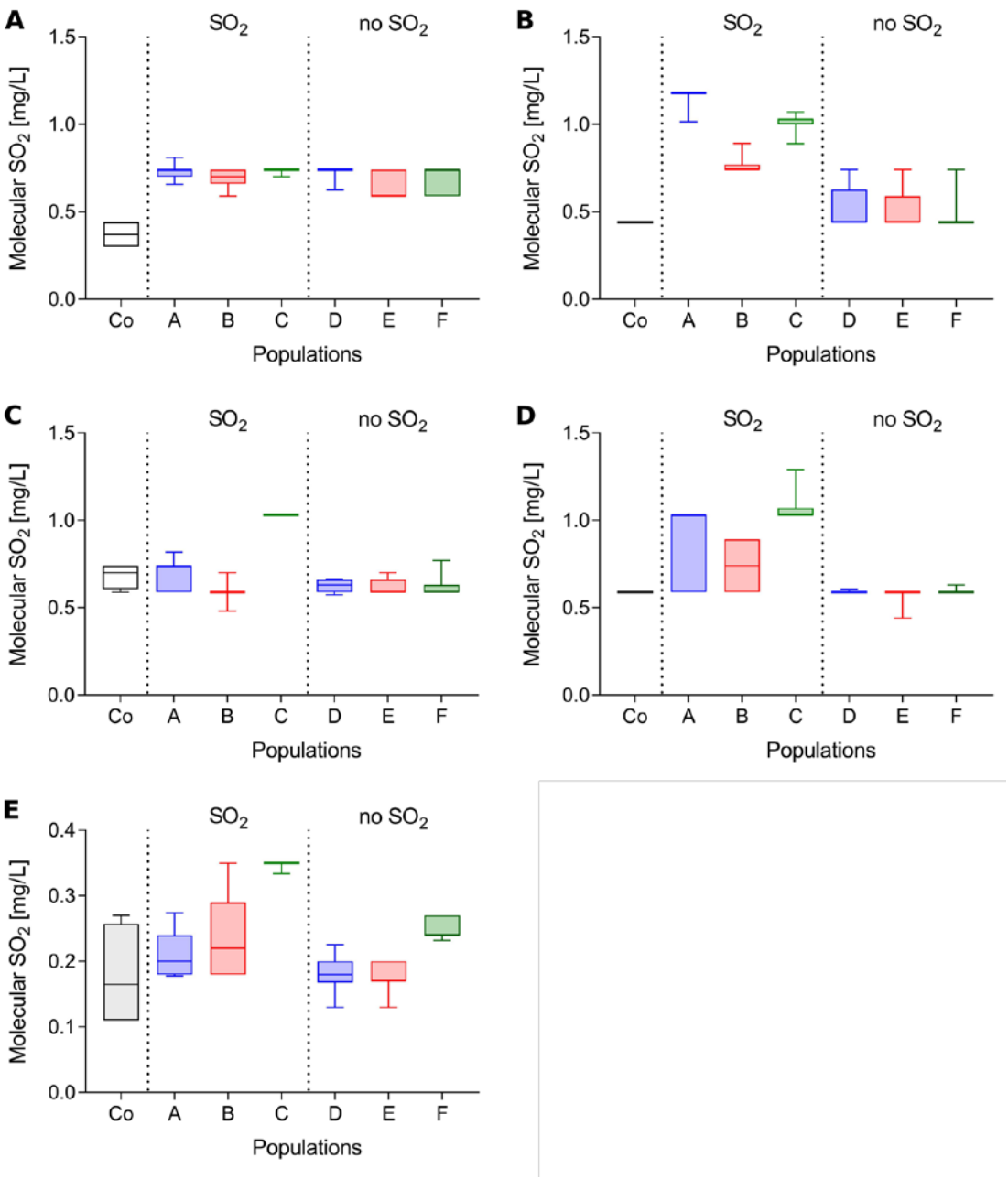


Figure 6.2.1. Long-term batch culture of *B. bruxellensis* under sulfite selection. Maximum sulfite tolerance was calculated for 15-17 isolates per populations grown under either increasing sulfite stress (populations A-C) or in the absence of sulfite (populations D-F) when compared to un-passaged controls (Co). Results are presented for **(A)** AWRI1613 passaged for 50 and **(B)** 100 generations; **(C)** AWRI1499 for 50 and **(D)** 100 generations (D); **(E)** AWRI2804 for 50 generations.

generations only isolates from populations subjected to sulfite stress showed a further increase in sulfite tolerance. Indeed, all three evolving populations (A, B and C) showed a remarkably increased sulfite tolerance (Figure 6.2.1A,B). These results indicate that an initial adaptation of AWRI1613 to the media conditions occurred early; this was then followed by a subsequent adaptation to the sulfite stress as the levels of the stressor were increased over time.

For AWRI1499 at 50 generations only population C showed an overall increased tolerance to sulfite (Figure 6.2.1C). At 100 generations, all three populations exposed to sulfite stress (A, B and C) were composed of individual isolates with higher sulfite tolerance than the control strain. However, once again, only AWRI1499 population C had reached a point where sulfite-tolerant individuals were dominant. As indicated by the broad distribution of individual sulfite tolerances, both populations A and B appeared to retain individuals that were not more tolerant to sulfite than founding strain

AWRI1499 and may therefore represent strains which have adapted to the general media conditions (and increased their overall growth rate) (Figure 6.2.1D).

Adaptive evolution experiments with AWRI2804 were not able to proceed for 100 generations due to reaching a maximum sulfite tolerance in this strain by 50 generations. Indeed, screening at 50 generations showed a modest increased in sulfite tolerance compared to the founding strain in only one of the three populations exposed to sulfite stress (Figure Boxplots 2804). However, isolates with increased sulfite tolerance were observed for populations B and C. Given the modest increase in sulfite tolerance it appears that AWRI2804 does not have the requisite genetic structure required to improve sulfite tolerance under the conditions tested here.

It is very interesting that different behaviours regarding the sulfite tolerance of individual isolates and evolution of this trait, were observed for the different strains. This suggest that the genetic makeup and ploidy levels of the strain influence the successful evolution of sulfite tolerance in *B. bruxellensis*.

After testing individual isolates, 5-6 isolates per population were selected for confirmation experiments in synthetic wine. Since adaptive evolution experiments were performed in minimal media to shorten *B. bruxellensis* generation time, these experiments enabled to evaluate if the enhanced sulfite tolerance was independent of the culture media. Confirmation experiments revealed isolates with a range of sulfite tolerances and a positive correlation between the two different culture media. These results showed the successful selection of sulfite tolerant isolates independent of the culture media. Results for the two most tolerant isolates for AWRI1613 and AWRI1499 in synthetic wine are shown in Figure 6.2.2.

Individual sulfite-tolerant clones derived from early time-points of morbidostat populations for AWRI1499, AWRI1613 and AWRI2804 have had their genomes sequenced (Figure 6.1.2). Analysis of AWRI1613 variants revealed several regions that have undergone changes relative to the original parent strain, which may be responsible for enhanced sulfite tolerance. At this time point, however, different clones displayed different changes, and a single sulfite-tolerant clone had not yet become dominant in the population.

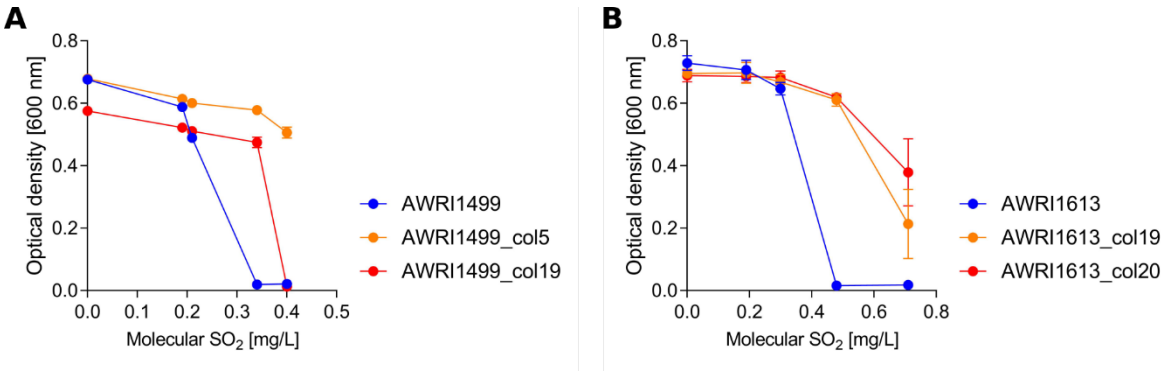


Figure 6.2.2 Sulfite tolerance in chemically defined wine for the two most tolerant isolates from the batch evolution populations. (A) *B. bruxellensis* AWRI1499 and (B) *B. bruxellensis* AWRI1613 (B).

6.2.3. Stability of sulfite tolerance

Repeat screening of individual clones from all morbidostats at later time points was completed, along with clones from an additional experiment where sulfite-tolerant populations were then freed of sulfite-stress for 50 generations. The latter revealed that after 50 generations of sulfite stress the populations were still heterogeneous, and it was possible for sulfite-sensitive but fast-growing clones to re-emerge. One population, that displayed very slow growth, did not display this heterogeneity, suggesting a fixed population had been reached within 50 generations (Figure 6.2.1C,D).

To test the stability of the sulfite tolerant phenotype the three AWRI1499 replicate populations that had been exposed to sulfite for 50 generations were then grown without sulfite-stress for additional 50 generations. Results showed that the sulfite tolerant phenotype displayed by population C was retained after 50 generations without sulfite stress (Figure 6.2.2). However, despite the relatively uniform population tolerance shown by population C after 50 generations of selection (Figure 6.2.1C), isolates from population C after relaxed selection displayed a far greater deviation in their individual levels of sulfite tolerance. This result suggests that the establishment of sulfite tolerance comes at a significant fitness penalty that likely to allows rare, sulfite sensitive cells to re-establish themselves following extended growth in sulfite-free media. This trade-off between resistance and fitness is commonly observed in other systems, such as the development of antibiotic or agrochemical resistance and may be exploited to 'purge' specific resistance phenotypes by rotating through control strategies. While this strategy could be difficult for the wine industry to adopt in the short term, due to its heavy reliance on sulfite, alternative control strategies (See Section 6.6) may provide a means to address this.

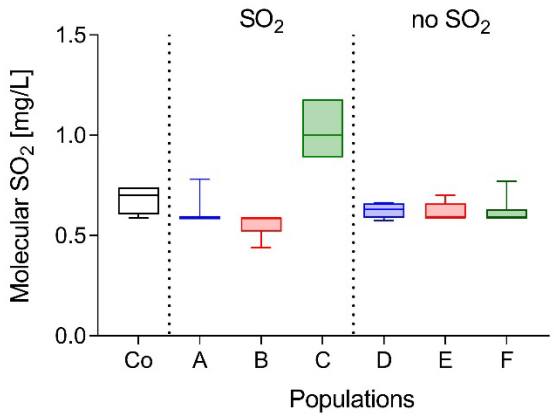


Figure 6.2.3. Limited stability of sulfite tolerance following relaxation of selection. Maximum sulfite tolerance was calculated for 15-17 isolates per population grown under either sulfite stress for 50 generations followed by 50 generations without stress (populations A-C) or in the absence of sulfite (populations D-F) when compared to un-passaged controls (Co). Results are presented for AWRI1499.

6.2.4. Pool-seq of sulfite tolerant populations

To attempt to define regions of the *Brettanomcyes* genome that were associated with the increase in sulfite tolerance, pooled-population sequencing was performed for each population that was established for each strain (with and without sulfite) at both 50 and 100 (AWRI1499 and AWRI1613 only) generations. DNA was prepared from frozen samples of each population and subjected to next-generation sequencing, with the subsequent data aligned against the AWRI2804 reference genome to determine SNPs and potential instances of copy-number variation that had occurred during the evolution experiment.

While it was possible to observe several instances of genomic variation that had occurred during the sulfite-culturing process, many alterations were also observed in the non-sulfite-passaged samples. Furthermore, there appeared to be no genomic alterations that were common amongst the sulfite-treated populations. This result mirrors that observed from the strain phenotyping and indicates that the populations remained highly heterogenous at the time of sample collection. As no selective population sweep had yet to occur in these populations, it makes the identification of the key genetic loci that are responsible for the sulfite tolerance impossible to reliably determine. Further long-term

culturing of these populations under stable, but high levels of sulfite may be required to allow the populations the requisite time to converge on a common (most fit) genotype.

6.3. Sulfite tolerance of new industry isolates

The results of laboratory-scale directed evolution provide the first direct evidence that *B. bruxellensis* strains have the capacity to adapt to the use of sulfite as a control agent. Could this happen in the field? Previous industry-based population surveys had already shown that the AWRI1499 clade, which displays the highest levels of sulfite resistance, was the most frequent *Brettanomyces* genotype isolated from Australian wineries in 2000 to 2004 (Curtin et al. 2012a, 2007)). Since then, Australian winemakers have become increasingly aware of the importance of sulfite management for *Brettanomyces* spoilage control. Their changing practices could potentially provide selective conditions analogous to those imposed in the laboratory, thereby causing evolution of sulfite tolerance.

Historical industry isolates were therefore sourced from the AWRI wine microorganism culture collection (including those isolated during the original Curtin et al. study), in addition to current industry isolates sourced from a commercial partner in 2016-2017. All of these strains were then subjected to high-throughput sulfite tolerance assays to determine if levels of sulfite resistance have changed over time (Figure 6.3.1). Isolates from 2000-2004 displayed levels of sulfite resistance that were ranged between those observed for AWRI1499 and AWRI1613, which broadly represented the range of tolerances observed in the original study by Curtin et al. (2012a). Isolates from 2010-2014 did not show a significant difference in their median sufite resistance, although there were a small number of isolates that did show higher levels of sulfite resistance than observed in the 2000-2004 cohort. Interestingly, the 2016-2017 isolates, sourced from 16 different wines, displayed greater tolerance to sulfite, growing at concentrations significantly higher than those observed from the two previous cohorts ($p<0.0001$). It should be noted that the 2016-2017 isolates were sourced from two wine companies (although one is a large, multi-site producer). As such, they represent a small part of the overall industry; however, these preliminary data suggest that strains with significantly higher levels of sulfite resistance are present in the field. It is noteworthy that these wineries follow industry best practice for *Brettanomyces* control. Further sampling is required to determine the broader extent of resistance development and to determine if this is a wide-reaching problem requiring significant attention by the Australian wine industry.

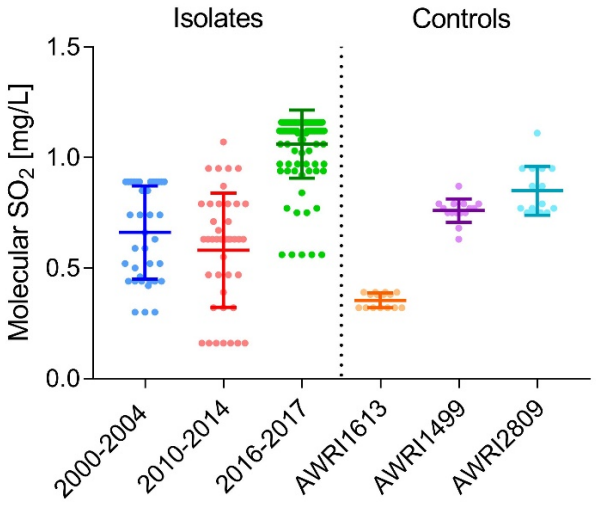


Figure 6.3.1 Sulfite tolerance of industry isolates of *B. bruxellensis* over time. Industry isolates were sourced from wineries and/or the AWRI wine microorganism culture collection during three periods. Maximum sulfite tolerance was calculated for each isolate. Maximum sulfite tolerance for AWRI1613, AWRI1499 and AWRI2809 all isolated during the 2000-2004 period are shown as controls.

6.4. Expression of *Brettanomyces SSU1* genes in *S. cerevisiae*

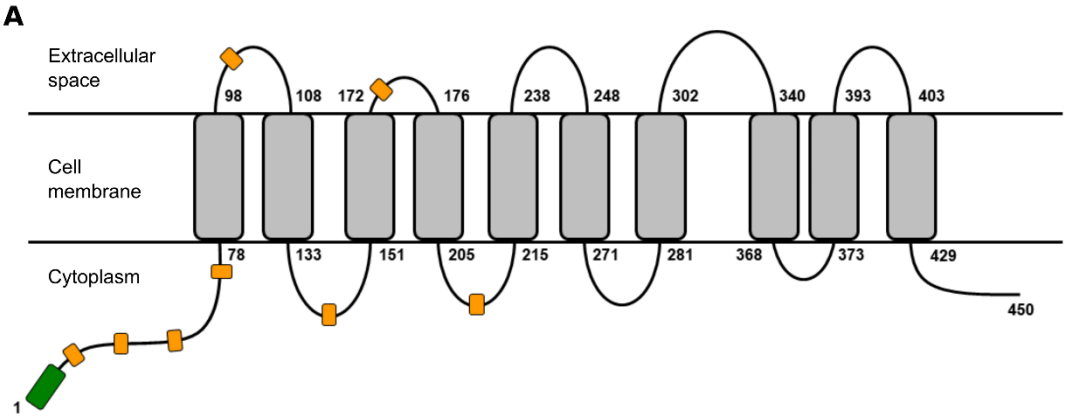
Genomic analysis described in 6.1 revealed differences in ploidy levels between *B. bruxellensis* AWRI1499 (triploid), *B. bruxellensis* AWRI1613 (diploid) and *B. bruxellensis* AWRI2804 (haploid). Analysis of gene sequences for these strains revealed four distinct gene sequences (haplotypes) for *SSU1*, the key gene involved in sulfite tolerance for *Saccharomyces cerevisiae* (Figure 6.4.1). AWRI1499 exhibited two *SSU1* haplotypes; AWRI1499_SSU1_hap1 and AWRI1499_SSU1_hap2 the latter being present in two copies. AWRI1613 also has two haplotypes, one identical to AWRI1499_SSU1_hap2 and a second annotated AWRI1613_SSU1_hap2. *B. bruxellensis* AWRI2804, as a haploid strain, therefore only contained a single version of the gene AWRI2804_SSU1_hap1. Comparison of the amino acid sequences for these four haplotypes revealed eight locations of amino acid variation and a deletion of 12 amino acids at the N-terminal of AWRI2804_SSU1_hap1. All variations were observed within the first 208 amino acids and located primarily within the cytoplasmic domains of the protein (Figure 6.4.1A). As a putative efflux pump, it would be anticipated that recognition of the correct molecule to export would be mediated by these domains.

To determine whether the *B. bruxellensis SSU1* sequences encoded functional sulfite efflux pumps, each haplotype was cloned into a high copy number expression vector pCV3, under the control of a strong *S. cerevisiae* promoter. The expression vector was then introduced into an *S. cerevisiae* strain that had its own copy of *SSU1* deleted (AWRI1631 ∆*SSU1)*. When expressed in high copy number all *B. bruxellensis SSU1* haplotypes increased the sulfite tolerance of the background strain approximately 4-fold, confirming their functionality, though none of the haplotypes fully complemented deletion of native *SSU1* in *S. cerevisiae.*

To facilitate direct comparison between the *B. bruxellensis* haplotypes, each was integrated into the *S. cerevisiae* chromosomal *SSU1* location, replacing the native *SSU1* sequence but leaving the promoter region intact (the only variable was amino acid coding sequence). The four haplotypes conferred significantly different tolerance to sulfite (Figure 6.4.2). *S. cerevisiae* AWRI1631 lacking *SSU1* exhibited no growth when molecular sulfite concentrations reached 0.26 mg/L. AWRI1499_SSU1_hap1 conferred the least sulfite tolerance, with no growth observed at 0.35 mg/L. AWRI1499_SSU1_hap2 was the strongest haplotype, enabling growth up to 0.88 mg/L of sulfite. AWRI1613_SSU1_hap2 conferred tolerance up to 0.53 mg/L of SO_2, while AWRI2804_SSU1_hap1 only allowed yeast growth until concentrations reached 0.44 mg/L. Therefore, in order of relative sulfite tolerance when expressed in *S. cerevisiae,* AWRI1499_SSU1_hap2 was the strongest haplotype followed by AWRI1613_SSU1_hap2, then AWRI2804_SSU1_hap1 and finally AWRI1499_SSU1_hap1.

Despite the *B. bruxellensis SSU1* proteins varying at only eight amino acid residues, these differences conferred a wide range in sulfite tolerance when expressed in *S. cerevisiae*. However, the relative importance of these eight substitutions could not be determined from these 'natural' haplotypes as some changes were only observed to co-occur across strains. To unravel the precise impact of each individual amino acid change, a selection of synthetic haplotypes were created. As described above, AWRI1613_SSU1_hap2 conferred a maximum sulfite tolerance to 0.53 mg/L whereas AWRI1499_SSU1_hap2 conferred growth in up to 0.88 mg/L of molecular SO_2. There are only two amino acid changes between these haplotypes; Asn143Asp and Thr208Arg (Figure 6.4.1.B). Single amino acid substitutions in AWRI1613_SSU1_hap2 were therefore evaluated for their impact on sulfite tolerance. Both Asn143Asp and Thr208Arg provided increased sulfite tolerance compared to AWRI1613_SSU1_hap2, allowing growth in up to 0.71 mg/L of SO_2. However, this increase in tolerance did not match the tolerance observed for AWRI1499_SSU1_hap2 indicating that both substitutions are required for maximum tolerance.

A



B

Position	AWRI1499 SSU1_hap1	AWRI1499 SSU1_hap2	AWRI1613 SSU1_hap2	AWRI2804 SSU1_hap1
1-12				deletion
25	Asn (N)	Asp (D)	Asp (D)	Asp (D)
31	Lys (K)	Gly (G)	Gly (G)	Arg (R)
39	Lys (K)	Ile (I)	Ile (I)	Lys (K)
67	Pro (P)	Leu (L)	Leu (L)	Leu (L)
102	His (H)	His (H)	His (H)	Tyr (Y)
143	Asp (D)	Asp (D)	Asn (N)	Asp (D)
173	Arg (R)	Arg (R)	Arg (R)	Lys (K)
208	Lys (K)	Arg (R)	Thr (T)	Lys (K)

Figure 6.4.1 Structural variation in the *SSU1* protein across *B. bruxellensis* strains. **(A)** protein topology for *B. bruxellensis* SSu1p indicating 10 transmembrane domains, interspaced with short cytoplasmic and non-cytoplasmic loops. Yellow boxes indicate positions of amino acid variation between the haplotypes and a green box indicates a deletion of 12 amino acids at the N-terminal of AWRI2804_SSU1_hap1. **(B)** Details on the position and amino acid variation between haplotypes.

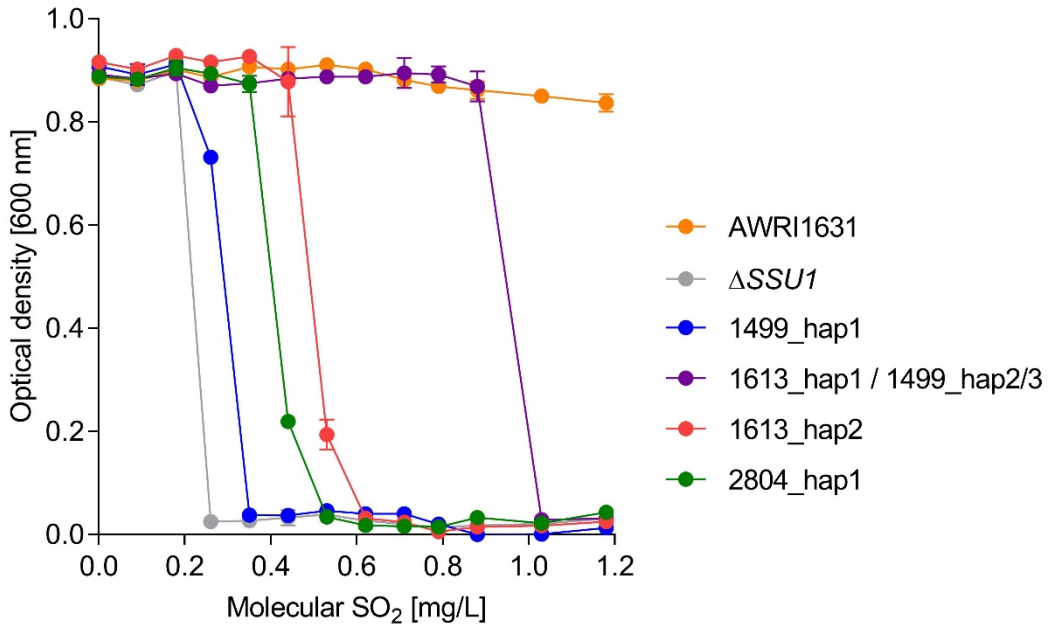


Figure 6.4.2. Functional analysis of *B. bruxellensis SSU1* variants in *S. cerevisiae*. Single copy expression of *B. bruxellensis SSU1* haplotypes in *S. cerevisiae*. *SSU1* haplotypes were expressed in *S. cerevisiae* strain AWRI1631 ΔSSU1 under the control of the native *S. cerevisiae* promoter.

Correlating the haplotype results with observed industrial phenotypes, *B. bruxellensis* AWRI1499, consistently shows the highest sulfite tolerance. This triploid isolate contains two copies of AWRI1499_SSU1_hap2 (the highly active haplotype) and one copy of AWRI1499_SSU1_hap1 (low acitivity). *B. bruxellensis* AWRI1613 (diploid) displays moderate sulfite tolerance and contains one copy each of AWRI1499_SSU1_hap2 (high) and AWRI1613_SSU1_hap2 (low). *B. bruxellensis* AWRI2804 showed the lowest sulfite tolerance and contained a single copy of AWRI2804_SSU1_hap1 (low).

These results suggest a clear correlation between the copy number and efficiency of the specific haplotype(s) in each strain and the observed sulfite tolerance. However, this may not be the entire explanation for the phenotypic variation between strains as it is not possible to rule out the potential role of gene expression or localised copy-number variation (as opposed to general ploidy differences) in sulfite tolerance as conferred by *SSU1* in *B. bruxellensis.* It is likely that a combination of haplotype efficiency, copy number and expression levels all contribute to the diverse sulfite tolerances observed in different *B. bruxellensis* strains.

6.5. Analysis of potential VBNC in *B. bruxellensis* and *S. cerevisiae*

Previous reports indicated the potential for *B. bruxellensis* and *S. cerevisiae* to present a VBNC phenotype (e.g. Agnolucci et al. 2010, Salma et al. 2013, Serpaggi et al. 2012, Willenburg and Divol 2012). However, none of these studies provided sufficient detail to establish the classical determination of true VBNC (Whitesides and Oliver 1997). Therefore, it was attempted to determine if VBNC is a phenomenon that winemakers need to be concerned about. Table 6.5.1 represents a summary of the multiple experiments undertaken to investigate VBNC in both *B. bruxellensis* and *S. cerevisiae*.

Table 6.5.1 Summary of VBNC experiments undertaken

Exp't	Spp.	Strain	Time (days)	[fSO2 mg/L]	[mSO_2 mg/L] pH 3.5	post pH 4.0 shift mSO_2	Recovered (no. of replicates)	Days to Recovery (max CFU/ml) [Divisions]	Doubling rate (days)
1	*B.b*	AWRI1499	450+	55, 30, 10, 0	1.1, 0.6, 0.2, 0	n/a	2 of 3 low SO_2	54 days, -4.6x10^4 [~16.5]	3.2
2	*B.b*	AWRI1613	420+	70, 45, 25, 0	1.4, 0.9, 0.5, 0	n/a	1 of 3 low SO_2	61 days, -5x10^4 [~16.5]	3.7
3	*B.b*	AWRI1613	400+	24, 15, 10, 0	0.48, 0.3, 0.2, 0	n/a	1 of 3 low & 2 of 3 mid SO_2	43-62 days, -1x10^5 [~17.5]	3.3-4.7
4	*B.b*	AWRI1613	110+	24, 15, 10, 0	0.48, 0.3, 0.2, 0	n/a	1 of 3 low SO_2	49 days, -3.4x10^5 [~23]	2.1
5	*B.b*	AWRI1613	50	24, 19, 13, 6, 0	0.48, 0.38, 0.26, 0.12	0.15, 0.12, 0.08, 0.04, 0	None	n/a	n/a
i	*S.c*	AWRI1631	53	4, 1.6 0.8, 0	0.08, 0.03, 0.15, 0	0.03, 0.01, <0.01, 0	None, unless culturable at pH shift	n/a	n/a
ii	*S.c*	AWRI1631	87	228, 100, 3.5, 0	4.56, 2, 0.07, 0	1.46, 0.64 0.02, 0	None, unless culturable at pH shift	n/a	n/a

The long-term experiments (more than 400 days) with *B. bruxellensis* did not reveal a VBNC phenotype (Table 6.5.1). FACS analysis detected viable cells above 10^4 CFU/mL, supporting classical culture determinations. However, once populations of culturable cells fell below this level, the background influence of non-culturable cells produced a signal that has been interpreted as VBNC in the literature. The experiments designed to explore this potential of VBNC did not establish that this was occurring. While culturable *B. bruxellensis* was detected, it appears to be as a stochastic response, rather than VBNC, whereby, a very low number of viable cells was present (one replicate in both Experiments 1 and 2 and two replicates in Experiment 3) which slowly grew to a culturable level (Table 6.5.1, Appendix 5 Figures 1-3).

The detection limit for culturablity via classical plating and using FACS is clearly highlighted in Figure 6.5.1 (Experiment 4). Here only one replicate out of three at the low SO_2 level recovered, albeit slowly (over 49 days). The FACS and CFU data only aligned once culturable population exceeded 5 x 10^4 cells/mL (at approximately day 50).

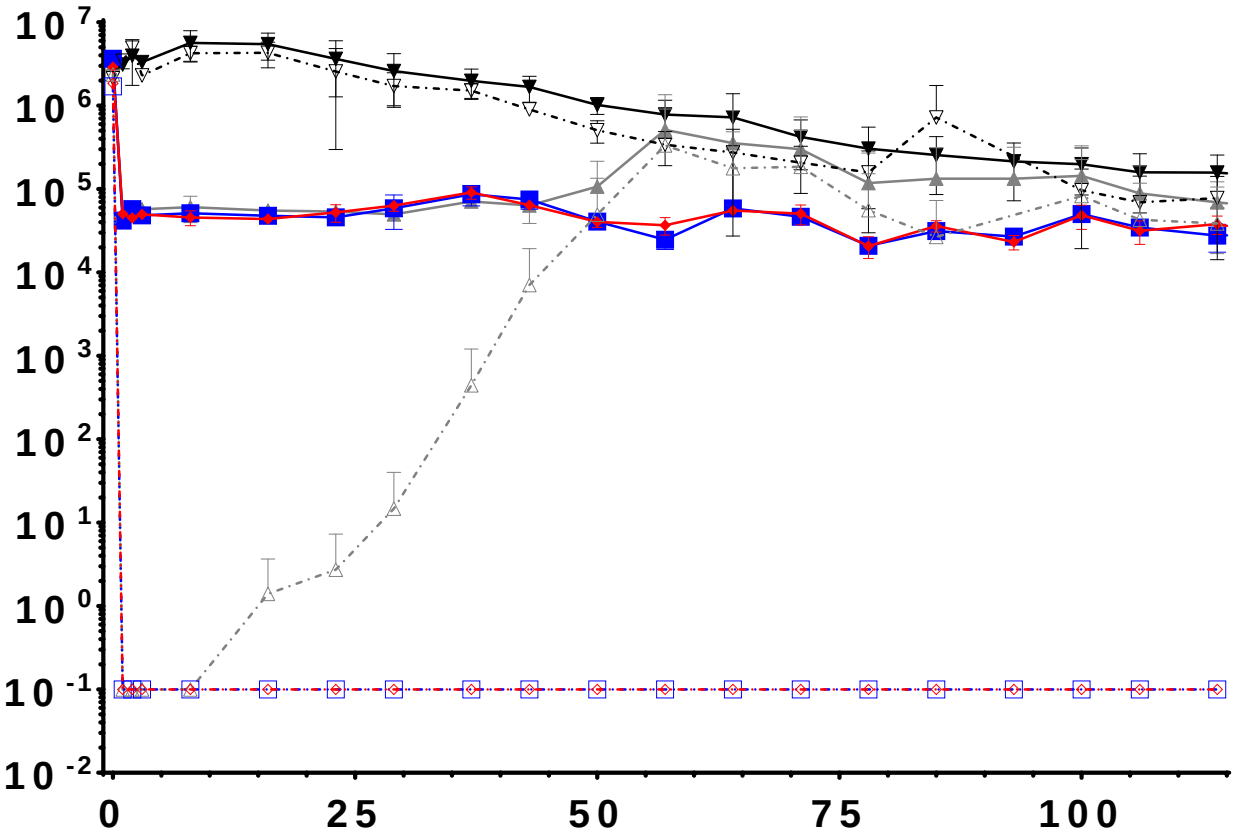


Figure 6.5.1. Experiment 4 *B. bruxellensis* AWRI1613 grown in WM: fSO_2 (mg/L) addition to lower levels at day 0 to ~24 ( ), ~15 ( ), 10 ( ) and untreated ( ), CFU values determined by plating (open symbols), solid symbols determined by FACS. Regrowth was observed in only the 10 mg/L culture after ~8 days. Plating again resolved lower levels of culturability than FACS.

Classical VBNC is displayed in microorganisms when a stress factor inducing the non-culturable phenotype is removed. Two studies have reported this previously (Salma et al. 2013, Serpaggi et al. 2012) for both *B. bruxellensis* and *S. cerevisiae* using sulfite addition and with pH shifting (pH 3.5 to pH 4.0) to reduce mSO_2 concentration to negligible levels. Attempts were made to replicate this approach. Using pH shifts, it was not possible to demonstrate an immediate return to a significant culturable population of either *B. bruxellensis* or *S. cerevisiae* (Appendix 5 Figure 5.5), further supporting that VBNC does not exist for these yeasts.

6.6. Investigation of alternative control and detection methods in *B. bruxellensis*

6.6.1. Alternative control methods

Several experiments were conducted to investigate the efficacy of two commercial products available in the Australian market which are reportedly alternatives for control of *B. bruxellensis*. These products were used as recommended by their manufacturer, using a range of *B. bruxellensis* strains (AWRI1499, AWRI 1613). Further, it was also investigated how effective these products were at limiting the growth of two sulfite-tolerant *B. bruxellensis* strains developed as part of this project (AWRI1499-38C, AWRI1613-13A). Figure 6.6.1 indicates a varied response to chitosan addition (further experiments are included in Appendix 5). Effectiveness of chitosan was demonstrated for AWRI1499 and its sulfite-tolerant clone AWRI1499-38C. AWRI1499 represents the most commonly isolated genotype of *B. bruxellensis* in Australian wineries. However, chitosan was not as effective against AWRI1613. Irrespective of which chitosan product was used, it was not able to restrict the growth of this strain. However, when applied to AWRI1613-13A, some variation was seen in response to the various products and concentration. This suggests that the effectiveness of chitosan may be strain dependent and that some chitosan products are not as effective as others. While these results suggest that chitosan has a fungicidal effect for AWRI1499, only a fungal-static effect was

demonstrated for AWRI1613, whereby populations initially reduced before recovering. If applied to wine and then racked immediately (within 1-2 days), chitosan would still reduce the AWRI1613 *B. bruxellensis* population.

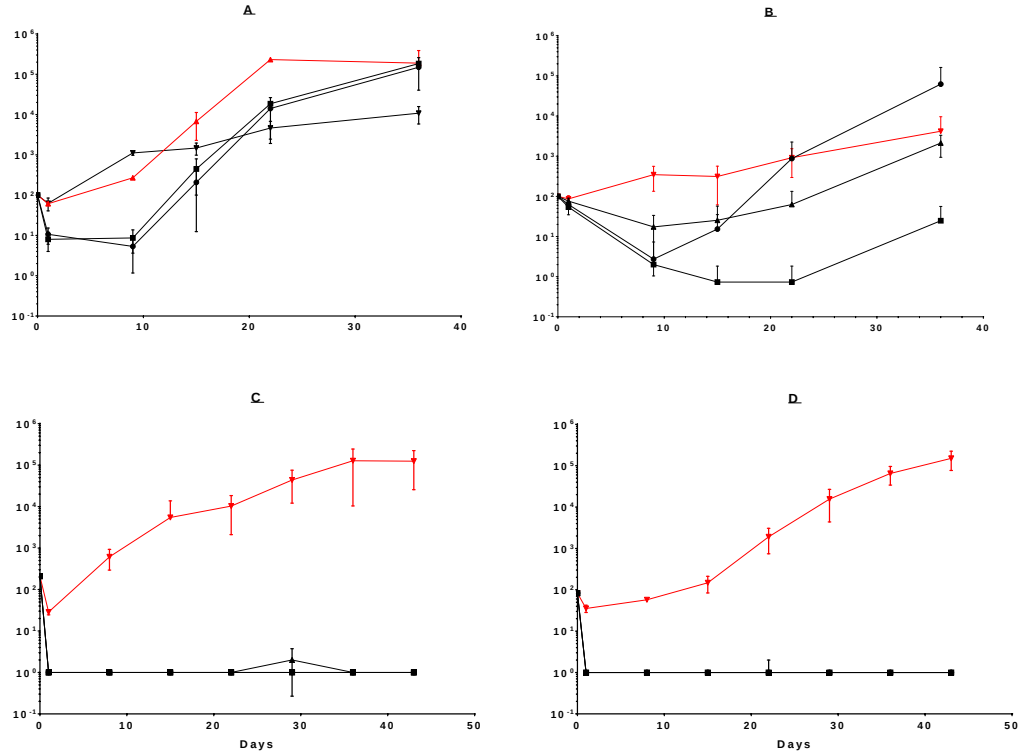


Figure 6.6.1. Chitosan treatments of *B. bruxellensis* strains. (A) AWRI1613 **(B)** AWRI1613-13A **(C)** AWRI1499 **(D)** AWRI1499-38C in 10 mL final of WM in glass COREX tubes, supplemented with commercial chitosan preparations in at 10^2 cells/mL
(No Chitosan ● OenoBRETT ■ NoBrettInside 8 g/hL ▲ NoBrettInside 4 g/hL ▼)

6.6.2. Alternative detection methods

Both flow cytometry and QPCR approaches were developed in an attempt to analyse both live and dead *B. bruxellensis* cells. While flow cytometry was an effective tool for monitoring and analysing live/dead *B. bruxellensis* populations, the limit below which interference occurred from dead or non-culturable cells was around 10^4 CFU/mL (Figure 6.6.1). This limit has been interpreted as VBNC by Salma et al. (2013) and Serpaggi et al. (2012).

The standard method for QPCR detection and quantification of *B. bruxellensis* was developed by Phister and Mills (2003). Since this publication, further progress in improving QPCR technology has occurred, improving its cost effectiveness. We applied this to develop new PCR primers, to improve sensitivity and cost-effectiveness. An accurate detection limit in wine was determined as 25 cell/100 mL (approximately 2.5 cells/mL) (see Appendix 5). The technology does allow detection of cells below this limit; however, a number of factors could potentially interfere or confound the results at very low cell densities.

When comparing the detection of live vs dead *B. bruxellensis* in WM, DNA extracted from cultures that had been heat killed (65ºC for 20 mins) was less amplifiable (approximately 22-fold less) than DNA extracted from cultures not heat-treated prior to DNA extraction (Table 6.6.1). Cell number for DNA extraction was normalised, such that it represented either a loss of DNA in the extraction method or a reduction in amplifiabilty of recovered DNA. Which of these possibilities represents the reason for this has not been determined; however, this may be of no significance, as dead cells are contributing to the QPCR response. This factor is more important than the cause of the reduced signal from dead cells. Furthermore, a QPCR signal with an absence of a culturable population does not represent VBNC, rather genomes from dead cells (which QPCR detects) contribute to the result.

Table 6.6.1. QPCR analysis of live vs dead *B. bruxellensis*

	Ct* (SD)	ΔCt (Log_2 vs Live)	Fold difference
Dead 100% (heat killed)	34.02 (+/- 0.3)	4.5	~22x
Mix (~50:50)	31.09 (+/-0.15)	1.59	~3x
Live 100%	29.49 (+/- 0.05)	0.00	0

*Ct (cycle threshold):-The number of PCR cycles required for the fluorescent signal to exceed background level.

6.7. Transformation of *B. bruxellensis*

6.7.1. Development of a set of high-efficiency transformation vectors

S. cerevisiae was the first yeast strain for which a genetic transformation protocol was developed. As such, this remains the yeast for which the majority of tools and vectors have been developed and there is now a large collection of selectable marker cassettes that can be used in this (and related) species. Accordingly, the original transformation protocols developed for *B. bruxellensis* made use of one of these pre-existing *S. cerevisiae*-specific cassettes; however, it was shown to being very inefficient and capable of producing transformants only when used at high concentrations in the transformation process.

Unlike many other yeast species in which the *S. cerevisiae* vectors have been shown to function with high efficiency, *B. bruxellensis* is relatively distantly related. Gene promoters that work in *S. cerevisiae* (and related species) are therefore likely to function less effectively in *B. bruxellensis* and it was hypothesised that this could be the cause of the problems experienced in the previous transformation attempts.

A highly expressed gene promoter was therefore sought from *B. bruxellensis* that could be used to drive the expression of the drug-resistant cassette. Previously obtained gene expression data for *B. bruxellensis* growing on standard YPD medium was interrogated for a promoter that provided high and consistent activity and the promoter of the *B. bruxellensis TDH1* gene was chosen to fit these requirements.

A set of DNA vectors was then created in which the *S. cerevisiae*-specific promoter (Ag_TEF2p) was exchanged with the *TDH1* promoter (*TDH1p*), such that this new promoter would drive the expression of three different drug-resistant genes, KanMX, NatMX and hygMX, encoding resistance to G418, CloNAT and hygromycin, respectively (Goldstein and McCusker 1999).

6.7.2. Functional confirmation of multiple drug-resistant markers in *B. bruxellensis*

To confirm the function of the high-efficiency transformation vectors reported in Section 6.7.1, electroporation-based genetic transformation was performed according to the protocol developed by Miklenič et al. (2015). *B. bruxellensis* strain AWRI2804 was transformed with both standard yeast (Ag_TEF2p) and *B. bruxellensis*-specific (Db_TDH1p) versions of the KanMX, NatMX and hygMX vectors, with transformation efficiency determined through plating on the relevant selective media for each resistance cassette, G418, CloNAT and hygromycin, respectively (Figure 6.7.1).

The original study by Miklenič et al. (2015) made use of the standard yeast KanMX vector and was shown to produce ~3 x 10^3 transformants per microgram of DNA. Lower results were found using standard KanMX vector in this study (5.5 x 10^2 transformants per microgram DNA); however, a 10-fold increase in transformation efficiency was observed when using the *Brettanomyces*-specific promoter developed in this study. Furthermore, while the hygMX vectors produced similar results to their

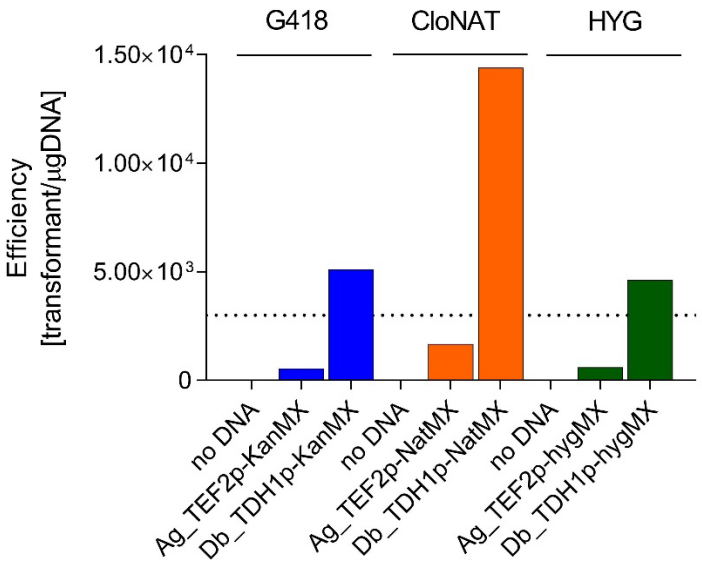


Figure 6.7.1. Functional confirmation of drug-resistant markers in *B. bruxellensis*. *B. bruxellensis* strain AWRI2804 was transformed with resistance cassettes for the drugs G418 (KanMX), CloNAT (NatMX) or Hygromycin (HYG; hygMX) expressed using either a standard yeast promoter (Ag_TEF2p) or a *Brettanomyces*-specific promoter (Db_TDH1p). The number of transformants obtained per microgram of DNA for each construct is shown.

KanMX counterparts, the NatMX-series vectors were observed to be 300% more efficient than either of the other markers, producing approximately 1.5 x 10^4 transformants per microgram of DNA. This higher efficiency combined with multiple transformation markers unlocks molecular applications such as strain tagging (See Section 6.7.4), insertional mutagenesis and potentially targeted gene deletion (See Section 6.7.3), which were not available, or practical using the original technique.

6.7.3. Targeted gene knockout in *B. bruxellensis*

The use of genetic transformation for performing targeted gene deletion relies on homologous recombination to integrate a selectable marker at a precise genomic location. This location is specified by flanking DNA sequences that match those sites in the genome where integration is to take place. A subset of yeasts, such as those of the *Saccharomyces* group, preferentially make use of homologous recombination, such that targeted genomic integration can be performed at very high efficiency using very short-flanking DNA (40 – 50 bp) (Wach et al. 1994). However, the majority of other eukaryotes (including *B. bruxellensis*) primarily use NHEJ processes to integrate transformed DNA. In these situations, the DNA is inserted into the genome at random locations and homologous recombination occurs very rarely. In these situations, long-flanking DNA (1000 – 2000 bp) can increase the rate of homologous versus non-homologous recombination; however, these events still typically occur at <1% of the total transformants obtained.

This very low homologous integration efficiency limited the broader applicability of targeted gene deletion and led to investigations to improve this process in species that did not favour homologous integration. This led to the discovery that disruption of key members of the NHEJ cellular machinery (Ku proteins) significantly shifted the balance towards homologous integration (Cen et al. 2015, Goins et al. 2006, Koh et al. 2014, Nayak et al. 2006).

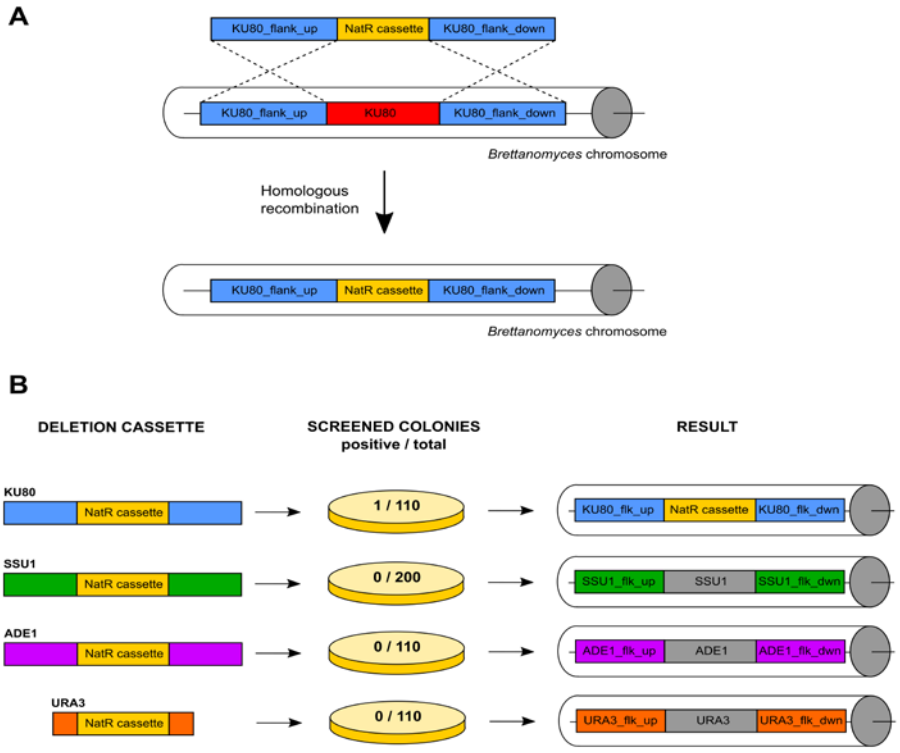


Figure 6.7.2 Homologous recombination-mediated gene deletion in *B. bruxellensis*. (A) To delete the KU80 gene, a vector was used that fused genomic DNA sequences adjacent to the KU80 open reading frame to the NatR resistance gene cassette. Homologous recombination between the vector flanking regions and the genome deletes the KU80 gene from the genome, replacing it with NatR. **(B)** Four deletion cassettes were assembled to test homologous gene deletion. Up to 200 colonies were screened by PCR for correct integration.

So as to produce a homologous-recombining *B. bruxellensis* strain, the *KU80* gene was targeted for gene deletion using low-efficiency, long-flanking targeting. A construct was created in which 1.2 kb of flanking DNA was placed either side of the Db_TDH1p-NatMX selectable marker. This construct was transformed into AWRI2804, with transformants selected on CloNAT (Figure 6.7.2A).

PCR-based screening was performed on 110 transformants to determine if homologous recombination had exchanged the *KU80* gene with the NatMX marker (Figure 6.7.2). This would be expected to disrupt non-homologous recombination in this strain and produce *S. cerevisiae*-like genomic integration for subsequent gene deletions. Of those transformants screened, only one isolate produced a PCR result that was consistent with the replacement of the *KU80* locus with NatMX (Δ*KU80*). Whole genome sequencing confirmed this result, with genomic analysis indicating that two copies of the Db_TDH1p-NatMX cassette had integrated into the *KU80* locus in a tandem array, with no other copies of the cassette detectable in the genome.

To assess the suitability of the Δ*KU80* strain as a platform for homologous recombination, this strain was transformed with several DNA cassettes that targeted four different genomic loci (Figure 6.7.2). Despite targeting four different genomic loci and screening over 100 colonies for each locus, it was not possible to detect homologous recombination at any of the targeted loci, either by PCR or using phenotypic tests for those loci with clear auxotrophic markers (*ADE1* and *URA3*, data not shown).

Disruption of the NHEJ system does not appear to be a viable option for the development of homologous recombination in *B. bruxellensis*. Further work is therefore required to investigate additional genetic means to increase the rate of homologous recombination in this system. Recently the CRISPR/Cas9 system has emerged as an effective methodology for accomplishing this task (Cen et al. 2017, Krappmann 2017, Numamoto et al. 2017). Development of this system for engineering of the *B. bruxellensis* genome would be the logical next step.

6.7.4. Application of drug-resistant markers for competition experiments in *B. bruxellensis*

The utility of the multiple selectable marker system was explored through its application in performing pairwise competitive growth experiments between diverse *B. bruxellensis* strains. Previous work has shown that while strains such as AWRI1499 demonstrate far higher sulfite tolerance, they generally grow at a far slower rate than strains such as AWRI1626, in the absence of this compound. As both types of strains are readily isolated from wine, this then leads to questions regarding competition in a wine environment between strains with opposing strengths and weaknesses.

In order to begin to investigate the dynamics of this competition, genetic transformation was used to 'tag' three different strains of *B. bruxellensis*, AWRI1499 AWRI1608 and AWRI1613. Each parent strain was separately tagged with either the NatMX or KanMX drug-resistance cassettes. As DNA integration in *B. bruxellensis* is untargeted, three individual transformants were chosen for each marker, with each transformant also being screened to ensure that the integration of the marker gene had not resulted in any negative phenotypic impacts relative to the parental strain.

Once a set of six tagged isolates had been established for each strain (3x NatMX, 3x KanMX), pairwise competition experiments were performed between isolates from two different parental strains, with parental origin indicated by the KanMX or NatMX marker. This enabled the enumeration of cells during the competitive growth phase by plating on the relevant selective media (CloNAT for NatMX and G418 for KanMX) (Figure 6.7.3). These competition experiments clearly showed that AWRI1613 was able to outcompete AWRI1499 when sulfite concentrations were below 0.4 mg/L; however, AWRI1499 was shown to dominate at higher sulfite levels. A similar situation was observed between AWRI1499 and AWRI1608, although AWRI1499 could only outcompete AWRI1608 at high concentrations of sulfite (0.6 mg/L). Interestingly, even at these high sulfite concentrations, the populations displayed significant variation in the proportions of both strains, suggesting that the competitive advantage afforded to AWRI1499 over AWRI1608 under these conditions was relatively minor.

These results highlight the important balances that exist between strains with very different phenotypes; mixed populations of *B. bruxellensis* (such as those expected to be encountered in a winery situation) will, therefore, adapt to a particular environment through selecting for 'fit' genotypes within the population that are then able to expand their abundance.

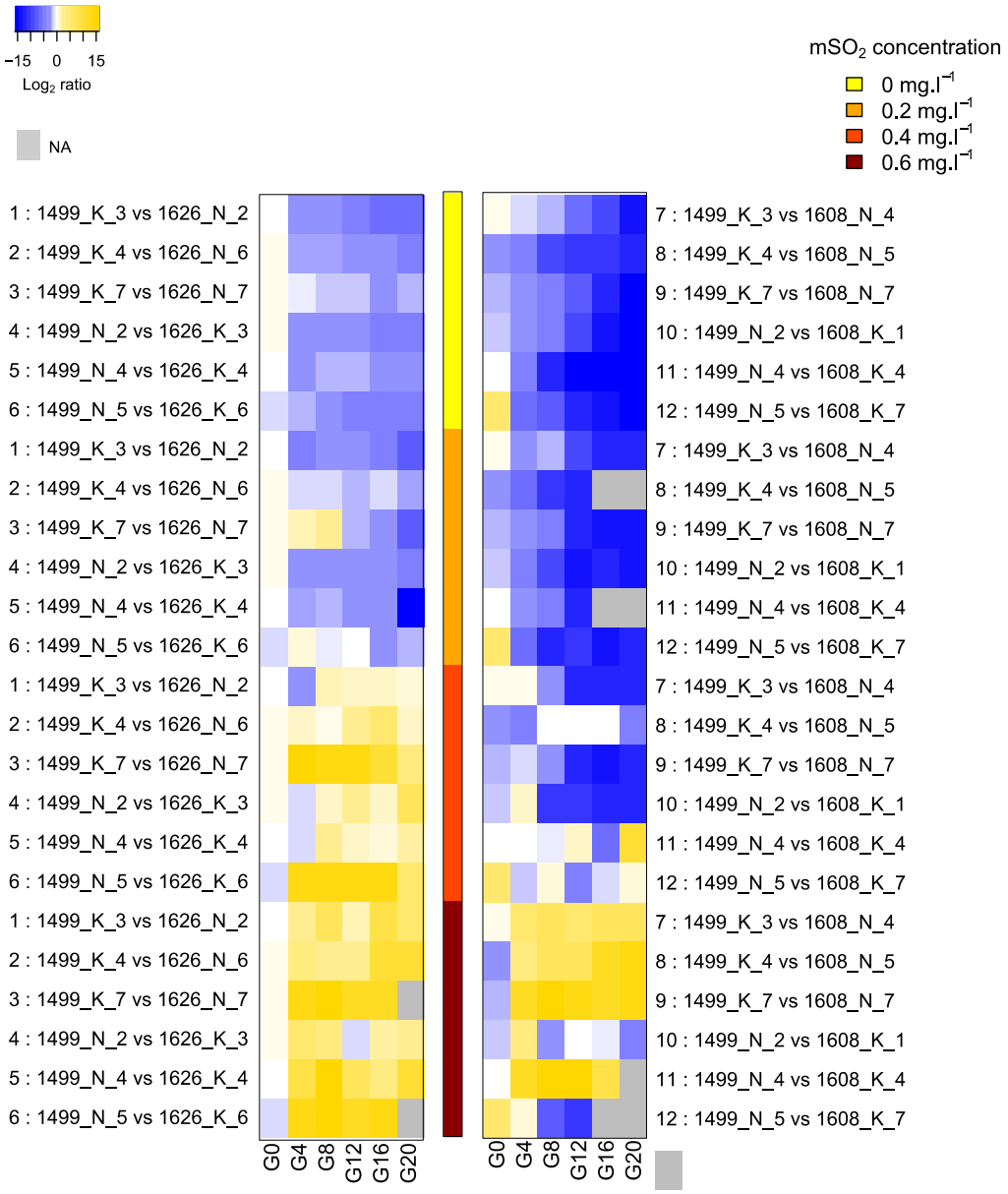


Figure 6.7.3. Competitive growth measurements under sulfite stress. Transformants for three *B. bruxellensis* strains (AWRI1499, AWRI1608 and AWRI1626) were assessed by pairwise competition experiments (AWRI1499 vs AWRI1626, and AWRI1499 vs AWRI1608) under different molecular SO_2 concentrations. N indicates transformants containing the NatMX cassette, whereas K denotes transformants carrying the KanMX cassette. Colours indicate the $\log_2$ of the ratio between KanMX and NatMX transformant cell numbers.

7. Outcome/conclusion:

7.1. Performance against planned output

7.1.1. Develop knowledge of mechanisms that enable *Brettanomyces* to adapt to environmental conditions, particularly in response to sulfite stress

Adaptive evolution experiments conclusively demonstrated that *Brettanomyces* strains have the capacity to evolve greater tolerance to sulfite, though the extent to which this is possible is dependent upon genetic makeup. Accordingly, a sensitive strain may become more tolerant, but it is unlikely to surpass a strain that already expresses a high level of tolerance, when both are exposed to the same selective conditions. This suggests that experimentally evolved tolerance is occurring through mechanisms independent of those responsible for differences between original strains.

Two parallel approaches were taken to generate specific knowledge of these mechanisms. The first approach focused upon the membrane protein Ssu1p, shown in other fungal species to be central to sulfite tolerance. Different versions of this protein can be found in sensitive and tolerant strains, and their performance was characterized through heterologous expression in *S. cerevisiae*. This demonstrated that Ssu1p sequence and copy number represent one mechanism that may explain *Brettanomyces'* variable sulfite tolerance.

The second approach employed genome sequencing to systematically compare the genetic composition of *Brettanomyces* strains before and after laboratory evolution. While this data revealed a range of genetic changes, they were not found to be consistent across tolerant populations. In the absence of genetic modification tools for *Brettanomyces* this type of result does not allow for clear conclusions to be drawn, though it is interesting as it suggests there may be several mechanisms that contribute to sulfite tolerance of this species.

7.1.2. Develop molecular diagnostics and apply them to new industry isolates as an early warning system for emergence of sulfite tolerant strains

While no consistent patterns were observed across the genomes of sulfite-tolerant populations when compared to sulfite-sensitive strains, the data generated in this project linking certain Ssu1p sequences to their efficacy as sulfite pumps provides one potential marker for use in molecular diagnostics.

As an adjunct to this output the throughput of physiological screening for sulfite tolerance was refined and increased, and the method then applied to laboratory-evolved populations and new industry isolates. A pilot sampling from two wineries revealed that isolates from 2016-2017 were on average significantly more sulfite-tolerant than isolates obtained during earlier studies (2000-2014).

7.1.3. Determine whether *Brettanomyces* enters a VBNC state, through rigorous experimentation that meets criteria previously described for VBNC bacteria

Long-term experiments (in excess of 400 days) were monitored for resumption of *Brettanomyces* growth following sulfite treatment. In addition, pH shift experiments in which mSO_2 stress was removed for both *Brettanomyces* and *Saccharomyces* also failed to demonstrate that VBNC can occur in these yeasts. Multiple rigorous experiments were undertaken, using various genera and strains. Specific experiments that had been previously published were also replicated; however, the reports of VBNC in the literature were not reproducible. This calls into question the concept of VBNC in yeasts. The classical VBNC response in bacteria is a rapid return to culturabilty of large populations, so rapid that it can cannot be explained by vegetative growth. This phenomenon was not observed in any experiment conducted.

7.1.4. If VBNC can be demonstrated, generate knowledge of what wine relevant conditions trigger this state, and under what conditions *Brettanomyces* exits its VBNC state and resumes growth

While the results in this project did not support existence of VBNC *Brettanomyces*, nevertheless work performed elsewhere was repeated that had shown recovery of culturability following a pH shift to relieve sulfite stress. The only samples in which this 'recovery' was observed contained already culturable cells; hence, this can be explained by normal vegetative growth rather than entry and exit from VBNC status.

7.1.5. Develop new, or improve existing, rapid tests (QPCR or flow cytometry) that enable accurate assessment of spoilage risk

This project has established that flow cytometry is an accurate and rapid method for the determination of viable *B. bruxellensis* populations. It compared well with classical microbiological testing relying on viable plating above a threshold of 10^4 cfu/mL. This technology, while requiring modest capital expenditure, represents a suitable and adaptable platform for rapid microbiological testing in winery laboratories; however, it cannot be used on its own for *B. bruxellensis* detection and quantification, particularly at very low cell densities. The most promising detection method for *B. bruxellensis* is QPCR. Here an updated version was developed of the most common QPCR method applied in winery laboratories for *B. bruxellensis* detection. This method could detect *B. bruxellensis* to 50 cell/mL. It was also shown that dead cells (at 10^4 cells/mL) were detectable, but not differentiated clearly from live cells, suggesting that using QPCR technology could potentially lead to a false positive determination of the presence of viable *B. bruxellensis*. Detection of viable *B. bruxellensis* might result in winery intervention, which in some cases could be unnecessary and costly. Further work is required to determine a more accurate detection and estimation method for viable *B. bruxellensis* using a QPCR approach.

7.1.6. Investigate alternatives to sulfite for control of *Brettanomyces* during wine maturation

The fungicidal effectiveness of various commercial chitosan preparations on several strains of *B. bruxellensis* was determined. Chitosan was effective in reducing growing populations of AWRI1499, the most common Australian genotype of *B. bruxellensis* and importantly, a sulfite-resistant evolved clone of this strain as well. However, while chitosan was effective in reducing culturable cell numbers of AWRI1613 (and an evolved clone), this yeast was not as sensitive. This suggests that chitosan may not always be an effective tool for winemakers to control the growth of *B. bruxellensis.* Further work is required to investigate why this might be the case and to investigate other alternatives to sulfite.

7.1.7. Provide Australian winemakers with an updated *Brettanomyces* control strategy

The results from this project, while reinforcing the potential limitations of the existing *Brettanomyces* control strategy, are not sufficiently developed to underpin an updated strategy. Specifically, more work is required to determine the extent to which strain variation in response to sulfite alternatives (such as chitosan) might limit their efficacy. With this knowledge, an amended control strategy could be designed that involves reduced used of sulfite, but only when supported by alternatives that provide additional growth barriers to ensure spoilage does not occur. In this way, the selective pressure on *Brettanomyces* in the field can be minimised, in turn reducing the risk of new sulfite-tolerant strains emerging.

7.2. Could changing the methodology/technology have improved the outcome?

While continuous culturing is generally the best methodology to apply towards directed evolution, the batch cultures could have been implemented simultaneously during a scoping experiment to determine which experimental set-up was most suited to sulfite-based selection, given the difficulties that were encountered when using sulfite under continuous selection.

Implementation of the Pool-seq strategy would have ideally been performed once the population phenotypes had stabilised. However, within the time constraints of the project, it was necessary to proceed with sequencing in order to identify genomic regions as potential markers for evolution of sulfite tolerance.

7.3. Practical implications

While the importance of sulfite in the control of *Brettanomyces* is well known to industry, any trend towards production of higher pH wines with limited amounts of sulfite could be detrimental to the long-term control of *B. bruxellensis,* as it provides the ideal conditions for the development of sulfite tolerance.

Reported instances of VBNC *Brettanomyces* are likely to represent false-positive errors in molecular tests that do not distinguish live versus dead cells. A wine that produces a high positive molecular test, but which is negative for plating may therefore simply be due to the presence of dead *Brettanomyces* cells (which still contain intact DNA).

7.4. Benefits to the industry

A high throughput sulfite tolerance assay has been developed. This assay can be used to rapidly assess the sulfite tolerance of *Brettanomyces* strains that are isolated from wine. This assay could therefore be used by Australian wineries to assess the sulfite resistance status of *Brettanomyces* strains isolated from their wines. Knowledge about the industry-wide occurrence of sulfite-resistant *Brettanomyces* provides a foundation for the potential refinement of best-practice sulfite control strategies. Information on the effectiveness and mode-of-action of alternative control methods, such as chitosan, provides winemakers with additional means to control *Brettanomyces*.

The establishment of a high-efficiency genetic transformation system in *B. bruxellensis* unlocks numerous molecular-genetic techniques that can be used to study the biology of this important spoilage yeast and to find ways to control its effects.

An understanding of the limitations of current molecular tests for live versus dead *B. bruxellensis* cells has been gained. This can be used in combination with plating results to allow wineries to decide if expensive *Brettanomyces* remediation steps (sterile filtration etc.) are required when a positive molecular test is obtained.

7.5. Recommendations

The results of the preliminary screening of current industry isolates presents data to suggest that levels of sulfite resistance may be increasing. As this current data presents findings from only two wineries, it is recommended that further isolates be sourced from a wide range of Australian wineries to determine if this is a generalised phenomenon. Furthermore, an ongoing general surveillance program should be established to ensure that this is monitored into the future.

Further passaging of the long-term sulfite evolution cultures should be pursued in order to determine if genetic homogeneity can be reached. This will then provide a solid framework for genetic studies which, combined with information from new, sulfite-tolerant industry isolates, may provide the means to establish early-warning molecular tests. An initial target may be the *SSU1* haplotype(s), although further work is required to establish the broader correlation between *SSU1* allele, copy number and level of sulfite resistance in a larger cohort of strains.

Additional work should be carried out on molecular tests that can distinguish live and dead *Brettanomyces* cells.

Appendix 1: Communication

Communication of the outcomes

Outcomes and knowledge generated during the progress of this project have been communicated to peers through peer-reviewed publications and conference presentations.

Information has been extended to industry stakeholders through articles in trade journals, presentations that form part of the AWRI roadshow program, webinars and workshops at both 2013 and 2016 Australian Wine Industry Technical Conferences.

Journal articles written during project

Borneman, A. R., Zeppel, R., Chambers, P. J., & Curtin, C. (2014). Insights into the *Dekkera bruxellensis* genomic landscape: comparative genomics reveals variations in ploidy and nutrient utilisation potential amongst wine isolates. *PLoS Genetics*, *10*(2), e1004161. http://doi.org/10.1371/journal.pgen.1004161.s014

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Appendix 2: Intellectual Property

Various new *Brettanomyces* strains were isolated or generated during the course of this project. A set of high-efficiency transformation vectors and a homologous recombination protocol to integrate a selectable marker at a precise genomic location were generated during the course of this project.

Appendix 3: References

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Appendix 4: Staff

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Appendix 5: Additional data

The information in this section is provided in confidence, not for publication.

Brettanomyces strains using in genome sequencing study (section 6.2)

AWRI number	Source collection strain names*	Source of isolation
1103	CBS5512	Brewery equipment, South Africa
1130	CBS6055	Ginger ale, USA
1499		Red wine, Mudgee, Australia
1546	CBS74	Beer, Belgium
1605		Red wine, Yarra Valley, Australia
1606		Red wine, Swan Valley, Australia
1607		Red wine, Murchison, Australia
1608		Red wine, Margaret River, Australia
1609		Red wine, Margaret River, Australia
1613		Red wine, Barossa Valley, Australia
1615		Red wine, Mornington Peninsula, Australia
1626	CBS2499	Wine, France
2800	UCD615	Wine, USA
2801	UCD738	Wine, USA
2802	UCD752	Wine, France
2803	UCD2030	Red wine, USA
2804	UCD2041	Fruit wine, Thailand
2805	UCD2049	Red wine, New Zealand
2806	UCD2050	White wine, New Zealand
2807	UCD2053	Red wine, USA
2808	UCD2054	Red wine, USA
2809	UCD2060	Red wine, Chile
2811	UCD2076	Wine, Canada
2812	UCD2077	Red wine, Chile
2813	UCD2082	Wine, USA
2814	UCD2091	Wine, France
2815	UCD2093	Wine, USA
2816	UCD2397	Wine, Portugal
2817	UCD2399	Wine, Portugal
2818	UCD2485	Red wine, USA
2819	UCD2493	Wine, USA
2820	UCD2494	White wine, USA
2821	UCD2504	Wine, South Africa

2822	UCD2506	Wine, South Africa
2823	UCD2841	Kombucha tea
2836	L0505	Wine, Bordeaux, France
2837	L0516	Wine, Bordeaux, France
2838	L0611	Wine, Bordeaux, France
2839	L0620	Wine, Bordeaux, France
2841	L0463	Wine, Bordeaux, France

*CBS = Centralbureau von Schimmelcultures collection, UCD = University of California Davis, L = Universite de Bordeaux

Brettanomyces industry isolates used for sulfite tolerance screening (section 6.3)

Period	Strain	Details
2000-2004	AWRI 1499	McLaren Vale 2002
2000-2004	AWRI 1603	Mudgee Shiraz 1999
2000-2004	AWRI 1604	Mudgee Shiraz 1999
2000-2004	AWRI 1605	Yarra Valley 2002
2000-2004	AWRI 1606	Shiraz, Swan Valley 2002
2000-2004	AWRI 1607	Cabernet Sauvignon, Victoria, 2003
2000-2004	AWRI 1608	Merlot, Margaret River, 2001
2000-2004	AWRI 1609	Shiraz, Margaret River, 2003
2000-2004	AWRI 1610	Queensland, 2003
2000-2004	AWRI 1611	Cabernet Sauvignon, Coonawarra, 2003
2000-2004	AWRI 1612	Cabernet Sauvignon, Coonawarra, 2003
2000-2004	AWRI 1613	South-East Australia
2000-2004	AWRI 1614	Cabernet Franc, 2004
2000-2004	AWRI 1615	Pinot Noir, 2003
2000-2004	AWRI 1643	Mudgee, Shiraz 1999
2000-2004	AWRI 1644	McLaren Vale
2000-2004	AWRI 1645	McLaren Vale
2000-2004	AWRI 1646	Yarra Valley
2000-2004	AWRI 1647	2001 Cabernet Merlot, Adelaide Hills
2000-2004	AWRI1648	Barossa Valley
2000-2004	AWRI1649	Rutherglen 2001 Shiraz
2000-2004	AWRI1650	Strathbogie Ranges 2002 Shiraz
2000-2004	AWRI1651	Tasmania 2002 Pinot Noir
2000-2004	AWRI1652	Margaret River 2002 Shiraz
2000-2004	AWRI1653	Queensland
2000-2004	AWRI1654	McLaren Vale Grenache

2000-2004	AWRI1670	Yarra Valley
2000-2004	AWRI1671	Wine isolate
2000-2004	AWRI1672	Wine isolate
2000-2004	AWRI1673	Wine isolate
2000-2004	AWRI1674	Wine isolate
2000-2004	AWRI1675	Wine isolate
2000-2004	AWRI1676	Wine isolate
2000-2004	AWRI1677	Wine isolate
2000-2004	AWRI1678	Wine isolate
2000-2004	AWRI1679	Wine isolate
2000-2004	AWRI1680	Wine isolate
2000-2004	AWRI1681	Wine isolate
2010-2014	AWRI1448	Bendigo, Victoria
2010-2014	AWRI1455	Granite Belt, Stanthorpe, Qld
2010-2014	AWRI1457	Cabernet Sauvignon
2010-2014	AWRI1458	Cabernet Sauvignon
2010-2014	AWRI1459	King Valley, Victoria
2010-2014	AWRI1460	Adelaide Plains, SA
2010-2014	AWRI1877	Wine isolate
2010-2014	AWRI1878	Wine isolate
2010-2014	AWRI1879	Wine isolate
2010-2014	AWRI1880	Wine isolate
2010-2014	AWRI1881	Wine isolate
2010-2014	AWRI1882	Wine isolate
2010-2014	AWRI1883	Wine isolate
2010-2014	AWRI1884	Wine isolate
2010-2014	AWRI1885	Wine isolate
2010-2014	AWRI1886	Wine isolate
2010-2014	AWRI1888	Wine isolate
2010-2014	AWRI1889	Wine isolate
2010-2014	AWRI1890	Wine isolate
2010-2014	AWRI1891	Wine isolate
2010-2014	AWRI1892	Wine isolate
2010-2014	AWRI1893	Wine isolate
2010-2014	AWRI1894	Wine isolate
2010-2014	AWRI2261	Wine isolate
2010-2014	AWRI2262	Wine isolate
2010-2014	AWRI2263	Wine isolate
2010-2014	AWRI2747	Barossa Valley

2010-2014	AWRI2748	Barossa Valley
2010-2014	AWRI2842	Coonawarra
2010-2014	AWRI2843	Coonawarra
2010-2014	AWRI2844	Wine isolate
2010-2014	AWRI2915	Wine isolate
2010-2014	AWRI2916	Wine isolate
2010-2014	AWRI2917	Wine isolate
2010-2014	AWRI2919	Wine isolate
2010-2014	AWRI2971	Wine isolate
2010-2014	AWRI2972	Wine isolate
2010-2014	AWRI2973	Barossa Valley
2010-2014	AWRI2974	Barossa Valley
2010-2014	AWRI3106	Bendigo, Victoria
2010-2014	AWRI3107	Bendigo, Victoria
2010-2014	AWRI3108	Bendigo, Victoria
2016-2017	AWRI 3927	838 B Barossa Valley Cabernet V16
2016-2017	AWRI 3928	838 B Barossa Valley Cabernet V16
2016-2017	AWRI 3929	838 B Barossa Valley Cabernet V16
2016-2017	AWRI 3930	838 B Barossa Valley Cabernet V16
2016-2017	AWRI 3931	838 B Barossa Valley Cabernet V16
2016-2017	AWRI 3932	838 B Barossa Valley Cabernet V16
2016-2017	AWRI 3933	903 B V16 Langhorne Creek Cabernet
2016-2017	AWRI 3934	903 B V16 Langhorne Creek Cabernet
2016-2017	AWRI 3935	903 B V16 Langhorne Creek Cabernet
2016-2017	AWRI 3936	646 B V17 McLaren Vale Shiraz
2016-2017	AWRI 3937	646 B V17 McLaren Vale Shiraz
2016-2017	AWRI 3938	646 B V17 McLaren Vale Shiraz
2016-2017	AWRI 3939	MRJ 2912 7824 26/6
2016-2017	AWRI 3940	MRJ 2912 7824 26/6
2016-2017	AWRI 3941	MRJ 2912 7824 26/6
2016-2017	AWRI 3942	829 B V16 McLaren Vale Shiraz
2016-2017	AWRI 3943	829 B V16 McLaren Vale Shiraz
2016-2017	AWRI 3944	829 B V16 McLaren Vale Shiraz
2016-2017	AWRI 3945	829 B V16 McLaren Vale Shiraz
2016-2017	AWRI 3946	829 B V16 McLaren Vale Shiraz
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2016-2017	AWRI 3950	829 B V16 McLaren Vale Shiraz

2016-2017	AWRI 3953	863 B Barossa Valley Shiraz V16
2016-2017	AWRI 3954	863 B Barossa Valley Shiraz V16
2016-2017	AWRI 3955	863 B Barossa Valley Shiraz V16
2016-2017	AWRI 3956	863 B Barossa Valley Shiraz V16
2016-2017	AWRI 3957	519 B V16 Barossa Valley Shiraz
2016-2017	AWRI 3958	519 B V16 Barossa Valley Shiraz
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2016-2017	AWRI 3966	519 B V16 Barossa Valley Shiraz
2016-2017	AWRI 3967	990 B V16 Adelaide Hills Cabernet
2016-2017	AWRI 3968	990 B V16 Adelaide Hills Cabernet
2016-2017	AWRI 3969	990 B V16 Adelaide Hills Cabernet
2016-2017	AWRI 3970	990 B V16 Adelaide Hills Cabernet
2016-2017	AWRI 3971	990 B V16 Adelaide Hills Cabernet
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2016-2017	AWRI 3976	990 B V16 Adelaide Hills Cabernet
2016-2017	AWRI 3977	497 B V16 McLaren Vale Shiraz
2016-2017	AWRI 3978	497 B V16 McLaren Vale Shiraz
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2016-2017	AWRI 3981	497 B V16 McLaren Vale Shiraz
2016-2017	AWRI 3982	497 B V16 McLaren Vale Shiraz
2016-2017	AWRI 3983	497 B V16 McLaren Vale Shiraz
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2016-2017	AWRI 3985	497 B V16 McLaren Vale Shiraz
2016-2017	AWRI 3986	497 B V16 McLaren Vale Shiraz
2016-2017	AWRI 3987	497 B V16 McLaren Vale Shiraz
2016-2017	AWRI 3988	497 B V16 McLaren Vale Shiraz
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2016-2017	AWRI 3991	V17 BV Cabernet Sauvignon
2016-2017	AWRI 3992	V17 BV Cabernet Sauvignon
2016-2017	AWRI 3993	V17 BV Cabernet Sauvignon
2016-2017	AWRI 3994	V16 Clare Valley Cabernet Merlot
2016-2017	AWRI 3995	V16 Clare Valley Cabernet Merlot
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2016-2017	AWRI 3998	V16 Clare Valley Cabernet Merlot
2016-2017	AWRI 3999	V16 Clare Valley Cabernet Merlot
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2016-2017	AWRI 4006	V17 BV Shiraz
2016-2017	AWRI 4007	V17 BV Shiraz
2016-2017	AWRI 4008	V17 BV Shiraz
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2016-2017	AWRI 4011	V17 BV Shiraz
2016-2017	AWRI 4012	V16 MV Shiraz
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2016-2017	AWRI 4024	2015 MV Shiraz

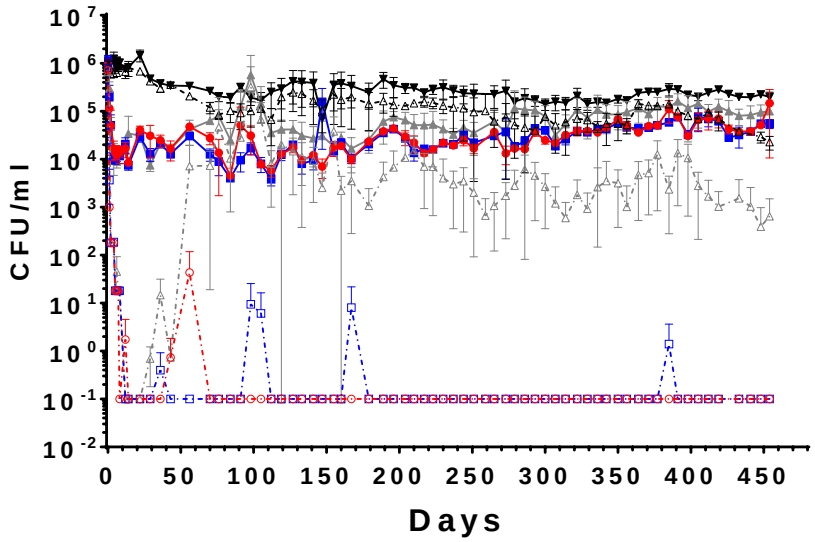


Figure Appendix 5.1. Expt 1: *B. bruxellensis* AWRI1499 grown in WM. fSO_2 addition at day 0 to:- ~55 (⊖), ~30 (⊟), 10mg/L (⧋) and Untreated (▽), CFU values determined by plating, solid symbols determined by FACS. Regrowth seen by plating observed without pH shift and at levels below those resolved by FACS even after 450 days.

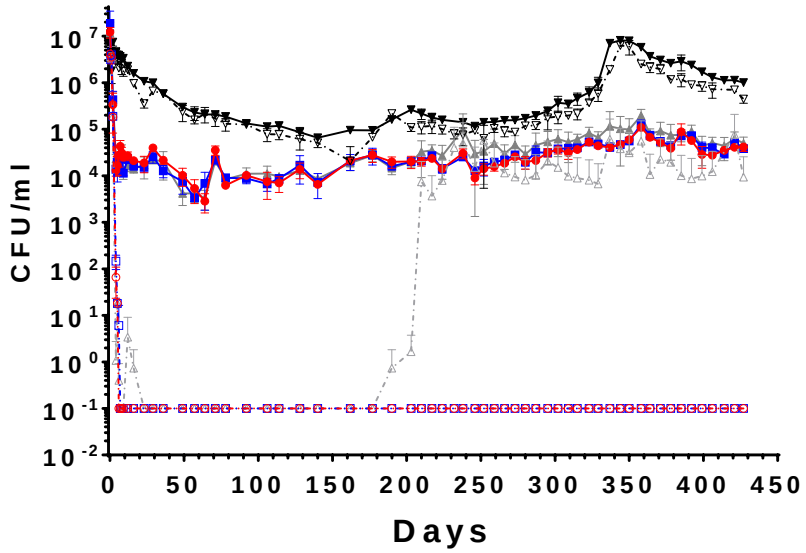


Figure Appendix 5.2. Expt 2 *B. bruxellensis* AWRI1613 grown in WM. fSO_2 addition at day 0 to: ~70 (⊖), ~45 (⊟), 25mg/L (⧋) and untreated (▽), CFU values determined by plating, solid symbols determined by FACS. Regrowth seen in low fSO_2 sample after ~180 days, once again plating was able to discern lower levels of culturability than FACS. Air intrusion (~330 days) led to a significant outgrowth response in cultures, but no recovery of culturability to samples treated with SO_2.

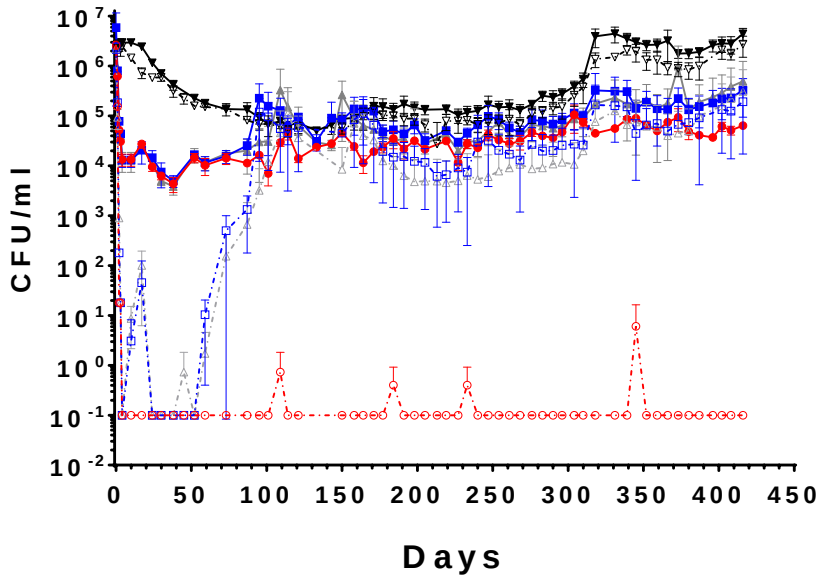


Figure Append 5.3. Expt 3 *B. bruxellensis* AWRI1613 grown in WM. fSO_2 addition to lower levels at day 0 to: ~24 (⊖), ~15 (⊟), 10 mg/L (⧋)and untreated, CFU values determined by plating, solid symbols determined by FACS. Regrowth seen in 24 and 10 mg/L cultures after ~52 days. Plating again resolved lower levels of culturability than FACS. Air intrusion (~310 days) led to a significant outgrowth response in cultures already growing, but no recovery of culturability to samples treated with SO_2 that were dormant/dead.

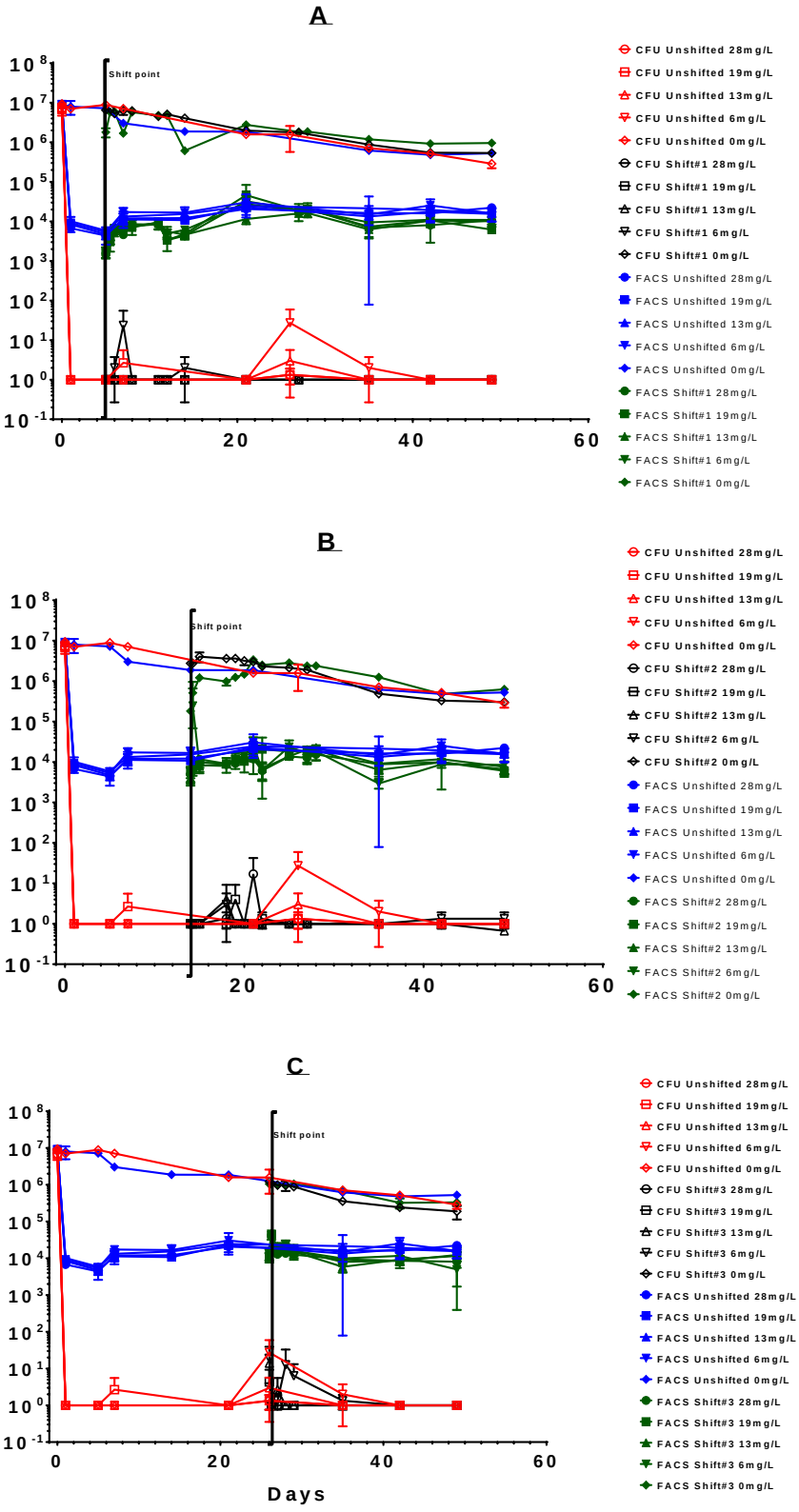


Figure Appendix 5.4. Expt 5 *B.bruxellensis* AWRI1613 grown in WM. fSO_2 addition at day 0 to: ~24 ( ), ~19 ( ), 13 ( ), 6 ( ) and untreated ( ) CFU values determined by plating (Red un-shifted, Black pH shifted), solid symbols determined by FACS (Blue un-shifted, Green pH shifted). Panels A-C: 50 mL culture was removed and pH was adjusted to 4.0. at **A**: 5 Days, **B**: 14 Days and **C**:26 days post-SO_2 addition.

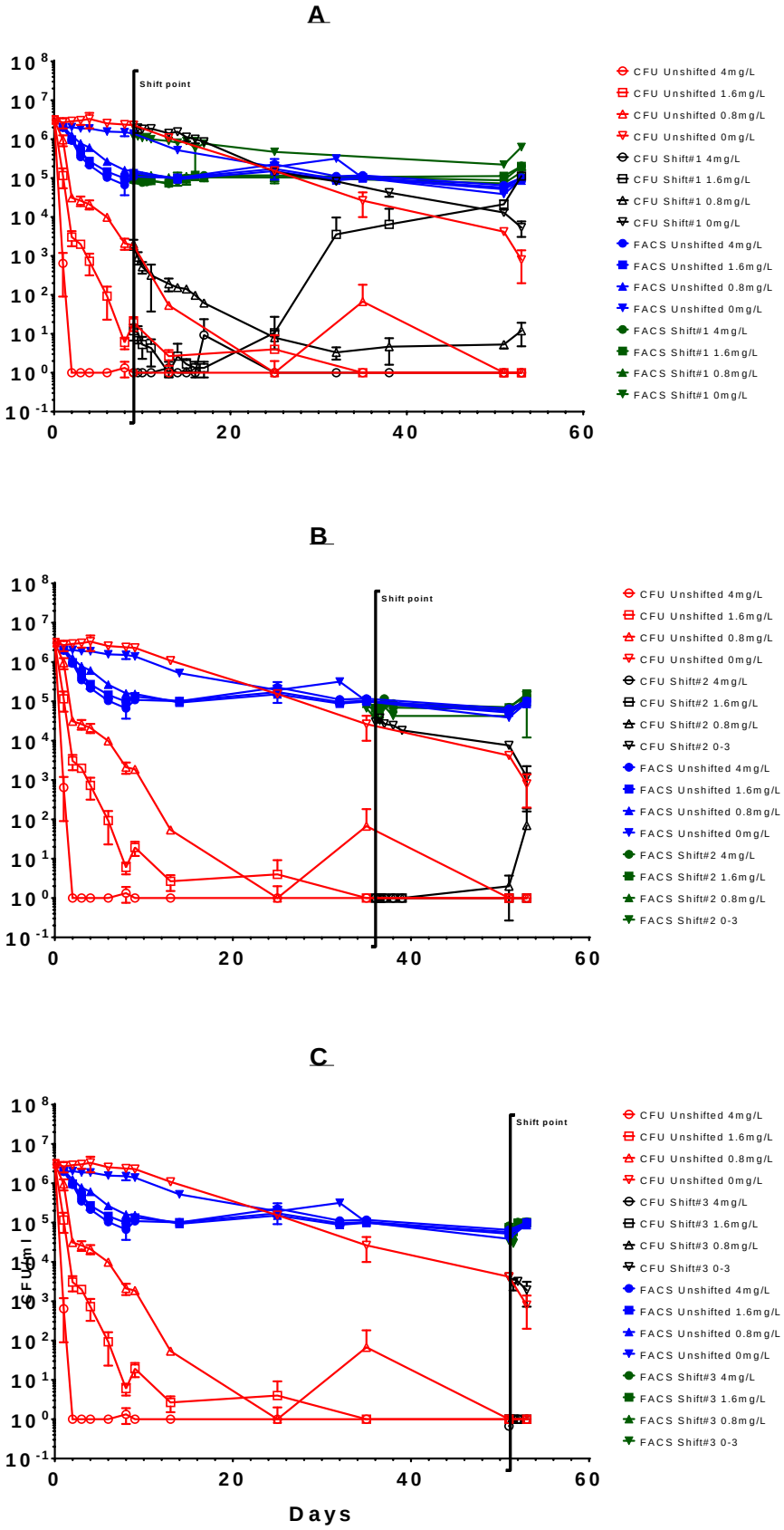
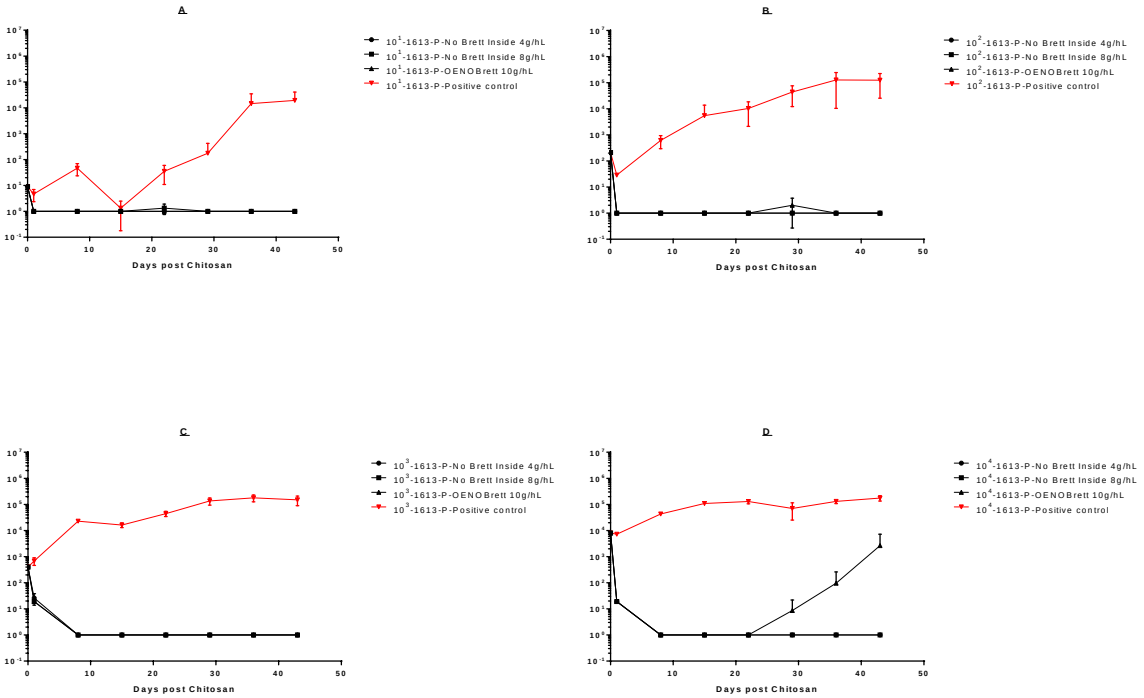
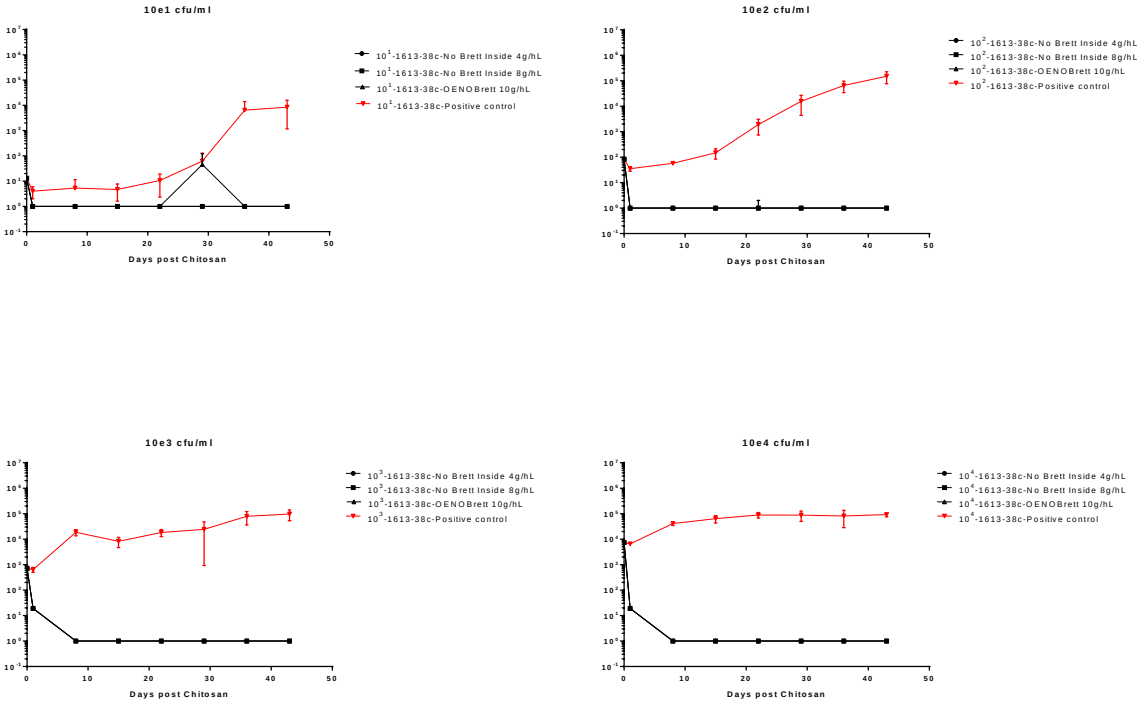


Figure Appendix 5.5 *S. cerevisiae* S288c grown in synthetic wine (Salma et. al. 2013). fSO_2 addition to levels at day 0 to: ~4 (⊖), ~1.6 (⊟), 0.8 mg/L (⧋) and untreated (▽), CFU values determined by plating, solid symbols determined by FACS. No recovery of culturability to samples treated with SO_2 that were dormant/dead.

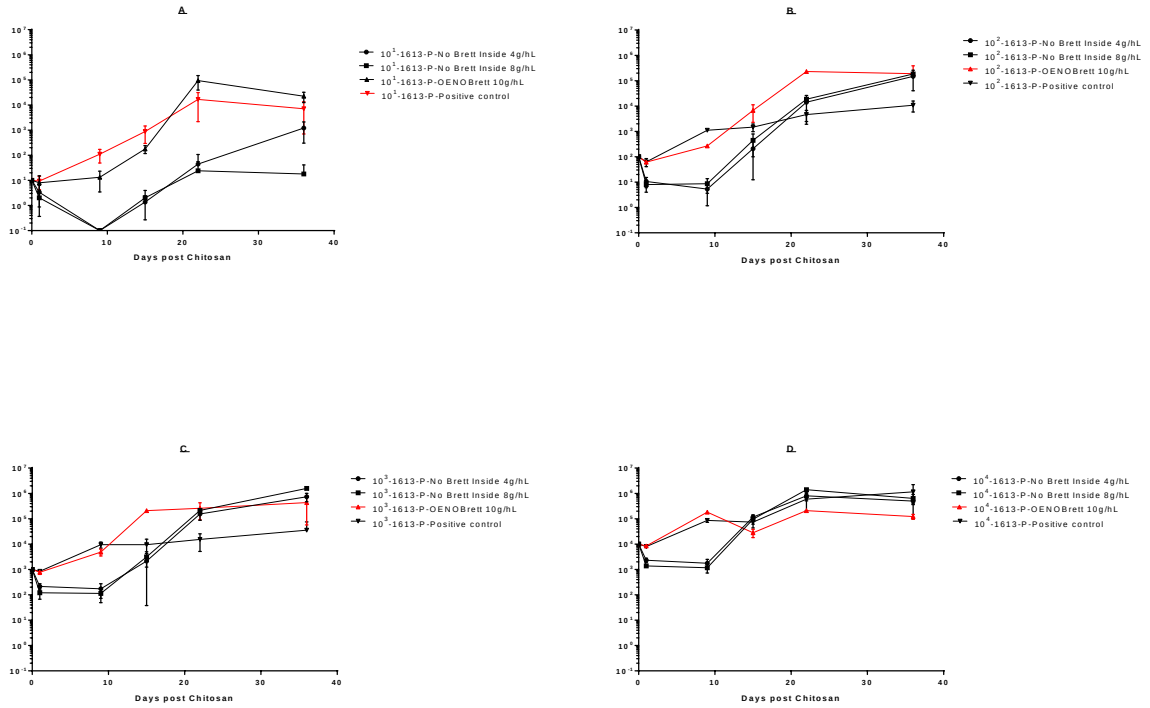


i:-AWRI1499-Parent

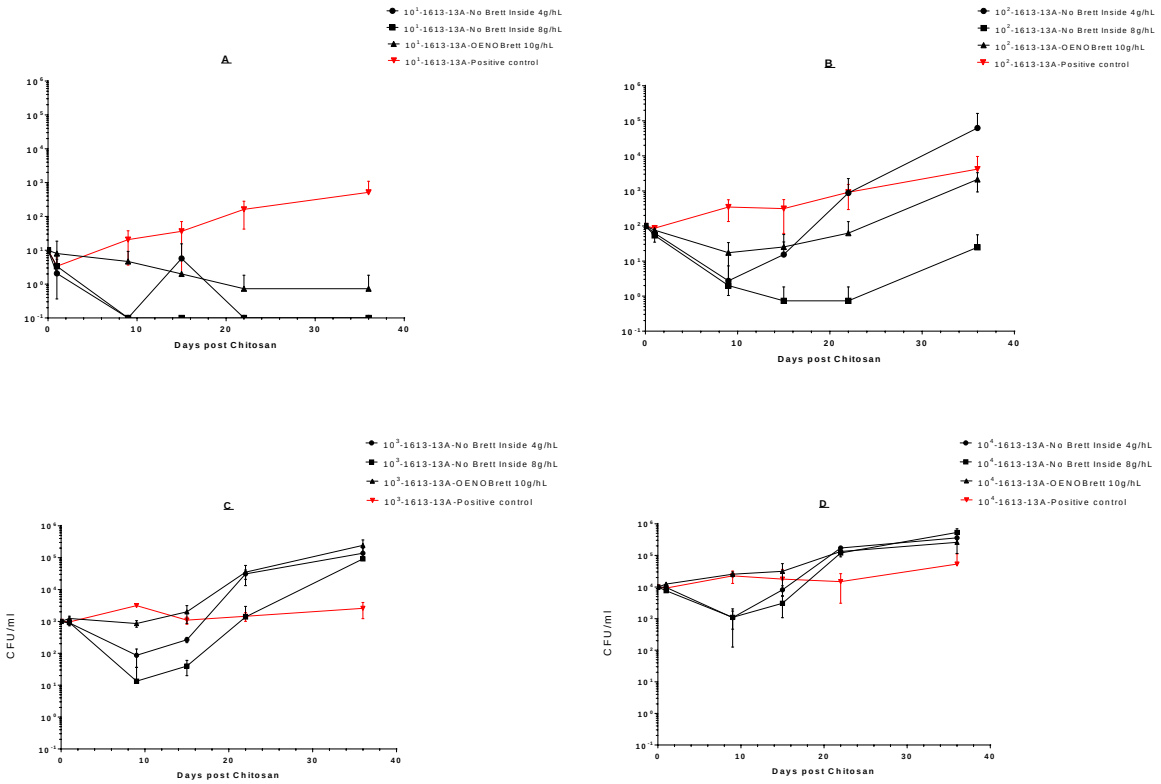


ii) AWRI1499-38C evolved strain

Figure Appendix 5.6 Chitosan addition. 10 mL final of WM in glass COREX tubes, supplemented with commercial chitosan preparations (No Chitosan ● OenoBRETT ■ NoBrettInside 8 g/hL ▲ NoBrettInside 4 g/hL ▼) were inoculated initially with *B. bruxellensis* AWRI1499 at **A** 10^1 ,**B** 10^2 ,**C** 10^3 or **D** 10^4 . Inoculation was achieved by mixing 5 mL of a 2x stock suspensions of cells and chitosan in WM.



i: AWRI1613-Parent



ii) AWRI1613-13A evolved strain

Figure Appendix 5.7 Chitosan addition. 10ml final of WM in glass COREX tubes, supplemented with commercial chitosan preparations (No Chitosan ● OenoBRETT ■ NoBrettInside 8 g/hL ▲ NoBrettInside 4 g/hL ▼) were inoculated initially with *B. bruxellensis* AWRI1613 at **A** 10^1 ,**B** 10^2 ,**C** 10^3 or **D** 10^4 . Inoculation was achieved by mixing 5 mL of a 2x stock suspension of cells and chitosan in WM.

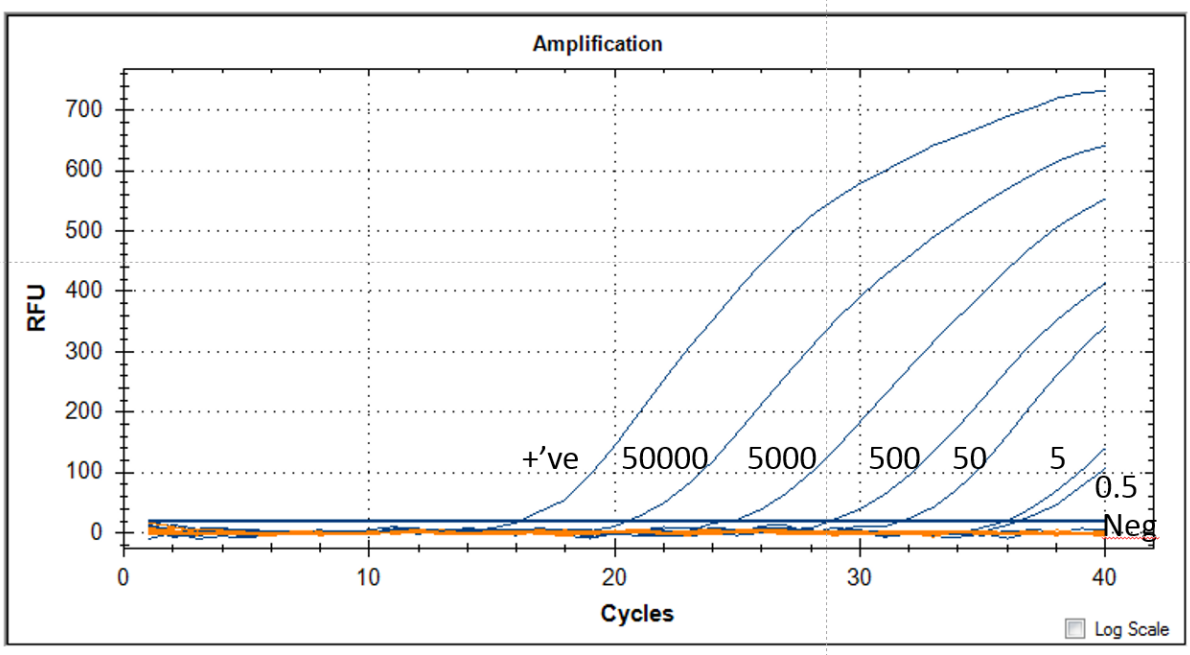


Figure Appendix 5.8. QPCR limit of detection in wine. RFU is relative fluorescence units; Cycles represents the number of PCR amplifications. Limit of detection of 25 cell/100 mL.

Appendix 6: Budget reconciliation

The project's budget reconciliation statement will be submitted separately via Wine Australia's Clarity Investment Management System.