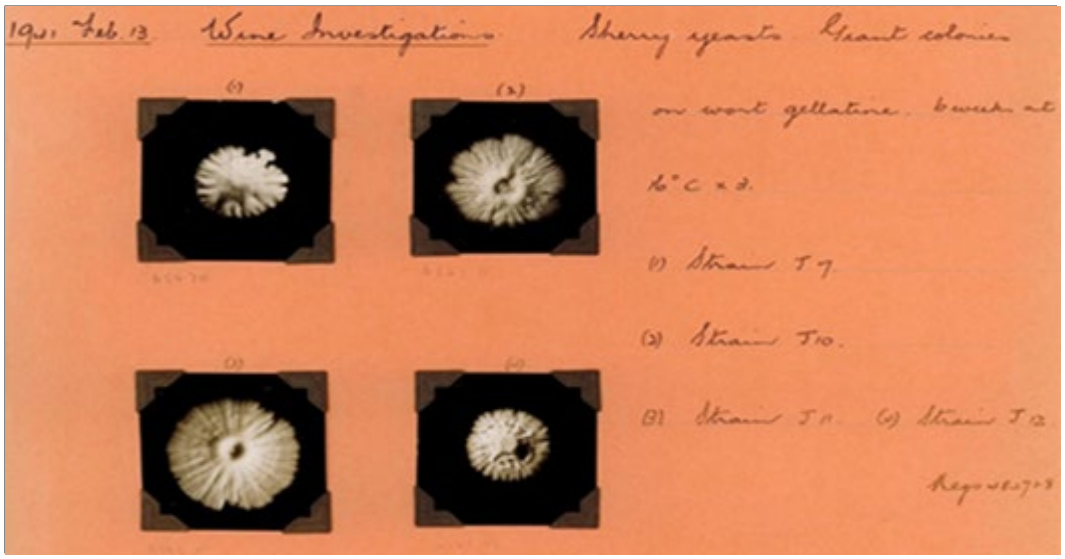
Australian Government
Wine Australia

AWRI Australian Wine Research Institute

Management and optimisation of the AWRI Wine Microorganism Culture Collection



FINAL REPORT TO WINE AUSTRALIA

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1. Abstract

The AWRI Wine Microorganism Culture Collection (AWMCC) contains an extensive collection of yeast and bacteria largely associated with winemaking. The AWMCC has been created from research collections, yeast deletion libraries, and commercial or environmental isolates. Between 2017 and 2022, the AWMCC nearly doubled in size. Microbial strains within the collection are available to winemakers and researchers, enabling product experimentation and differentiation. This project has refined and optimised identification, receival and distribution procedures to improve quality control. In addition, new custom collections have been developed to facilitate high throughput screening of specific microbial genera.

2. Executive summary

The AWRI Wine Microorganism Culture Collection (AWMCC) contains more than 20,000 yeast and bacterial strains, including laboratory strains, yeast gene deletion libraries, proprietary strains, fermentation isolates and environmental isolates from Australian wineries. The collection underpins current and ongoing Wine Australia projects and secures investments in germplasm creation and discovery.

The collection significantly increased in size from 2017 to 2022, due to ongoing research activities and isolation of microorganisms from environmental samples. A large proportion (55%) of these isolates were received from Project 4.4.3 – Bioprospecting Australian microbial genetic diversity.

Project 3.4.1 – Understanding *Brettanomyces* and its adaption to control measures, isolated or generated 779 strains stored in the collection, contributing 71% of the total number of *Brettanomyces* strains in the collection since its inception. The number and diversity of the *Brettanomyces* strains span all winemaking regions in Australia and this collection is an invaluable resource for studies to better understand and control this wine spoilage yeast.

The increased demand for AWMCC facilities and the broadening scope of its activities over the project term created some operational challenges. These challenges were overcome by procedural optimisation driving productivity gains. In addition to efficiency gains, these changes, specifically the creation of dedicated screening arrays of defined microbial genera, have created opportunities for the collection, allowing greater access for individuals wishing to study the physiological characteristics of these organisms using higher throughput technologies.

Storage facilities were reorganised to promote more efficient use of the collection. Off-site storage was moved to Adelaide Biobank. The relocation of the AWMCC back-up collection ensures that irreplaceable specimens are archived in a monitored facility, and that specimen data are also archived via the Adelaide Biobank Laboratory Information Management System. The redundancy of physical specimens and associated information ensures that this resource is secured for future generations of researchers and wine industry practitioners.

Quality control (QC) procedures were reviewed and enhanced during the project. Yeast and bacterial submissions and retrievals are now processed through selective media before being entered into the collection or delivered to external parties. The documentation sent to external parties with each requested strain was revised and formalised. Together, these operational improvements ensure that end users can use AWMCC specimens with confidence.

The Australian wine industry invests significant resources into research projects that require microbiological material of known provenance, purity and stability, both for the duration of the project and for potential future projects. Readily available characterisation data for biological research material results in increased opportunity, faster start-up and reduced costs for projects that would otherwise need to perform additional sample selection, identification, and

quality assurance. The research outcomes of all microbiological research projects are dependent on the quality and reliability of the microbiological starting material. The AWMCC has underwritten the investment in these projects by ensuring that researchers have access to identified, pure, stable microbiological material of known provenance.

3. Acknowledgements

The University of Adelaide's Biobank is thanked for providing access to the biobanking facilities located at its North Terrace campus. Discussions with Biobank staff in relation to harmonisation of procedures and quality of South Australian biological collections has helped the AWMCC become an important part of the Australian biobanking community, in which it has a special place as one of the few multi-user (commercial and research) non-medical banks in Australia. This in-kind support will allow the secure and ongoing storage of the AWMCC for many years to come.

4. Background

At the project outset, the AWRI Wine Microorganism Culture Collection contained more than 10,000 yeast and bacterial strains, including yeast gene deletion libraries, proprietary strains and natural isolates from Australian wineries. The culture collection was initiated by the inaugural director of the AWRI, John Fornachon, to retain the cultures he obtained for the Australian wine industry. Those seminal cultures were isolated in the course of research on flor wine production and wine spoilage. Since that time, the collection has grown to include yeast for primary fermentation, malolactic bacteria, research strains constructed in the laboratory, acetic acid bacteria for vinegar production, and isolates of non-*Saccharomyces* yeasts for research into spoilage or wild fermentation.

Since its inception, the collection has supported many projects and continues to underpin research to benefit the Australian wine industry. The *Brettanomyces bruxellensis* collection at the AWRI is one of the world's largest collections of this species. It has proven to be invaluable for our current understanding of the physiology and genetics of this organism. *Brettanomyces* research, supported by the collection, has highlighted the diversity of these organisms and contributed to their control in Australian winemaking.

Likewise, the AWMCC has accumulated a significant collection of wine bacteria that has been used to determine the genetic diversity and winemaking characteristics of these enigmatic organisms. Additional research into various non-*Saccharomyces* yeast has also been undertaken using strains from the collection.

Non-GMO techniques for strain development, including hybridisation and directed selection, have given rise to several new strains useful for imparting differentiated characteristics to wine. Safeguarding these investments in a secure and monitored collection is fundamental to the role of the AWMCC.

The AWMCC has several high throughput collections available to support research. The laboratory yeast and wine yeast gene deletion libraries are part of the assets used for research into gene function, or as a comparison between laboratory and wine yeast.

In summary, the collection is resource that has the potential to broaden the diversity of organisms available to winemakers.

5. Project aims

This project, together with others underway in the Australian grape and wine community, contributed to the following broad aims:

- New or enhanced yeast and bacterial germplasm for the sector to enhance winemaking processes and realise performance and economic gains, through improved fermentation efficiency and differentiation of wine products or styles.
- Secure hosting and growth of the AWRI Wine Microorganism Culture Collection

The project objectives were:

- Quality control checks (identity and purity) of new accessions and existing strains in the collection.
- Storage of, and access to, wine-associated microorganisms from Wine Australia-funded research projects and the Australian wine industry.
- Maintenance and back-up of the existing collection.
- Evaluation of storage formats compatible with high throughput screening.
- A reformatted collection that is compatible with high throughput screening technologies.
- A review of operational procedures, DNA extraction, quality control and screening methods, and opportunities from emerging sequencing technologies.
- Application of identified opportunities from emerging sequencing technologies for improved identification and quality control of strains.

6. Methods

6.1 Quality control checks (identity and purity) of new accessions and existing strains in the collection.

6.1.1 Selective media

Quality control improvements during receival and distribution of cultures were achieved by increasing the selectivity of culture media. Antibiotics were introduced for both yeast and bacterial culture plates.

The use of a broad-spectrum antibiotic (chloramphenicol 50 mg/L) was initiated for all plates requiring yeast cultures to be grown on them. Similarly, fungal antibiotics cycloheximide 100 mg/L or natamycin 50 mg/L were introduced into bacterial plates to eliminate yeasts.

In addition to the inclusion of antibiotics, use of differential media enabled disparate groups of microorganisms to be visually distinguished. WL Nutrient Agar (Difco, Bayswater, Australia) containing a pH indicator (bromocresol green) was used routinely for yeast plating. Colour variation can indicate differences in an organism's underlying biochemistry, showing that a strain is different than expected or that the culture is mixed (Figure 1).



Figure 1. The appearance of various yeast species on WL Nutrient Agar plate

Isolation of yeast from wine samples can be problematic as they may also contain moulds and other filamentous fungi. A commercially available media, Dichloran Rose Bengal Agar with chloramphenicol (Oxoid, Scoresby, Australia) was trialled with several yeast species for the ability to eliminate bacteria through antibiotics and restrict the growth of filamentous fungi and moulds.

In assessing acetic acid bacteria, differentiation can be improved using similar pH indicator compounds (bromocresol green, bromocresol purple) to indicate contamination or for physiological identification of species based on the metabolism of a set of carbon sources (sugars). The production of acid from sugars has been well documented and can help identify new cultures or problems with older cultures.

Typically, all bacterial cultures were grown on MRS agar (De Man, Rogosa and Sharpe agar, Oxoid, Scoresby, Australia) with 20% apple juice (MRS+A), which is applicable for *Oenococcus oeni* cultures, other lactic acid bacteria and acetic acid bacteria.

6.1.2 Genetic identification

Restriction fragment length polymorphism (RFLP)(Esteve-Zarzoso et al. 1999) and internal transcribed spacer (ITS) sequencing were used to identify yeast (White et al. 1990).

The multi-well plate DNA extraction, ITS polymerase chain reaction (PCR) and clean-up procedures used in this project are provided below. They describe the process of PCR once DNA has been extracted and purified before it is analysed by an external laboratory. The purpose of this procedure is for PCR clean-up in 96 well microplate to remove excess deoxynucleotide triphosphate (dNTP) and unincorporated primers.

96-well DNA extraction and PCR of yeast strains for ITS sequencing

1. Quick genomic DNA preparation from cryogenic stocks

   - In 96 well plate place 100 µL of YPD + chloramphenicol (50 mg/mL) and use a tip or loop to inoculate from frozen stocks grown in 28°C overnight
   - In a 96 well PCR plate place 50 µL of a Zymolyase solution (e.g. 2.5 mg/mL in 0.1 M sorbitol) (order prepared from manufacturer, or 18.2 g/L sorbitol) then transfer 100 µL of cell culture into the plate and let incubate for 5 minutes at 37°C.

2. PCR Master Mix using hot start polymerase Kappa2G Robust HotStart PCR kit

- Aliquot 22.5 µL of PCR master mix (below, or variation using other polymerases) into each PCR tube/well.

Reaction components	**Final concentration**	**Per 25 µL reaction**
PCR grade water	1x	14.3 µL
5x KAPA2G Buffer B with MgCl2		5.0 µL
dNTP mix (10 mM each dNTP)	0.2 mM each dNTP	0.5 µL
Forward primer (10 µM)	0.5 µM	1.25 µL
Reverse primer (10 µM)	0.5 µM	1.25 µL
KAPA2G Robust HotStart (5 U/µL)	1 unit per 25 µL rxn	0.20 µL
TOTAL		**22.5 µL**

- Add 2.5 µL of the resuspended colony/overnight culture (mixed with Zymolyase) to the appropriate PCR well.
- Seal the plate using PCR film. Perform the PCR using the following profile

Cycling step	**Temperature and time**
Initial denaturation	5 min at 95°C
Denaturation	30 sec at 95°C
Annealing	15 sec at 56°C
Extension	15 sec at 72°C for fragments ≤500 bp; 30 sec/kb at 72°C for fragments >500 bp
Number of cycles	30°C
Final extension	Not required

3. PCR clean-up (if needed)

Use procedure for 'Yeast ITS PCR clean up with Exo-SAP method'

4. Long-term storage of genomic DNA preparations

Addition of glycerol to a final concentration of 20% and freezing at -80°C can be performed if the genomic DNA (step 1) is needed to be held for an extended period with little degradation.

Yeast ITS PCR clean up with EXO-SAP method

Materials	**n=1**	**n=25**	**n=100**
Antarctic Alkaline Phosphatase buffer	0.4 µL	10 µL	40 µL
Water	2.72 µL	68 µL	272 µL
Exonuclease I	0.08 µL	2 µL	8 µL
Antarctic Alkaline Phosphatase	0.8 µL	20 µL	80 µL
Total	**4 µL**	**100 µL**	**400 µL**

1. Add 4 µL of Exo-SAP mix to 10 µL of PCR products (10 µL per 25 µL PCR prod)
2. Incubate at 37°C for 20 minutes (degradation of primers and dephosphorylation of dNTPs)
3. Incubate at 80°C for 15 minutes (enzyme degradation)

Quantification using the QUBIT can be performed after clean-up. A gel should also be run to make sure there is only one band. Sequencing works better with a higher concentration of products. The DNA is now cleaned and ready for further processing (such as sequencing).

Recently, documents relating to the contents of the cultures sent to winemakers or researchers have been requested as part of quality assurance within the recipient organisations. A safety data sheet associated with the live culture agar samples sent to recipients and a strain certification document have both been developed and sent on an as needs basis (see Appendix 5).

6.2 Storage of, and access to, wine-associated microorganisms from Wine Australia-funded research projects and the Australian wine industry

6.2.1 Collection storage and access

Culture collection storage and access conditions have undergone minor but important changes over the last five years. Access to the routine collection at Urrbrae and the back-up collection at the Thebarton site was restricted to culture collection staff. Such restrictions mitigate the risk of equipment failure due to misuse or overuse and provide security for genetically modified organisms (GMOs), ensuring a chain of custody is maintained. In addition, these restrictions reduce the potential for contamination.

The back-up collection stored at Thebarton was relocated to the University of Adelaide's Biobank facility, which will provide additional benefits for the collection. This facility is monitored and attended by trained biobanking staff and is housed in a purpose-built (PC2 standard) temperature-controlled facility equipped with a reticulated liquid CO_2 backup. On-site freezer access is available in case of breakdown.

6.2.2 Collection reformatting to create sub-collections for high-throughput analysis

High throughput sub-collections were created for *Saccharomyces*, *Brettanomyces* and other non-*Saccharomyces* yeasts. Bacterial sub-collections were created for *Oenococcus*, other lactic acid bacteria and acetic acid bacteria. These sub-groups were created based on genus or function.

Reformatted cultures were prepared using different media described below:

- Yeasts were cultured on YPD agar (10 g/L yeast extract, 20 g/L bacto peptone and 20 g/L D-glucose). It provides the components essential for yeast growth in excess.

- Bacterial species were cultured on MRS+A, which is a standard medium for storing lactic acid and acetic acid bacteria and a common medium for the cultivation of *Oenococcus* oeni (Kellyet al. 1989). The other lactic acid bacteria (non-*Oenococcus* LAB)/acetic acid bacteria (AAB) were also tested on this medium and were found to be capable of luxuriant growth.

After incubation on the above solid media for 3- or 7-days (for yeasts and bacteria, respectively), microorganisms were transferred to the respective liquid media containing 20% glycerol, sealed and frozen at -80°C. These plates are now a source for retrieval of microbial arrays. Retrieval can be undertaken with specific array tools such as steel pins.

6.2.3 Reference images

Several yeasts were prepared for scanning electron microscopy using osmium tetroxide fixation (see method in Appendix 5c). Micrographs were taken and stored as digital copies for usage in research publications and communications involving microorganisms. These images also serve as a resource for identification and QC functions.

6.2.4 Intellectual property management

Intellectual property rights management is a consistently evolving area within microbial culture management. The effort of individuals or organisations in microbial germplasm isolation and characterisation require recognition and protection. To this end, the AWMCC has developed a revised database of cultures whose property rights are controlled by the AWRI, Wine Australia or both, and those owned or potentially owned by external parties. This database aims to clarify the usage rights associated with organisms within the collection, protect wine industry investment from exploitation, and create opportunities for return on investment by mediating access to microbial germplasm.

The AWMCC uses a Material Transfer Agreement (and accompanying Terms & Conditions) that explicitly requires users to:
(1) use bioresources for non-commercial research purposes only
(2) not distribute strains to third parties.

6.3 Maintenance and back-up of the existing collection

Protect vials are used as the standard vessel for all yeast and bacterial culture storage in the collection. The high throughput plates (see 6.2.2) were created in addition to these and essentially replicated parts of the collection. Culture revitalisation was conducted throughout the project and was considered vital for samples regularly used or associated with intellectual property (IP) rights. During culture revitalisation, purity was assessed and strains were re-identified using molecular techniques (see 6.1).

Database records of revitalised cultures were reviewed and aligned with original documentation when available. Information pertaining to intellectual property rights and historical data on uses and performance was updated. These reviews were performed on an as-needs basis, usually when a customer requested the culture or when a search of online databases revealed new information.

6.4 A review of operational procedures, DNA extraction, quality control and screening methods, and opportunities from emerging sequencing technologies

Previous DNA extraction methodology consisted of an incubation with Zymolyase (MP Biomedicals. Irvine , CA, USA), freezing the sample at –80°C then boiling the sample straight after freezing. This was a crude method for DNA extraction where the cell wall was degraded by enzymes present in the Zymolysase, and then the cell was fractured with a freezing step followed by a boiling step, which released the DNA. While this was a quick and cheap method, substantial numbers of yeast were resistant to the process (especially non-*Saccharomyces* yeasts). A high throughput array system for DNA extraction was seen as a desirable advancement of the process, however, the resistant yeasts made the use of a plate array with the original method intractable due to the number of failed samples in an array. Each failed sample would need to be repeated individually and the re-arraying of the successful samples would take more time and could lead to errors.

The main component of the new procedure (which makes DNA extraction more rapid) was the use of a high throughput array to allow culturing of the individual strains in DNA extraction plate wells without defrosting the yeast sample plate, and then using the wells for the steps of

DNA extraction, ITS PCR and clean-up and the subsequent sequencing of the DNA. This procedure is described in section 6.1.2.

A review of all procedures used within the culture collection was needed to align new protocols, include new or improved procedures and review methods that were to be made available to winemakers for scientific content and technical accuracy. Some of the procedures in the culture collection had been used for many years without formal collation of updates on methodology or technological advances. A collection of documents was also needed to help with the training of new personnel and to serve as a basis for continuous improvements.

7. Results and discussion

7.1 Quality control checks (identity and purity) of new accessions and existing strains in the collection.

Different growth media were trialled as alternatives to the previously standard YPD media. The commercially prepared Dichloran Rose Bengal Agar marketed as optimal for detection of yeast, elimination of bacteria and restriction of moulds proved inadequate. This media was less suitable for the growth of non-*Saccharomyces* yeast such as *Brettanomyces* and was rejected as an isolation media for wine samples.

Use of WL agar instead of YPD agar allowed subtle or gross phenotypic differences in yeast growth to be visually determined. The addition of a broad-spectrum bacterial antibiotic chloramphenicol at 50 mg/mL to WL agar helped eliminate bacterial contamination. Microscopic analysis was used to confirm the elimination of bacteria from cultures before storage. WL agar + chloramphenicol was made standard for all yeast cultures being requested or resuscitated for external research or industry customers.

The ability to detect non-target yeast in the culture allowed the deconstruction of previously presumed pure cultures. Purified yeast were repackaged into new storage vials and any contaminant yeasts were given a new culture collection number and stored separately.

The addition of cycloheximide or natamycin to MRS+A agar became a standard practice for propagation and revival of lactic acid and acetic acid bacteria. Although yeast contamination of bacterial cultures is uncommon, the addition of a yeast antibiotic to the MRSA plates was considered a prudent change in the process.

A 50% WL agar and 50% MRS+A agar mix was used to culture yeast and bacteria for delivery to external customers on agar slopes. A unified media for yeast and bacteria saves time and reagents. Antibiotics are not included in live agar slopes for delivery to winemakers, as this may be seen as contamination by some end users.

A Safety Data Sheet (Appendix 5a) and a Culture Certification Document (Appendix 5b) were developed during the project. These are sent to recipients with each live culture to facilitate the safe use of materials provided by the AWMCC and represent a step toward formalising best practices.

7.2 Storage of, and access to, wine-associated microorganisms from Wine Australia-funded research projects and the Australian wine industry

During the project, 13,877 yeast strains were deposited into the AWMCC. This number comprised non-*Saccharomyces* yeasts, research *Saccharomyces* and *Brettanomyces* strains, strains obtained from other AWRI activity (e.g. Affinity Labs) and strains from Project 4.4.3 - Bioprospecting (Figure 2).

Strains were deposited into the collection as either individual isolates or bulk submissions derived from ecosystem surveys. The greatest number of individual strains added to the collection were *Brettanomyces* strains (Figure 2). Additions of *Brettanomyces* yeast to the collection reflect the efforts of Project 3.4.1 – *Brettanomyces*, supported by the Affinity Labs Applied Biosciences team and the AWMCC.

The largest overall number of submissions was from Project 4.4.3 - Bioprospecting. The submissions from Project 4.43 vastly increased the AWMCC's non-*Saccharomyces* wine yeast collection and the culture collection's size by 400% relative to its pre-2017 size. The strains isolated by Project 4.4.3 were collected from samples of non-inoculated ferments and comprise an assemblage of novel genetic material, adding diversity to that already within the collection. The expanded collection of yeasts is an extensive germplasm source that will support future winemaking production and research projects.

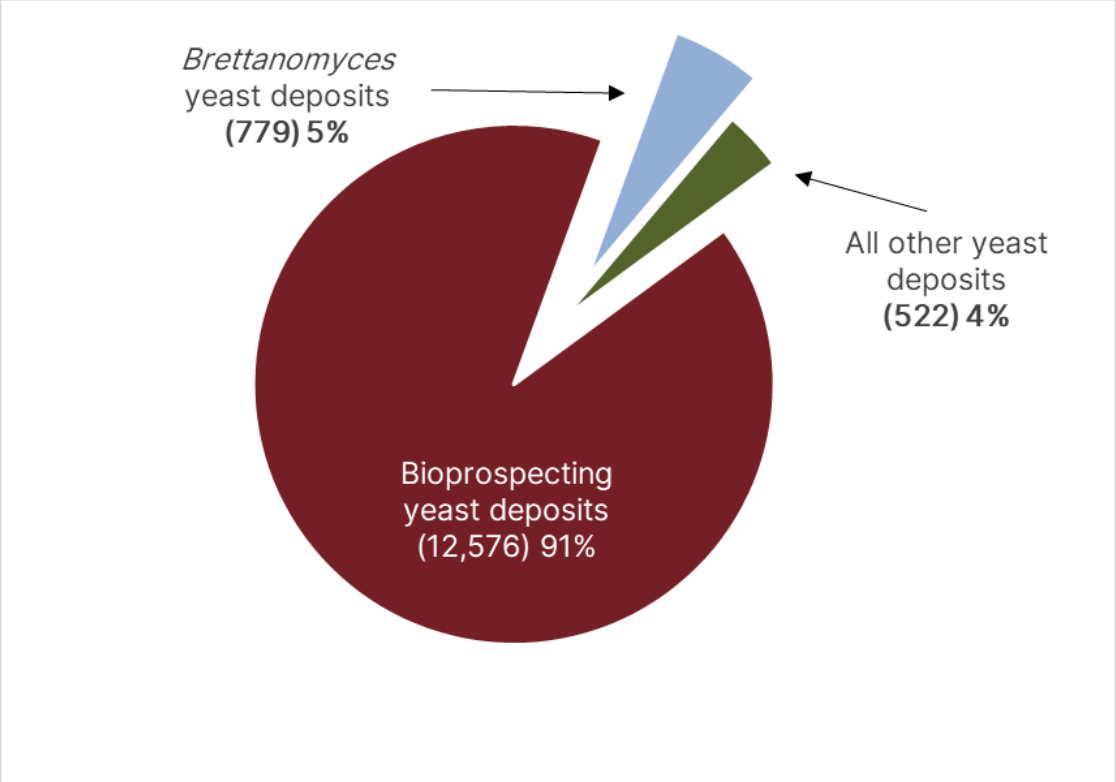


Figure 2. Yeasts deposited into AWMCC from July 2017 to July 2022

The total number of yeast and bacterial strains delivered to customers from July 2017 to July 2022 was 3,002. Of these, 2,550 were distributed to research organisations, and the remainder were provided to industry via an MTA in accordance with third-party IP requirements (Figure 3). This number of requests is approximately 24% above the previous reporting period (2013-2017) and represents the growing importance of the AWMCC to wine researchers and industry alike.

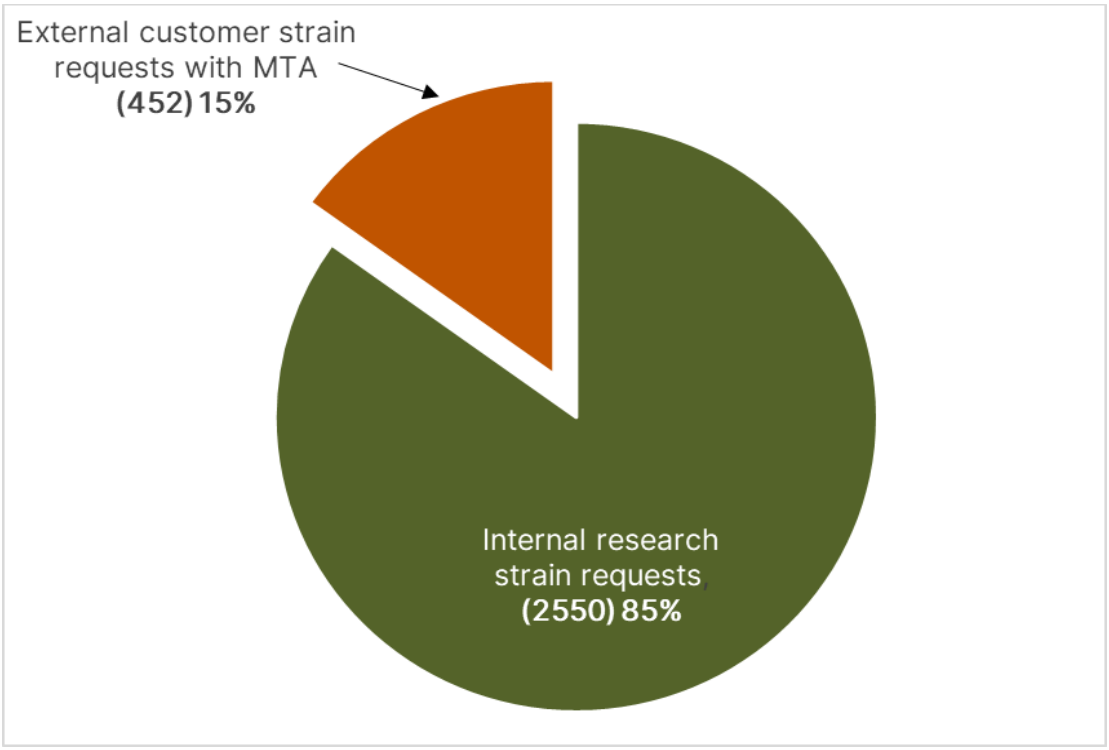


Figure 3. Requested yeast and bacterial strains July 2017- July 2022

The deposition of *Oenococcus oeni* strains into the collection primarily resulted from Project 3.2.2 (Figure 4). The large increase in *Oenococcus* numbers in the AWMCC represents a significant addition to the genetic diversity of this critical winemaking microbe. The expansion of the bacterial collection cements the AWMCC as a central resource underpinning wine research. Specifically, studies on fermentation performance, flavour differentiation and the critical aspects of yeast and bacterial interactions will benefit from the increased diversity available within the collection.

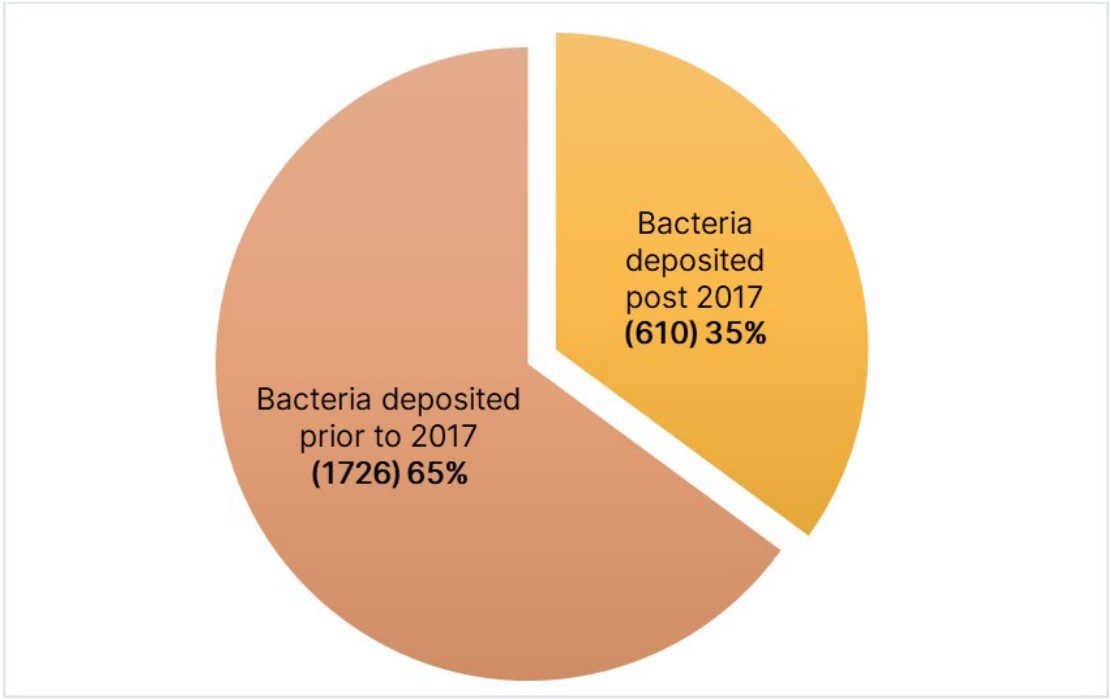


Figure 4. Bacteria deposited into AWMCC from July 2017 to July 2022

Contact with the AWMCC was commonly driven by word of mouth or via the 'Available microbial strains' webpage on the AWRI website. The culture collection webpage was updated

during the project to reflect the range of strains available for purchase. Popular strains such as the flor yeast strains and acetic acid bacterial strains for wine vinegar were updated along with strains for primary fermentation.

A website section about yeasts commercialised by the AWRI was also updated. The section provides an example of research activities contributing to wine microbiology development. Information was added to a section of the AWMCC webpage targeting yeast researchers. Terms and conditions, publications and other guides were also renewed on this page to provide the information researchers need.

The project team sought to continually improve the information available about strains held within the collection. These annotations highlighted strain potential for specific uses, increasing the collection's utility for research or industry.

The creation of high-throughput microbial collections also facilitated access and increased utility. To this end, 96-well arrayed collections of *Saccharomyces*, *Brettanomyces,* non-*Saccharomyces* yeasts, and lactic acid and acetic acid bacteria were created. Carbon source utilisation in non-*Saccharomyces* yeasts was evaluated using the 96-well array format. The efficiency and functionality of the array format were apparent relative to the usually slow and tedious method of screening from individual cultures. By necessity, the array format compromises identity assurance for speed and functionality. This trade-off in quality is appropriate for screening operations. However, the data obtained cannot be a conclusive step for any research project. Confirmation with individual cultures is still required for all findings from using the array collection. The details and composition of the high-throughput arrays are available online at DOI https://doi.org/10.5061/dryad.dfn2z355b.

The range of vinegar bacteria species available to winemakers was expanded during the investment period. This expansion helped facilitate the diversion of previously downgraded or damaged wine into saleable products contributing to cost recovery.

Several images of yeast and bacteria from the AWMCC were recorded using scanning electron microscopy. The digital images were used for at least one publication in the brewing industry. This collection has great value in communications to winemakers by researchers who require detailed images of yeast and bacteria to illustrate various concepts. Images of *Saccharomyces cerevisiae*, *Brettanomyces bruxellensis*, *Oenococcus oeni, Aureobasidium pullulans* and *Schizosaccharomyces pombe* were retained and made available to AWRI researchers for education and engagement with winemakers or for research publications. Some of these images will be used in future versions of the AWMCC website (example in Figure 5).



Figure 5. Scanning electron micrograph of *Saccharomyces* yeast (micrograph colourised)

7.3 Maintenance and backup of the existing collection

Regeneration and re-identification of older cultures (>7 years) using the latest diagnostic methods continued throughout the project. Several cultures were identified that contained multiple strains of yeast and/or bacteria. Regeneration into new freezer vials was completed in duplicate, and the cultures were then re-identified to confirm the species. This procedure enabled the retention of historical cultures, which were obtained at varying levels of quality, securing them for future use.

Information was updated on an ongoing basis for cultures where data were missing. During the project, strains that required continued investigation of IP encumbrance were annotated. The origins of selected cultures were further clarified, allowing metadata updates to be completed, such as from old publications that referenced the strain. The new data have assisted in determining intellectual property ownership and genetic modification status, both of which have ramifications for intellectual property assignment. Additional fermentation data and performance attributes were included, which will benefit Australian winemakers when it comes to providing cultures that meet their requirements.

The relocation of the AWMCC back-up collection to the University of Adelaide's Biobank facility was a significant move forward in the security of the AWMCC. It strengthened and secured the unique investment that Wine Australia has made in the AWMCC, by ensuring the collection's continuation and accessibility for the Australian wine industry. Improved monitoring and support systems for the AWMCC back-up collections are now available, contributing to the overall sustainability of the culture collection.

7.4 A review of operational procedures, DNA extraction, quality control and screening methods, and opportunities from emerging sequencing technologies

New DNA extraction and PCR methods were developed to enable the simultaneous processing of samples for molecular identification. This method was adapted to work around the DNA extraction difficulties encountered with some species. The poor-quality DNA extracted from some microorganisms can affect the quality and reliability of downstream molecular identification.

The updated method uses 96-well plates for cell lysis and DNA extraction. This technique produces DNA of suitable quality for ITS-PCR, which is the method of choice for yeast identification and the genomic library preparation required for whole genome sequencing. The DNA extract could be stored at -80°C for long periods until needed.

A review of all operational procedures was undertaken. Procedures were harmonised into a standard template, reviewed for outdated information and collected into a document titled the 'AWMCC Handbook of Policies and Procedures'. The handbook was made available to researchers looking for standard media or propagation protocols. New policies or procedures were added to make the handover of culture collection management more efficient and less ambiguous. It could also be used when training personnel to run the culture collection. These procedures include the methods, day-to-day operational tasks, troubleshooting guidelines and descriptions of less frequently encountered operations.

Retrieving and delivering cultures to customers is now a cleaner and more straightforward process (Figure 6).

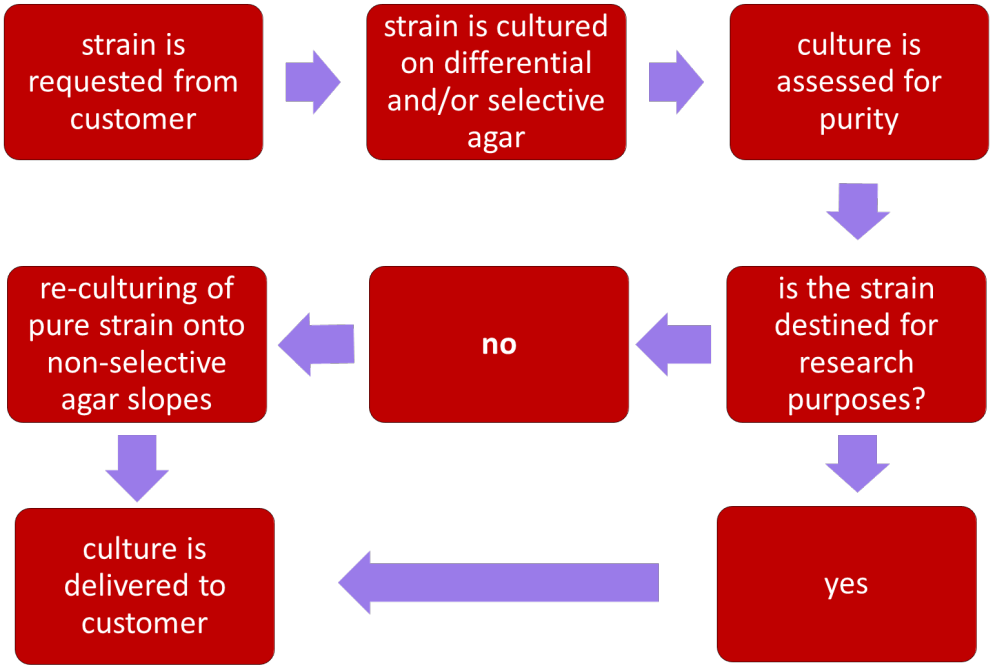


Figure 6. Flow chart of dealings with a request from a research or industry customer

One procedure that previously resided with the culture collection and was made available to winemakers as needed (vinegar bacteria propagation) was transitioned to the AWRI website as a fact sheet by the IDS team. This fact sheet is now available for all interested winemakers

without the requirement to contact culture collection staff, as was previously the case. Other relevant protocols will be made available in a similar way, increasing the availability of the knowledge we currently supply. Winemakers will benefit from the increased access via the website, which allows printing or saving this document if needed.

Opportunities for next-generation sequencing (NGS) were reviewed as a way to enhance the information about yeast and bacterial identity and relationships between isolates. However, the use of next-generation sequencing for the identification of yeast or bacterial species needs more laboratory hours than the 96-well plate assay to process due to the multiple pipetting and PCR steps required to construct a DNA library. Data from the sequencing run needs training in the next-generation sequencing pipeline analysis to complete the speciation and quality control of the data. Sequencer manufacturers have determined that for one area of interest (i.e. ITS), the use of NGS is not cost-effective. The use of NGS for whole genome sequencing has been mooted as a possibility for sequencing of the entire bacterial genome if the need for such data arises. Information about the current methods and previously used protocols for species analysis used by the AWMCC was published as an industry article in *AWRI Technical Review* in April 2021 (Dillon 2021).

8. Outcome and conclusion

8.1 Quality control checks (identity and purity) of new accessions and existing strains in the collection

The quality of cultures coming into the AWMCC was greatly increased at a small input cost by using antimicrobials and differential media for the culturing of pure strains. This affected both the receival of new cultures and the regeneration of older cultures, which may have had existing contamination or have been a risk of concurrent contamination due to slow growth (e.g. *Oenococcus* or *Brettanomyces* cultures). No drawbacks from these procedural changes have become apparent to date and their adoption has made the retention of pure cultures much simpler and more robust.

The preservation of the genetic diversity of the strains in the AWMCC is paramount to the utility of the collection. The potential for contamination puts at risk the genetic resource assembled over more than 80 years which represents the collective efforts of, and investment into, winemaking research. Methods for isolating pure strains from mixed cultures containing filamentous fungi will continue to be developed. Such methods will make isolating yeast and bacteria from these sources easier and may help with wine spoilage diagnostics.

8.2 Storage of, and access to, wine-associated microorganisms from Wine Australia-funded research projects and the Australian wine industry

Cultures are available to levy payers at a discounted rate to maximise the accessibility of the AWMCC to Australian winemakers. The AWMCC is not only a source of microbiological material for winemakers but is also a secure storage facility for individuals or organisations that have isolated or developed their own genetic resources. The preservation of microbiological material for Australian wine industry participants ensures ongoing access to resources that are important to those participants.

The total number of cultures being handled via deposits, distribution and storage has increased to levels far above those experienced in the AWMCC's history. The numbers alone represent a large increase in workload. They have also allowed some consolidation of strains into high-throughput sub-collections, increasing accessibility to many of these newly deposited isolates.

8.3 Maintenance and back-up of the existing collection

For most of the current project and the project period preceding it, the AWMCC maintained back-up freezer storage at a general-purpose warehouse. While this back-up mitigated the risk

of a catastrophic failure or natural disaster at the Urrbrae facility, it had several drawbacks. Primary among these was the poor situational monitoring available at that location. Because of those limitations, alternative back-up facilities were sought.

The University of Adelaide Biobank was identified as an ideal location for storing archival material. The facility provides storage freezers, with qualified staff responsible for monitoring freezer status. Freezer contents can be rapidly moved to alternative freezers in the event that a freezer fails or needs maintenance. At the time of the writing of this report, a copy of the AWMCC is housed at the University of Adelaide Biobank.

8.4 Evaluation of storage formats compatible with high throughput screening

Different tube and microplate formats were assessed for suitability as high throughput storage formats for microorganisms. A combination of standard 96-well microplates and Nunc OmniTrays were found to be most suitable for short-term processing and storage of microorganisms. FluidX Cluster tubes were deemed to have the best storage integrity and high-throughput sample tracking reliability for ULT freezer storage and high throughput handling. However, the cost of this system rendered it economically unsustainable.

Creating high-throughput sub-collections increased the utility and accessibility of the AWMCC by enabling high-throughput analysis of target genera for a range of attributes (carbon source utilisation, enzyme activity, resistance to xenobiotics etc.). In all, more than 4,000 strains (3,032 yeast and 1,096 bacteria) were transferred to a 96-well format using a combination of manual processing, the Tecan liquid handler and the PIXL single-colony picker. The high-throughput collection is split into distinct screening populations of *Saccharomyces*, *Brettanomyces*, other non-*Saccharomyces*, *Oenococcus*, lactic and acetic acid bacteria, and other species.

These screening populations represent the subset of the strains in the culture collection that were previously stored only in individual storage tubes. The reformatted strains are known to grow at a comparable rate on universal media such that it is practical to screen them with standardised high-throughput protocols. The alternate format is a gateway to many isolates which are currently underutilised. Increasing the accessibility of these isolates can unlock a greater return on investment for the Australian wine industry.

8.5 A review of operational procedures, DNA extraction, quality control and screening methods, and opportunities from emerging sequencing technologies

A review of all operational procedures was undertaken. Procedures were harmonised into a standard template, reviewed for outdated information and collected into a document titled the 'AWMCC Handbook of Policies and Procedures'. The handbook was made available for researchers looking for standard media or propagation protocols.

8.6 Could changing the methodology/technology have improved the outcome?

Sequencing technology is constantly evolving. For example, throughout this project, USB sequencing technology became ubiquitous, increasing both the capacity it can deliver and the accuracy of the data it generates to the point that it has displaced the sequencing technologies that were dominant at the start of the project for many applications. The evaluation that next-generation sequencing was not an economically viable approach to strain identification will almost certainly be incorrect within the next five years. It is possible that alternative solutions to Sanger sequencing of PCR-generated amplicons from the ITS regions of yeast exist already. Therefore, it is possible that changing the methodology could have provided different outcomes for strain identification. However, the technology environment and rate of change in this space made an exploration of all possible alternatives to existing methods challenging with the resources available.

8.7 Practical implications

The recognition of the role that microorganisms play in the many aspects of wine production has never been greater than it is now. Microbial culture collections have a crucial part to play in understanding the microorganisms of the winemaking ecosystem and enabling future wine industry development and sustainability. Culture collections underpin research by providing secure storage and identification services, and providing access to microorganisms with unique genetic features and limited distribution. Culture collections underpin investment in research by securing project outputs beyond the duration of the individual project, enabling access by future generations of researchers and industry participants. Culture collections underpin peer-reviewed publications ensuring effective dissemination of information.

The maintenance and development of the AWMCC has fulfilled these fundamental roles of a culture collection by continual procedural development, creation of new formats to broaden access and utility, and improving security through building and improving system redundancy.

The misidentification of research samples is a well-known and costly problem for biological research and many thousands of scientific articles have reported research with misidentified cells. The cost of repeating primary and downstream research that has involved misidentified material, and of identifying and correcting erroneous results, can be large. Misidentification can have serious and lasting consequences for the ownership of intellectual property. The potential for misidentification is increased when the responsibility for sample maintenance is distributed to individual researchers. Problems such as inconsistent use of storage protocols, inadequate record-keeping, lost samples, freezer breakdown risks, handling and labelling errors are all multiplied by the number of individuals involved in maintaining biological samples. These risks have been greatly mitigated by the centralised management of biological material through the AWMCC, the use of which has underwritten the success of many past and present industry-funded projects.

9. Recommendations

Wines, especially in storage, often contain mould spores and, although harmless, these make the analysis of associated microbes impractical. Ingredients that limit the spread of filamentous fungi have been trialled in conjunction with the Affinity Labs Applied Biosciences team, and variations to the isolation procedure for yeast from wine or environmental sources need to be formalised into AWMCC standard practice.

Advanced databases are crucial to knowledge transfer. The database currently used to maintain the AWMCC is limited in its ability to accommodate certain types of information and in the way that information can be accessed. In the medium term, the way that AWMCC is housed needs to be reviewed, the underlying data structure needs to be aligned with best-practice systems for descriptions of biological diversity and the interface modernised to facilitate access to a wider community of researchers and winemakers.

Discussions with other culture collection and biobank facilities are underway to determine the most appropriate path forward in the management of culture collection metadata. The AWMCC is not alone in facing this issue. Nevertheless, transitioning and modernising culture collection software is imperative for future functionality and accessibility.

Appendix 1: Communication

Online resources:
Simon Dillon is currently a co-author in the development of *the ISBER Best Practices: Recommendations for Repositories*, Fifth Edition

AWRI fact sheet. 2021. *Vinegar production – inoculation of a base wine with live acetic acid bacteria*. Available from: https://www.awri.com.au/wp-content/uploads/2022/04/Inoculation-of-wine-with-acetic-acid-bacteria-for-vinegar-production.pdf

Industry articles:
Dillon, S. 2021. How the identification of wine microorganisms has evolved within the AWRI Wine Microorganism Culture Collection. *AWRI Tech. Rev.* 251:4-10.

Forgan, A., Dillon, S. 2017. The AWRI Wine Microorganism Culture Collection. *AWRI Tech. Rev.* 231:12-15.

Images:
Several images kept as a part of the AWMCC's database have been used for publications in *Wine and Viticulture Journal* articles on spoilage yeast and in *Saccharomyces cerevisiae* and indigenous yeast interaction, both in 2017.

One image of a *Brettanomyces bruxellensis* strain (AWRI 1499) was gifted in 2021 for inclusion in the second edition of *Scientific Principles of Malting and Brewing* by Charlie Bamforth and Glen Fox.

Presentations:
Simon Dillon was an invited speaker to the 2021 Annual Meeting of the International Society for Biological and Environmental Repositories (ISBER) and gave a talk entitled 'Fermentation microbes -the real workers in innovative products for the future'

Simon Dillon was an invited speaker to the Australasian Biospecimen Network Association (ABNA) 18th Annual Conference 2021 and gave a talk entitled 'Fermentation Microbes-the real workers in future product innovation'

Simon Dillon gave a talk for a Science Week event in 2018 titled 'Grape Expectations! The Future of Wine'. This event aimed to give wine drinkers an opportunity to learn more about the science of wine.

Simon Dillon gave a talk for a Science Week event in 2017 titled 'The Complete Wine Science Spectrum'. This event adapted wine science research for wine drinkers to learn more about the science of wine.

Poster:
AWITC poster 2019 *The AWRI Wine Microorganism Culture Collection – Securing the future of Australian wine microorganisms.* Angus Forgan and Simon Dillon

Appendix 2: Intellectual property

Program IP has been communicated through presentations and publications and resides in the public domain. Copyright in any reports, posters or presentations is held by the AWRI. Copyright in publications remains with the publisher. No inventions or patentable discoveries have been identified from the work. No Program IP has been commercialised or identified for commercialisation at the time of writing this report.

Appendix 3: References

Bokulich, N. A. and Mills, D.A., 2013. Improved Selection of Internal Transcribed Spacer-Specific Primers Enables Quantitative, Ultra-High-Throughput Profiling of Fungal Communities. A.E.M. 79(8): 2519 – 2526

Dillon, S. 2021. How the identification of wine microorganisms has evolved within the AWRI Wine Microorganism Culture Collection. *AWRI Tech. Rev.* 251:4-10

Kelly, W.J., Asmundson, R.V., Hopcroft, D.H., 1989. Growth of *Leuconostoc oenos* under anaerobic conditions. *Am. J. Enol. Vitic.* 40(4): 277-282.

Esteve-Zarzoso, B., Belloch, C., Uruburu, F., Querol, A., 1999. Identification of yeasts by RFLP analysis of the 5.8 S rRNA gene and the two ribosomal internal transcribed spacers. *Int. J. System. Evol. Microbiol.* 49(1): 329-337.

White, T.J., 1990. Application and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Innis, M.A., Gelfand,D.H., Sninsky, J.J., White, T.J. (Eds.) *PCR protocols: a guide to methods and applications*San Diego, California: Academic Press, Inc: 315-325.

Appendix 4: Staff

Simon Dillon – Technical Officer (2017-2020); Culture Collection Manager (2020-2022)
Angus Forgan -Culture Collection Manager (2016-2020)
Laura Hale – Technical Officer (February 2021- December 2021)
Caroline Bartel - Technical Officer (August - October 2021)

Appendix 5: Other supporting material (including raw data, if appropriate)

Appendix 5a. Safety Data Sheet for culture distribution

Chemwatch: 9-716202

Chemwatch Hazard Alert Code: **0**

MINI SDS

Yeast Culture Slope

INGREDIENTS	CAS NO	%	8HR OEL
D-glucose	50-99-7	2	-
peptones	73049-73-7	2	-
yeast extract	8013-01-2	1	-
yeast, dried	68602-94-8	1	-

Description: Yeast culture cultured on agar
Product use: Wine making or research
Supplier name and address:

The Australian Wine Research Institute

GHS **DG**

UN No: **Not Applicable**
DG Class: **Not Applicable**
Not ApplicableSubsidiary Risk: **Not Applicable**
Packing Group: **Not Applicable**

HEALTH HAZARD INFORMATION

Signal word:	
Hazard statement(s):	Not Available

PRECAUTIONS FOR USE



Appropriate engineering controls:	General Exhaust Ventilation adequate.
Glasses:	Consider chemical goggles.
Gloves:	When handling larger quantities: General purpose rubber glove.
Storage and Transportation:	Store in cool, dry, protected area.

PROPERTIES



Liquid.Does not burn.

EMERGENCY



FIRST AID

Swallowed:	Rinse mouth with water.
Eye:	Wash with running water. For discomfort seek medical advice.
Skin:	Wash with soap
Inhaled:	Fresh air. Rest, keep warm.
Advice To Doctor:	Treat symptomatically.
Fire Fighting:	Keep surrounding area cool. Water spray/fog.
Spills and Disposal:	Absorb with dry agent. Stop leak if safe to do so.

SAFE STORAGE WITH OTHER CLASSIFIED CHEMICALS



x — *Must not be stored together*
0 — *May be stored together with specific preventions*
+ — *May be stored together*

Product & useage

Product name: Agar Yeast slope
PO Box 197, Glen Osmond, SA, 5064, Australia
Contact telephone: +61 8 313 6600
FAX :+61 8 8313 6601

Date of MSDS preparation: January 2020 Print Date: 05/02/2020

Chemwatch: 9-716202

Issue Date: 28/01/2020

Appendix 5b. AWMCC culture specification sheet

Date: 23/11/2021	**IP rights:** This document is the property of the AWRI
Accession number and species	**AWRI B545** ***Acetobacter pasteurianus***

REVISION HISTORY				
Review	Description of change	Pages	Author	Date
0	Document creation	3	Simon Dillon	23/11/2021
1	Document revision	3	Simon Dillon	19/07/2022

Check	Requirement
Material Transfer Agreement	Available under MTA only
Customs and Importation	Responsibility of recipient
Genetically Modified Organisms	Ability to handle GMO required
IP, strain ownership and commercialisation	Remains property of AWRI according to MTA

1. PRODUCT

GENERAL SPECIFICATIONS

1. Origin

Culture Origins	Details
Culture Received	30/05/2006
Culture Source	Winery
Sample Type	Cabernet Sauvignon
Region	Victoria, AUSTRALIA
References	N/A

2. Strain

Taxonomy	
Genus	Acetobacter
Strain	pasteurianus
Subspecies or biotype	N/A

Other collections	-

3. Identification

Identification	Details
Identification method	16S sequencing
Date of Identification	2018
Identification performed by	Dillon, S.

4. Culturing

Variable	
Culture medium	MRS+A agar, GYC agar, WL agar
Temperature	28° C
Aerobic or Anerobic	aerobic
Incubation time	3-5 days

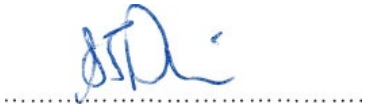
5. Defects in Packaging and Internal Contents

a. GENERAL SPECIFICATIONS

Description of defect	category		
	critical	major	minor
leaking export package		X	
internal polycarbonate packaging damaged and contents open to environment	X		
contamination with bacteria in slope			X
contamination with yeast or other fungi in slope		X	

b. RESPONSE TO DEFECTS

Upon recognition of a major or critical defect, the recipient should decontaminate and destroy all contents, including packaging. Other defects can be remediated. Contact AWRI for further information.



Signature of authorised person

Simon Dillon

Culture Collection Manager-AWRI

- --- End of document - ---

Appendix 5c. Processing Schedule – Fixation and dehydration for SEM

Adelaide Microscopy

PROCESSING SCHEDULE
FIXATION AND DEHYDRATION FOR SEM









The following SEM protocol incorporates a Safe Operating Procedure for fixation and dehydration. Note that contact lenses may pose a special hazard; soft contact lenses may absorb and concentrate irritants.

All processing must be carried out in a fume cupboard.

Personal protective equipment must be used











Danger

Formaldehyde (paraformaldehyde) is corrosive (Class 8) and a suspected human carcinogen. It is toxic by inhalation or ingestion and in contact with skin, and can cause eye burns or blindness. Formaldehyde is a strong sensitizer and can cause irritation to skin, eye and respiratory tract. Avoid exposure and do not breathe vapour/fumes. Dispose of liquid and solid waste in appropriate waste container in the fume cupboard.

Glutaraldehyde is corrosive (Class 8) and toxic (Class 6.1). Glutaraldehyde is toxic by inhalation; Avoid exposure and use it in a fume cupboard. Do not breathe vapour/fumes. Glutaraldehyde causes burns and may cause serious damage to eyes. It may cause sensitisation by inhalation and skin contact. Very toxic to marine life. Dispose of liquid and solid waste in appropriate waste container in the fume cupboard.

Osmium Tetroxide is extremely hazardous (DG Class: 6.1). Authorised use only. It is fatal if swallowed, comes in contact with skin or inhaled. Osmium can cause severe skin burns and eye damage. Avoid exposure and use ONLY in a fume hood. Keep triple contained, tightly closed and stored in a locked fridge. Dispose of liquid and solid waste in appropriate waste container in the fume cupboard.

Ethanol is highly flammable (Class 3). Do not breathe vapour/spray. Ethanol is irritating to eyes. Dispose of ethanol waste in the appropriate waste container in the fume cupboard.

Hexamethyldisilazane (HMDS) is extremely hazardous. It is flammable (Class 3). It is toxic by inhalation and very toxic in contact with skin (Subsidiary risk 6.1). It is irritating to eyes and skin. Avoid exposure and do not breathe vapour/fumes.

Adelaide Microscopy

PROCESSING SCHEDULE AND DEHYDRATION FOR SEM

All processing must be carried out in a fume cupboard. Personal protective equipment must be used

1. Fix for at least 30 minutes in 4.0% Paraformaldehyde/1.25% Glutaraldehyde in PBS, 4% sucrose, pH7.2.

   All steps involving glutaraldehyde and formaldehyde are carried out in a fume hood. Discard solid and liquid formaldehyde-contaminated waste into the appropriate waste container in fume cupboard.

2. Wash in PBS + 4% sucrose – 2 x 5 mins.

   Discard formaldehyde-contaminated waste into the appropriate waste container in fume cupboard

3. Post-fix in 2% OsO_4 in PBS - 1hr.

   Authorised use only. All steps involving osmium are carried out in a fume hood. Discard osmium-contaminated waste into the appropriate waste container in fume cupboard.

4. Wash in PBS + 4% sucrose – 2 x 5 mins.

   Discard osmium-contaminated waste into the appropriate waste container in fume cupboard.

5. Dehydration series

   70% ethanol - 3 changes of 15 mins each

   If solution is still grey it has osmium contamination. Discard osmium-contaminated waste into the appropriate waste container in fume cupboard.

   90% ethanol - 3 x 15 mins

   100% ethanol - 3 x of 15 mins

6. Drying samples

   a. Hexamethyldisilazane (HMDS).

      1:1 100% Ethanol:HMDS for 20 mins

   Discard HMDS-contaminated waste into the appropriate waste containers in fume cupboard.

      100% HMDS 2 x 20 mins each

      Pipette off HMDS and air dry samples, partly covered with lid of petri dish to protect from dust.

   b. Critical Point Dryer (AM operator)

7. Mount on aluminium stub with sticky tabs, carbon tabs, etc

8. Coat with carbon or platinum, etc