PROJECT TITLE: Determining the spoilage potential of *Brettanomyces* strains isolated from Oregon vineyards and cellars

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OBJECTIVES OF PROPOSED RESEARCH:
Specific project objectives will be to:

1) Comprehensively sample for *Brettanomyces* yeast strains from vineyards and winery-related surrounding environments from at least six collaborating wineries across two Willamette valley sub-AVAs
2) Isolate by enrichment culture and identify *Brettanomyces* yeast strains
3) Evaluate spoilage potential of Oregon *Brettanomyces* strains
4) Develop and convene two regional workshops summarizing project results and providing updated guidance on *Brettanomyces* control

The proposed research area addresses the strategic pillars of wine quality and foundational research, within the specific areas of:

- Viticulture 3B – the extended vineyard microbiome as a critical component of terroir and its impact on wine characteristics.
- Enology 1D – Develop a deeper understanding of the fungal and bacterial communities present during wine fermentation and aging, their effects on wine quality, and levers for the control of undesirable microbes in wine to minimize microbial defects.

JUSTIFICATION AND IMPORTANCE OF RESEARCH:
Oregon is known for production of high-quality Pinot Noir, with nuanced styles reflecting microclimates and geologies of AVAs – true terroir-driven winemaking. Microbial populations in vineyards and wineries have, over recent years, been shown to differ geographically and represent an important component of 'terroir' (Knight et al. 2015). Nonetheless there are some microbial influences on wine style that are widely considered undesirable, regardless of whether the offending species is part of the natural winemaking microflora. The most prominent of these undesirable microorganisms is the spoilage yeast *Brettanomyces*, which continues to cause significant loss of quality in premium, barrel-aged red wines (Curtin et al. 2015). Economic analysis shows this loss can be substantial, one Californian winery found to have lost 2% of its production value in one vintage through cost of downgrading of blends alone (Alston et al. 2021).

Avoidance of spoilage requires winemakers to implement an integrated strategy to minimize wine's exposure to viable *Brettanomyces* cells, through close attention to primary and malolactic fermentations, equipment sanitization, wine filtration, and careful management of pH and sulfite. Not all winemakers are able to implement all control measures due to cost and/or winemaking philosophies, but even amongst those with vigilant *Brettanomyces* control and detection strategies, damaging outbreaks can occur. This may, in part, be due to differences between *Brettanomyces* strains in terms of their spoilage potential. Characterized sulfite-tolerant strains have been observed in Australia, Europe and California (Avramova et al. 2018, Cibrario et al. 2019, Curtin et al. 2012a, Dimopoulou et al. 2019). Concerningly, recent work has shown that previously sulfite-sensitive strains can gain up to 2.5-fold greater tolerance through repeat sub-lethal dosage (Bartel et al. 2021). We do not know whether previously described, or novel, sulfite-tolerant *Brettanomyces* strains occur within Oregon. Furthermore, recent work shows that *Brettanomyces* strains may vary in response to sulfite-alternative, chitosan (Paulin et al. 2020). Assessing overall spoilage potential of Oregon's *Brettanomyces* strains is necessary to inform control strategies.

An additional explanation for variable efficacy of control strategies may be the degree to which incoming grapes from different vineyards harbor populations of *Brettanomyces*, particularly sulfite-tolerant strains, and the extent to which vineyard populations of yeast are able to colonize the cellar. Some evidence for cellar colonization by vineyard *Brettanomyces* was found in France, where similar DNA fingerprints were observed for enrichment-culture isolates from post-veraison and harvest berry samples, and those from a co-located winery (Albertin et al. 2014). Unfortunately, the DNA fingerprinting methodology used lacked sufficient resolution to be conclusive. This was also the case for a study performed in Italy (Oro et al. 2019), where enrichment-culture also yielded vineyard and winery isolates of *Brettanomyces*. Whole-genome sequencing has surpassed DNA fingerprinting methods, allowing for definitive detection of identical yeast strains. For example, Borneman et al. (2016) identified several instances where identical *Saccharomyces cerevisiae* wine yeast are marketed by different yeast suppliers. Whole genome sequencing has been applied to wine (and other beverage) isolates of *Brettanomyces* (Curtin et al. 2012b, Colomer et al. 2020, Eberlein et al. 2021), but has not yet been applied to any vineyard isolates.

The proposed work addresses a critical knowledge gap concerning Oregon's *Brettanomyces* populations and their spoilage potential. It also seeks to build upon work previously funded by the Oregon Wine Board (2018 – Elucidating *Brettanomyces* paths of entry into the cellar), where we optimized a *Brettanomyces* enrichment protocol and successfully isolated *Brettanomyces* from 12 of 149 grape berry cluster samples obtained from two vineyards in the Willamette Valley (Pigao et al. 2021). Our proposal will also leverage 108 already available cryopreserved aseptic must samples gathered during the 2022 harvest for another project. Analysis of these samples, in addition to expanded sampling during the 2023 and 2024 harvests will facilitate evaluation of

whether *Brettanomyces* prevalence in the vineyard or cellar varies within and between Willamette Valley sub-AVAs. Finally, through application of whole-genome sequencing we will determine whether vineyard populations of *Brettanomyces* are the same as those found in associated cellars.

Knowledge generated by the proposed research will have practical applications as well as contributing to a deeper understanding of microbial ecology of wine production. Anticipated applied outcomes will include:

- A new workshop based that will provide Oregon's winemakers with guidance on implementation of control strategies effective against *Brettanomyces* strains found in Oregon vineyards and cellars.
- An extensible enrichment-based detection method and sampling framework that can be used to assess vineyard/cellar environment prevalence of known/novel sulfite-tolerant *Brettanomyces* strains.

These outcomes will enhance the capacity of Oregon winemakers to detect and respond to *Brettanomyces* contamination in a timely manner, reducing economic losses due to wine spoilage. Furthermore, availability of validated scalable methods to detect vineyard and cellar populations of *Brettanomyces* will open up the possibility of further research to assess whether viticultural interventions can augment *Brettanomyces* control strategies in the future.

PROCEDURES TO ACCOMPLISH OBJECTIVES:

Objective 1:

During the 2022 harvest we cryopreserved laboratory-generated aseptic must samples (n=108) from 12 vineyards in two Willamette Valley sub-AVAs (Eola Hills and Dayton – 6 vineyards in each sub-AVA) that share similar viticultural management practices. The sampling strategy was designed in consultation with yeast ecologist Prof. Matthew Goddard (University of Lincoln, UK) to maximize our ability to detect within- and between-region differences for another project that will analyze the overall yeast community structure. This parallel project may provide informative data on yeast species that can serve as proxy indicators for much less abundant *Brettanomyces*. We will enrich these samples for *Brettanomyces* isolation prior to the 2023 harvest.

Our plan for the 2023 and 2024 harvest seasons is to return to the same vineyards and aseptically collect 216 cluster samples each year for washing and *Brettanomyces* enrichment. We will also sample from wineries associated with the 12 sampled vineyards during both years, collecting 240 environmental swabs while the cellars are actively processing grapes and 120 barrel samples post-harvest. In total, during this project we will attempt isolation of *Brettanomyces* from 900 samples.

Objective 2:

Grape cluster samples will be processed as described in Pigao et al. (2021) except for the 2022 samples, which consist of already processed grape must that was concentrated by centrifugation prior to cryopreservation. For all grape cluster/grape must samples, and environmental swabs, liquid enrichments in *Brettanomyces* Enrichment Medium (BEM) will be performed for two

weeks, then samples will be plated out onto selective medium (WLNA+cyclohexamide). Wine samples will be directly plated onto WLNA+cyclohexamide. Based upon colony and cellular morphology, putative *Brettanomyces* isolates will have their species identity confirmed using a published DNA fingerprinting technique (Guillamón et al. 1998). Frequency of isolation will be used to determine *Brettanomyces* prevalence across different sample types. Subsamples of all enrichments will be frozen for DNA extraction and direct PCR detection of *Brettanomyces* using species-specific and strain-specific primers, and results compared with isolation. In this way we will evaluate approaches potentially applicable for development of a fee-for-service method to compare Brettanomyces load between vineyards.

Objective 3:
Whole-genome sequencing and evaluation of spoilage-potential will be performed for *Brettanomyces* isolates recovered from all sample types alongside representative *Brettanomyces* strains from the Curtin and Osborne yeast collections. This will include isolates (n=17) from previously funded OWB project (2018 – Elucidating *Brettanomyces* paths of entry into the cellar). Based upon past rates of isolation we anticipate ~40 new vineyard isolates, along with ~70 new environmental/wine isolates across the two study years. Thus, we expect to work on ~150 isolates in total during this project.

For all new isolates (~130), sequencing libraries will be prepared from DNA extracts for genomic sequencing. Sequence data (2x150bp paired-end reads) will be generated on an Illumina NextSeq, to an average coverage >50-fold across two sequencing runs. Raw sequence reads will be QC'd and trimmed, then mapped to reference genomes using BWAmem. Other open-source software packages (SamTools, BamTools, Stampy and readDepth) will be used to process mapped assemblies for identification of copy number variations (CNVs) and single nucleotide polymorphisms (SNPs). Integrated Genome Viewer (IGV) will be utilized for data visualization. Maximum likelihood and neighbour-joining phylogenetic trees will be constructed using RaXML from genome-scale alignments of newly sequenced *Brettanomyces* isolates and pre-existing available genomic datasets, allowing each new isolate to be unambiguously assigned to strain groups.

To evaluate spoilage potential, high-throughput growth assays in defined laboratory medium will be performed varying factors that affect *Brettanomyces* growth in wine (pH, ethanol, residual sugar, nitrogen type and content, sulfite, fungal chitosan). Additionally, capacity for volatile phenol production will be assessed using a UV-vis spectrophotometric assay that detects consumption of hydroxycinnamate precursors (Mertens et al. 2017). Multivariate statistical analyses will be used to predict whether environmental isolates have the same potential to spoil wine as known spoilage strains. Follow-up tests will be performed in representative Pinot Noir wine with a subset of isolates predicted to have varying spoilage capacity.

Objective 4:
A half-day workshop will be developed that incorporates results generated by the project, updated guidance on *Brettanomyces* control and detection, and a tasting that trains participants on recognition of wine faults caused by *Brettanomyces*. This workshop will be held in Willamette Valley and Southern Oregon during the final summer of the project.

PRESENT OUTLOOK AND ESTIMATED SUCCESS:
The outlook and estimated success of the project are both excellent given the previous research experience of the investigators. Curtin was first to complete a genome assembly for *Brettanomyces* (Curtin et al. 2012) and has since published two comparative genomics studies probing variation in strain properties. Osborne has experience with the propagation of *Brettanomyces* (Schopp et al. 2013; Chescheir et al. 2015) and his lab is fully equipped to isolate, propagate, and store isolates of *Brettanomyces*. In his role as the statewide enology extension specialist for Oregon Osborne will be able to efficiently co-ordinate collection of samples from locations throughout Oregon. Curtin and Osborne collaborated on work previously funded by Oregon Wine Board (2018 – Elucidating *Brettanomyces* paths of entry into the cellar) that utilized enrichment culturing to isolate *Brettanomyces* from Oregon vineyards (Pigao et al. 2021).

All equipment and facilities necessary for this research are available in the Department of Food Science and Technology and at the Center for Quantitative Life Sciences Laboratories (CQLS), both located on the Oregon State University campus. Next-generation sequencing will be performed at the CQLS utilizing an Illumina NextSeq DNA sequencer. Winery sampling and sample collection will be coordinated by Osborne. Culturing, isolation, and maintenance of the *Brettanomyces* strain collection will be performed by Osborne and Curtin. Curtin's lab is fully equipped for DNA isolation, PCR, and bioinformatic analyses downstream of sequence data generation. Specific equipment to be utilized for this project includes a semi-automated 96-channel liquid handling workstation, a multi-mode 384-well compatible microplate reader with barcode scanner and automated plate-feeder.

Results from this project will be valuable to the wine industry in several ways. Based upon other large strain surveys using various DNA-typing techniques, it is expected that the majority of new isolates obtained from environmental and wine barrel samples will be unambiguously classified into known strain groups using genome sequence data and phylogenetic tools. Pending confirmation that Oregon representatives of these genetic groups display similar spoilage potential to isolates described in research literature, the outcome of this work will inform an updated control strategy based upon international research to be disseminated via a new workshop and fact sheet.

If vineyard isolates also conform to previously defined *Brettanomyces* strain groups and more importantly, that they are identical to isolates recovered in associated wineries, the outcome of this

work will point to the vineyard as the next frontier in understanding risk of wine spoilage by Brettanomyces. Our approach will incorporate feasibility assessment of a sampling and *Brettanomyces* enrichment protocol as a scalable approach for determining prevalence of high spoilage-risk *Brettanomyces* strains in the vineyard prior to harvest. Based upon results and demand, such an approach would be developed subsequently as a fee-for-service offering to Oregon's wine producers.

TIMETABLE FOR PROJECT:

	2023-2024				**2024-2025**				
	Su	Fa	Wi	Sp	Su	Fa	Wi	Sp	Su
Objective 1: Sampling from vineyards and wineries									
Objective 2: Enrichment isolation of Brettanomyces									
Objective 3: Whole-genome sequencing									
Objective 3: Evaluation of spoilage potential									
Objective 4: Develop and hold* workshops									*

OUTREACH AND EDUCATION:

Information from this research will be disseminated through peer reviewed publications such as the American Journal of Enology and Viticulture, trade journals such as Wines and Vines, presentations at technical meetings including the annual American Society for Enology and Viticulture meeting and the OWRI Grape Day Research meeting. The co-PI is the Extension Enologist specialist for Oregon and regularly organizes Enology technical meetings (Willamette Valley and Southern Oregon groups) at which research projects and results are discussed and industry feedback is sought. In addition, the OWRI website (http://owri.org) and newsletter will be used to inform industry of research results and applications. In the last year of the project (Summer 2025), results will be presented at newly developed regional workshops (Willamette Valley and Medford OR) addressing prevention of *Brettanomyces* wine spoilage.

BUDGET SUPPORT SUMMARY:

Funding for year one of this study is requested from OWB in the amount of $53,478. This study will leverage samples gathered during the 2022 harvest period for another project, adding substantial value through generation of early results and refinement of protocols ahead of the 2023 harvest.

TOTAL BUDGET REQUEST:

Funding is being requested in year one to provide support (0.49 FTE) for a MS graduate student to be co-supervised by Curtin and Osborne as well as wages for an experienced and trained undergraduate student (400hrs @ $15/hr inc. OPE) to assist with sample preparation, DNA extraction, and PCR analysis. Specific lab materials for Year 1 will be DNA extractions kits ($3,150), PCR reagents ($1,500), and general lab consumables including microbiological media

($2,500). Costs for DNA sequencing in year one ($6,095) includes library preparation, quantification and one lane of Illumina NextSeq P2-150bp PE sequencing. Travel costs ($550) and sampling supplies ($500) are requested to support sampling from vineyards and winery, across approximately 10 days throughout harvest and post-harvest periods.

Costs will be similar in year two of the project with the majority of the funding requested for continual support of a MS graduate student as well as costs associated with sequencing *Brettanomyces* isolates and performing PCR on all enrichment cultures to evaluate direct-detection methodologies. Additional travel costs are requested for the MS student to travel to the National American Society of Enology and Viticulture annual meeting to present research findings. Travel and associated costs are also requested to support delivery of two workshops.

Budget (tuition excluded)

	% Time on project	Request 2023-2024	Projected 2024-2025
Personnel			
Professional			
GRA	0.49 FTE	$25,896	$26,931
Employee benefits		$7,397	$7,986
Student assistant wages		$6,000	$6,000
Supplies & expenses			
DNA extraction kits		$3,150	$2,750
PCR reagents		$1,500	$1,200
General laboratory consumables		$2,500	$2,500
DNA sequencing		$6,095	$6,095
Travel costs for sample collection		$440	$440
Sampling supplies		$500	$500
Equipment (itemize when cost >$1000)			
Travel			
Trips/purpose/costs Results presented at 2025 ASEV meeting (student + PI)			$1500
Extension delivery			$605
Workshop costs			$500
Computer time			
Overhead (where appropriate)			
Indirect costs			

TOTAL REQUEST		$53,478	$57,007

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FOR OWB review purposes– Relevant wine focused publications and outreach:

Curtin Selected Refereed Journal Articles:

Bartel, C., Roach, M., Onetto, C., Curtin, C., Varela, C., & Borneman, A. (2021). Adaptive evolution of sulfite tolerance in *Brettanomyces bruxellensis. FEMS Yeast Research*, 21(5), foab036.

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Book Chapters (peer-reviewed)

Osborne, J.P. (2010). "Film Yeast" *In*: Winemaking Problems Solved (Ed: Butzke, C.). Woodhead Publishing, UK. Pp 381-382.

Osborne, J.P. (2010). "Microscope use in the Winery" *In*: Winemaking Problems Solved (Ed: Butzke, C.). Woodhead Publishing, UK. Pp 270-271.

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Workshops

Principles and Practices of Filtration	Feb. 15th, 2007
Principles of Wine Texture	May 17th, 2007
	May 18th, 2007
Vine Ventures: Guiding Vineyard Establishment, Management and Winemaking	Aug. 18th, 2007
Grape Maturity	Sept 14th, 2007
	Sept, 18th 2007
Winery Sanitation	Sept 18th 2007
Principles and Practices of Filtration	Feb 26th 2008
When Good Wine Goes Bad: Microbial Spoilage of Wine	April 23rd 2008
	April 25th 2008
	Sept 3rd 2008
Grape Maturity	Sept 19th 2008
Wine Microbiology: Using a microscope in the winery	June 17th 2009
Winery Sanitation	Sept 15th 2009
Basic Wine Microbiology	Dec 5th 2009
Harvest Workshop	August 4th 2010
	August 5th 2010
Recognizing and preventing common winemaking flaws	March 8th, 2011
	March 9th, 2011
	March 10th, 2011
Sulfides in Winemaking	April 12th, 2011
Wine Sensory Workshop	March 8th, 2012
	March 9th, 2012
Fundamentals of Wine Filtration	April 25th, 2012
Winemaking 101 – Assessing grape maturity	August 4th 2012
Recognizing and evaluating common wine faults	April 24th 2013
Tannin management	August 7th 2013
Use and Management of Barrels during Winemaking	January 30th 2014
Using a Microscope in the Winery	June 17th 2014
Using a Spectrophotometer in the Winery	May 28th 2015

Management of Malolactic Fermentation	June 22nd 2015
	June 23rd 2015
	June 24th 2015
Recognizing and Evaluating Common Wine Faults	July 29th 2015
Sparkling Wine Symposium	April 14th 2016
Getting Ready for Harvest	August 9th 2016
	August 11th 2016
Sulfides in Winemaking	April 27th 2017
Sparkling Wine Symposium	May 7th 2018
Unravelling the Mysteries of Cold Soak	June 13th 2018
Cold Hardy Grape growing and Wine making	July 17th 2018
OSU Red Blotch Workshop	Nov 29th 2018
Preventing Formation of Sulfur Off Odors During Winemaking	June 24th 2019
OSU Red Blotch Workshop	Nov 20th 2019