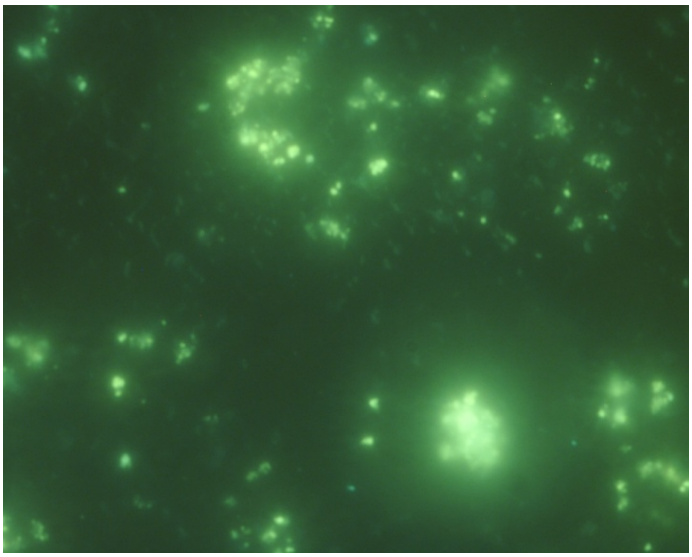
THE UNIVERSITY OF ADELAIDE
AUSTRALIA

Australian Government
Grape and Wine Research and Development Corporation

Identification of salt accumulating organisms from winery wastewater



FINAL REPORT to

GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

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Principal Investigator: **Paul Grbin**

Research Organisation: University of Adelaide

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Identification of salt accumulating organisms from winery wastewater

Dr Paul R Grbin

Dr Kathryn L Eales

Dr Frank Schmid

Assoc. Prof. Vladimir Jiranek

The University of Adelaide

School of Agriculture, Food and Wine

PMB 1, Glen Osmond, SA 5064 AUSTRALIA

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Abbreviations:

COD: Chemical oxygen demand
Ec: Electrical conductivity
FACS: Fluorescence activated cell sorting
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
OD: Optical density
PBFI: Potassium benzofuran isophthalate
PI: Propidium iodide
SAR: Sodium adsorption ratio
WWW: Winery wastewater

Abstract:

In an attempt to find microorganisms that would remove salts from biological winery wastewater (WWW) treatment plants, 8 halophiles were purchased from culture collections, with a further 40 isolated from WWW plants located in the Barossa Valley and McLaren Vale regions. These organisms were assessed for their ability to accumulate intracellular salt (in particular potassium), remove colour, reduce chemical oxygen demand, change pH and lower the electrical conductivity of a synthetic wastewater. Furthermore, fluorescence activated cell sorting (FACS) was employed to separate those cells that could accumulate higher concentrations of intracellular potassium. The outcomes of this study indicate that the many halophiles naturally occurring in WWW exhibit a large phylogenetic diversity, and some accumulated large concentrations of intracellular potassium, therefore further investigation is warranted to determine the applicability of these organisms in WWW treatment.

Executive summary:

- Halophilic synthetic winery wastewater (WWW) was designed to isolate halophiles from WWW. A total of 40 isolates were grown under aerobic and anaerobic conditions.
- These isolates were identified using 16S rRNA gene sequence analysis, and a large phylogenetic diversity was observed.
- The ability of these isolates to accumulate intracellular salts (particularly potassium, but also sodium, magnesium and calcium) was determined and some isolates accumulated 5 – 20 times more than others.
- Isolates were also evaluated for their effects on pH and Ec, and reduction in colour and chemical oxygen demand (COD). Generally, isolates did not affect pH or Ec, however there were slight changes in colour (hue) and COD (decrease).
- To eliminate the bias imposed by culture dependant techniques, environmental samples were directly evaluated for intracellular potassium concentration using the fluorescence indicator PBFI with fluorescence activated cell sorting (FACS). Those that gave very strong PBFI signals were separated from the remainder of the population. The DNA was then extracted, the 16S rRNA gene PCR amplified, amplicons separated by insertion into a plasmid and then identified by gene sequencing.
- Five WWW samples were examined by FACS/clone library. Four of the five clone libraries were dominated by a single species, *Burkholderia phytofirmans* strain PsJN.
- This study suggests that halophiles naturally occur in WWWs, with some (*Halomonas, Psychrobacter and Arthrobacter sp.*) exhibiting the potential to accumulate large concentrations of intracellular potassium.

Background:

High salt content often persists throughout the biological treatment of WWW and can remain too high to allow its use as recycled irrigation water for grapevines. In 2008, 520,393 ML of water was used for the irrigation of grapevines across Australia, with only 0.9% of that being recycled water (Australian Grape and Wine Industry 2008). The build up of salts, especially sodium and potassium, limits the application of WWW to land as it leads to poor soil health affecting plant growth and vitality. Although potassium is an essential nutrient for healthy grapevine growth, excess accumulation in the berry can negatively influence wine quality (Mpelasoka et al. 2003). In the report to the GWRDC (CSL02/03) Kumar et al. (2006) clearly established that salinity, sodicity and available potassium were elevated in soils treated with WWW. Potassium originates from the grape skins and sodium predominantly from cleaning products eg. sodium citrate and sodium hydroxide. In Australia, a sodium adsorption ratio (SAR) of 6 is considered the benchmark of sodicity. Winery wastewater was found to be highly sodic (SAR = 10-35) throughout the 2006 vintage (Kumar et al. 2006). Therefore, these elevated salt levels in WWW are a key limiting factor in its sustainable use in irrigation. Examples of current strategies to remove salts include reverse osmosis, which is expensive and clearly not economically viable or evaporation ponds, which results in no reuse of water.

Biological removal of organic nutrients and salt (in particular phosphorus) has been studied in depth for domestic biological wastewater treatment systems (Seviour et al. 2003). For example, organisms that accumulate inorganic phosphorus in excess of their physiological requirements have been identified (eg. *Candidatus* 'Accumulibacter phosphatis') and their growth encouraged by anaerobic/aerobic cycling of the biomass, increasing the overall quality of effluent and decreasing its harmful impacts on the environment. This demonstrates that improvements to these systems can be made through a sound understanding of their microbiology. A similar approach was undertaken to investigate the use of halophiles to reduce the salt concentration in WWW. Halophiles are organisms that thrive in high salt environments. In order for these organisms to survive their cytoplasm has to be isoosmotic with their surrounding environment. There are 3 main strategies employed by organisms to achieve this:

1. Biosynthesis or accumulation of "compatible solutes"; organic osmotic solutes
2. Accumulation of intracellular potassium and chloride
3. A combination of mechanism 1 and 2

Strategy 2 is largely restricted to the "extreme halophiles", those that require >2.5 M salt, much higher than WWW. Therefore in this study we targeted organisms that employ strategy 3, which includes moderate halophiles.

Halophiles have been used in other industrial wastewater treatment plants to improve nutrient removal and remove colours (Kargi 2002). To date, no research has been carried out on WWW to investigate the presence and use of such potentially beneficial bacteria. The identification of

such organisms could lead to the development of a novel treatment process for WWW and allow the sustainable reuse of a key water resource.

The aims of this project were to:

1. Study intracellular salt content in microorganisms from WWW through the use of molecular and fluorescent identification/screening techniques.
2. Investigate microorganisms capable of accumulating high levels of intracellular salt from the activated sludge of WWW.
3. Isolate and identify these organisms.
4. Explore the impact of inoculating WWW with wastewater isolates and halophiles known to accumulate high levels of salts.

Project Outputs and Performance Targets

Table 1. Project outputs and performance targets

Outputs	Performance Targets
1. Protocol for applying fluorescent dyes to control organisms and WWW biomass	Staining and visualization of organisms with high intracellular Na/K levels.
2. Identification of organisms with high intracellular salt levels	Separation of organisms containing high intracellular salt levels by flow cytometry and identification by gene sequencing.
3. Effect of dosing WWW with known species of halophiles	Compare salt concentrations of winery effluent in the presence and absence of halophiles
4. Dissemination of project findings.	At least one conference presentation or publication detailing the potential use of microorganisms to reduce salt concentrations.

Methods and materials:

1. Winery wastewater treatment plants examined in this study

Wastewater samples (50 mL) were collected from plants in the Barossa Valley and one plant in the McLaren Vale, and kept on ice for no longer than 24 h before analysis. Each sample was microscopically (Olympus BH fitted with an Olympus DP50 digital camera) examined after Gram staining (Lindrea et al. 1999).

2. Growth of purchased halophiles

The following strains were purchased and selected to reflect a large genetic diversity of halophiles.

- ACAM591 *Shewanella frigidimarina*^{T}
- ACAM35 *Halomonas elongata*^{T}
- ACAM547 *Pseudoalteromonas haloplanktis*^{T}
- DSMZ 13855 *Salinibacter ruber*^{T}
- DSMZ 2228 *Halanaerobium praevalens*^{T}
- DSMZ 3754 *Halobacter salinarum*^{T}
- DSMZ 17747 *Marinobacter vinifirmus*^{T}
- DSMZ 3050 *Pseudomonas halophila*^{T}

Each organism was cultured using the recommended media and conditions (http://www.dsmz.de/microorganisms/bacteria_catalogue.php). ^{T} = type strain

3. Isolation of halophiles from winery wastewater

Halophilic WWW (Table 2) was designed based on published formulations (Bories et al. 2005, Colin et al. 2005), and modified to include red wine colour compounds and elevated levels of sodium and potassium salts.

Table 2. Chemically defined halophilic winery wastewater.

Solids	g/L	Solutions	mL/L
tartaric acid	1	lactic acid	0.4
yeast extract	1	acetic acid	0.6
casamino acids	1	ethanol	5
fructose	1	glycerol	0.6
glucose	1	colour extract[1]	50
NaCl	20	trace elements[2]	0.1
K_2HPO_4	20	vitamins[2]	0.125
CaCl	5		
$MgSO_4$	5		

[1] Polyphenol powder from red grapes (0.1% in ethanol, Tppr, Oeno Prod, France).
[2] Trace elements and vitamins made up according to Jiranek et al (1995).
pH adjusted to 7.4 with KOH.

3.1 Microbial growth, pH and Ec measurments.

Microbial growth was monitored as OD_{550} (Spectronic Helios α UV-Vis spectrophotometer, Thermo Fisher Australia). Electrical conductivity was measured on cell free supernatants using a Cyberscan Con 11 conductivity/TDS/°C meter fitted with a conductivity electrode (ECCONSEN 91W, Eutech Instruments, Adelab). Culture pH was measured using a Cyberscan 1100 pH meter fitted with a Eutech pH electrode (Adelab).

3.2 COD measurements

COD was determined using the CHEMetrics COD (Mercury free) Vials kit (0-1500 ppm) as per manufactures instructions.

3.3 Colour measurements

Colour values (CIELab) of cell free supernatants were determined by measuring the visible transmission spectrum from 380 nm to 780 nm in 5 nm increments (75 µL in a 96-well plate, µQuant Microplate Spectrophotometer, Bio-Tek Instruments). The spectral data was then converted to L^* (lightness, 0 – black to 100 - white), a^* (-265, green to 265, magenta) and b^* (-265, blue to 265, yellow) using the conversion equations and tables developed by the Commission Internationale d'Eclairage. Results are expressed using a D65 illuminant at 10° standard observer. Hue (H, 0° - 360°) and chroma (C, 0 - 100) values were calculated using the following equations:

$$H = arctan\left(\frac{b^*}{a^*}\right) \times \frac{360°}{2 \times 3.142} \qquad C = (a^{*2} + b^{*2})^{0.5}$$

3.4 Salt and sodicity measurements

Intracellular salts were extracted by centrifuging a cell suspension (2.5 mL, OD_{550} = 0.75, 15,000 x *g*, 20 mins) through 2.5 mL of silicone oil (Xavier et al. 2008). Pellets were resuspended in H_2SO_4 (2.0 mL, 2.7 M) and mineralised (4 h, 120°C). Intracellular salt and supernatant salt concentrations were quantified by atomic absorption at Waite Analytical Services (The University of Adelaide, Waite Campus). Subsequently, sodicity or the sodium adsorption ratio (SAR) was calculated using the following equation:

$$SAR = \frac{[[Na]^+]}{\sqrt{\frac{[[Ca]^{2+}] + [[Mg]^{2+}]}{2}}}$$

4. Fluorescent staining of cells for intracellular potassium

A working solution of tetra[acetoxymethyl] ester of the K^+-binding fluorescent dye benzofuran isophthalate (50 µg, PBFI, Invitrogen) was prepared by re-suspension in DMSO (8.5 µL) and

pluronic acid F-127 (8.5 μL, Invitrogen). Cultures (OD_{550} = 0.3, 1 mL) were centrifuged (14,000 rpm, 5 min, Eppendorf Centrifuge 5417R), then resuspended in 996 μL loading buffer (10 mM HEPES, 0.5% glucose), and incubated in the dark for 2 h after the addition of PBFI working solution (4 μL). Samples were then washed 3 x 1 mL loading buffer and finally resuspended in 200 μl of loading buffer. Positive controls for intracellular potassium were prepared by incubating cells with the cell membrane disrupter valinomycin (final concentration of 5 μM, Sigma) at room temperature for 15 minutes. After this time a high potassium solution (final concentration 1 mM K^+) was added and due to the leaky membranes able to diffuse into the cell giving a positive fluorescence signal. Negative controls (non-dyed) were prepared to visualise any autofluorescence.

5. Fluorescence microscopy

Samples were visualised using an Olympus BH fluorescence microscope (excitation 340-380 nm, emission 500 nm). Images were captured using an Olympus DP50 digital camera.

6. Fluorescence Activated Cell Sorting (FACS)

Before flow cytometry, samples were sonicated for 2 x 45 sec (UP400S fitted with a radial probe, Hielscher Ultrasound Technology, Germany) at maximum setting to disrupt aggregates and floc structures. Suspensions were then filtered through 40 μm nylon mesh filters (353335 Falcon), and aliquots (1 mL) centrifuged (14,000 rpm, 5 min, Eppendorf Centrifuge 5417R), supernatants discarded and cells resuspended in loading buffer (996 μL). Before staining all cell samples were diluted to 0.3 OD_{550} (approx 1 mg/mL of dry cell weight). Cells were stained with PBFI as outlined above. Propidium iodide (PI, 1 μL, 10 mg/mL, Invitrogen), an indicator of dead cells, was added such that only viable cells with high intracellular potassium were gated for (see Results). Also included were positive controls (treated as outlined above), and PI controls were made by autoclaving samples and staining. The negative control was an unstained sample.

Cells were sorted on a fluorescence activated cell sorter (FACS) Vantage flow cytometer (Becton Dickson). Ten thousand cells from each sample were analysed. PBFI was excited by a multiline high UV output laser (Coherent, Innova 90C) and propidium iodide (viability stain) by a 488 nm argon ion laser. Gates were set on a PBFI *vs* PI dot plot. Sorted cells were stored on ice prior to further analysis.

7. DNA extraction and amplification

Pure cultures and FACS sorted cell DNA was extracted using a MoBio Laboratories UltraClean Soil DNA kit (Geneworks, Melbourne) in accordance with the manufacturer's instructions. Amplification of the 16S rRNA gene was based on the method of Felske et al. (1998) with 27f and 1525r primers. The Qiagen PCR Purification Kit was used to purify PCR products to improve the efficiency of subsequent cloning and sequencing.

8. Cloning of FACS sorted 16S rRNA fragments

In order to separate and isolate the 16S rRNA gene fragments of the mixed FACS populations, cloning was performed using the pGEM-T Easy Vector System II (Promega) and plasmids were extracted using the QIAprep Miniprep kit (Qiagen).

9. Sequencing of PCR products from pure cultures and FACS cells

16S rRNA gene amplicons from pure cultures and plasmid inserts were sequenced with the primers M13F, M13R, 27F, 530F, 907R and 1525R at AGRF Sequencing Facility (AGRF, Brisbane).

10. Construction of 16S rRNA gene phylogenetic trees

Phylogenetic trees were inferred by aligning nearly complete 16S rRNA gene sequences using clustal-W accurate (Thompson et al. 1994) and applying maximum likelihood (Felsenstein 1981) tree making algorithms.

Results and Discussion:

Output 1. Protocol for applying fluorescent dyes to control organisms and WWW biomass

A protocol was successfully developed for the labelling of intracellular potassium using the fluorescence indicator PBFI. Differences in the intensity of the fluorescence signal are proportional to the concentration of intracellular potassium (Fig. 1). The protocol is detailed in Methods Section 4: *Fluorescent staining of cells for intracellular potassium*. WWW samples were further evaluated by fluorescence microscopy or by Fluorescence Activated Cell Sorting (FACS) using this method.

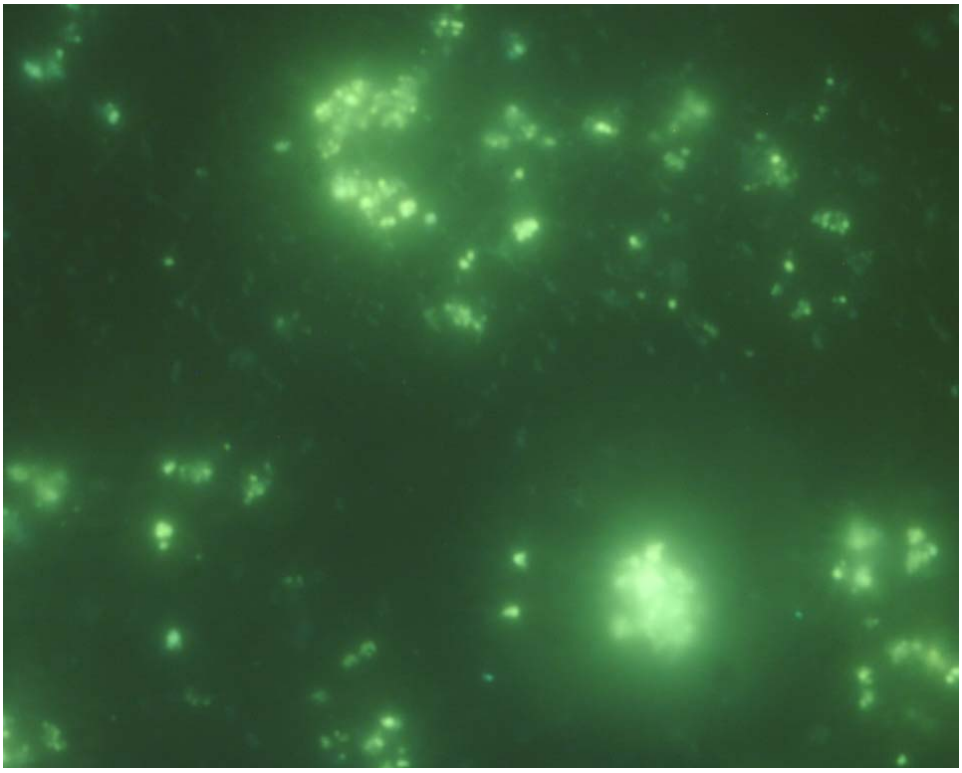


Figure 1. An environmental WWW sample stained with PBFI. The green fluorescent intensity is proportional to the intracellular potassium concentration.

Output 2: Identification of organisms with high intracellular salt levels

In order to maximise the probability of isolating viable halophiles, as soon as possible after sample collection, the biomass was examined microscopically to identify organisms and assess biomass vitality and structure. A balanced activated sludge consisting of single bacterial cells, some filamentous bacteria and protozoa is reported to be ideal (Seviour and Blackall 1999). Protozoa (Fig. 2a) are an excellent indicator of wastewater treatment plant health. If the population disappears it can be an early indicator of toxicity as they are more sensitive to water

changes than bacteria. Conversely, if the population increases and begins to dominate the activated sludge, the bacteria are most likely dead, which protozoa thrive on (Seviour and Blackall 1999).

Filamentous bacteria (Fig. 2b) are also good indicators of plant health. They are essential for good settling characteristics of activated sludge in clarifiers. However, if the system becomes unbalanced they can proliferate to extreme levels causing solid separation problems such as bulking and foaming.

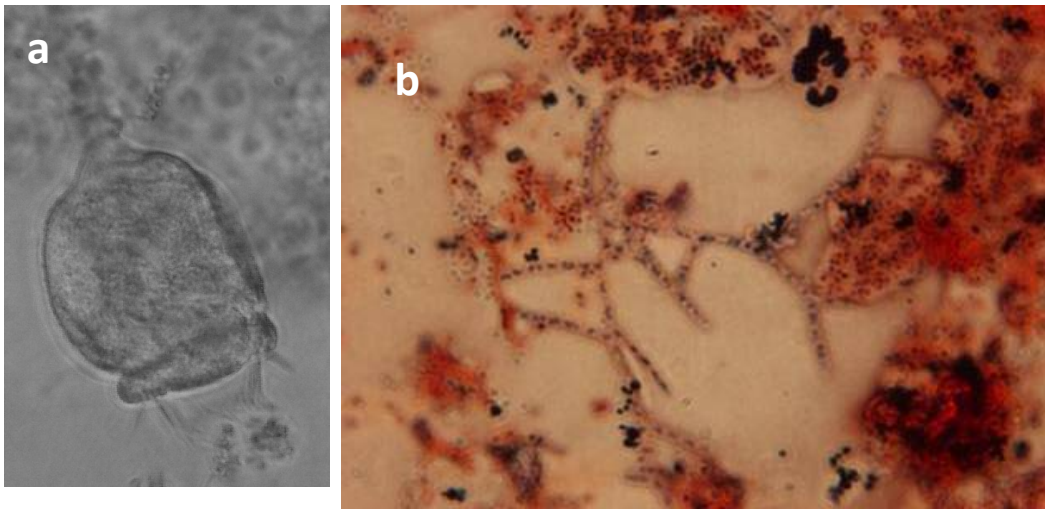


Figure 2. (a) Wet mount micrograph of a protozoa *Vorticella* spp., and (b) Sudan Black stain showing poly hydroxyl alkanoate storage material (black spots) in GALO and *Zoogloea* in a bulking WWW sample.

Healthy biomass samples were plated onto halophilic synthetic WWW media. Forty colonies were isolated and re-plated to obtain pure cultures. Each isolate was identified by comparison of its 16S rRNA gene sequence (BLASTn, Benson et al. 2004). The 16S rRNA gene is commonly used for the identification of bacteria as it is a mosaic of highly conserved and highly variable regions that can discriminate between organisms down to the species level (Bull et al. 2000). The phylogenetic tree (Fig. 3) generated by a subset of this data indicates a large diversity of halophiles naturally occurring in these treatment plants. Representatives from 16 genera from 4 classes namely *Betaproteobacteria*, *Gammaproteobacteria*, *Bacilli* and *Actinobacteria* were identified (Table 3).

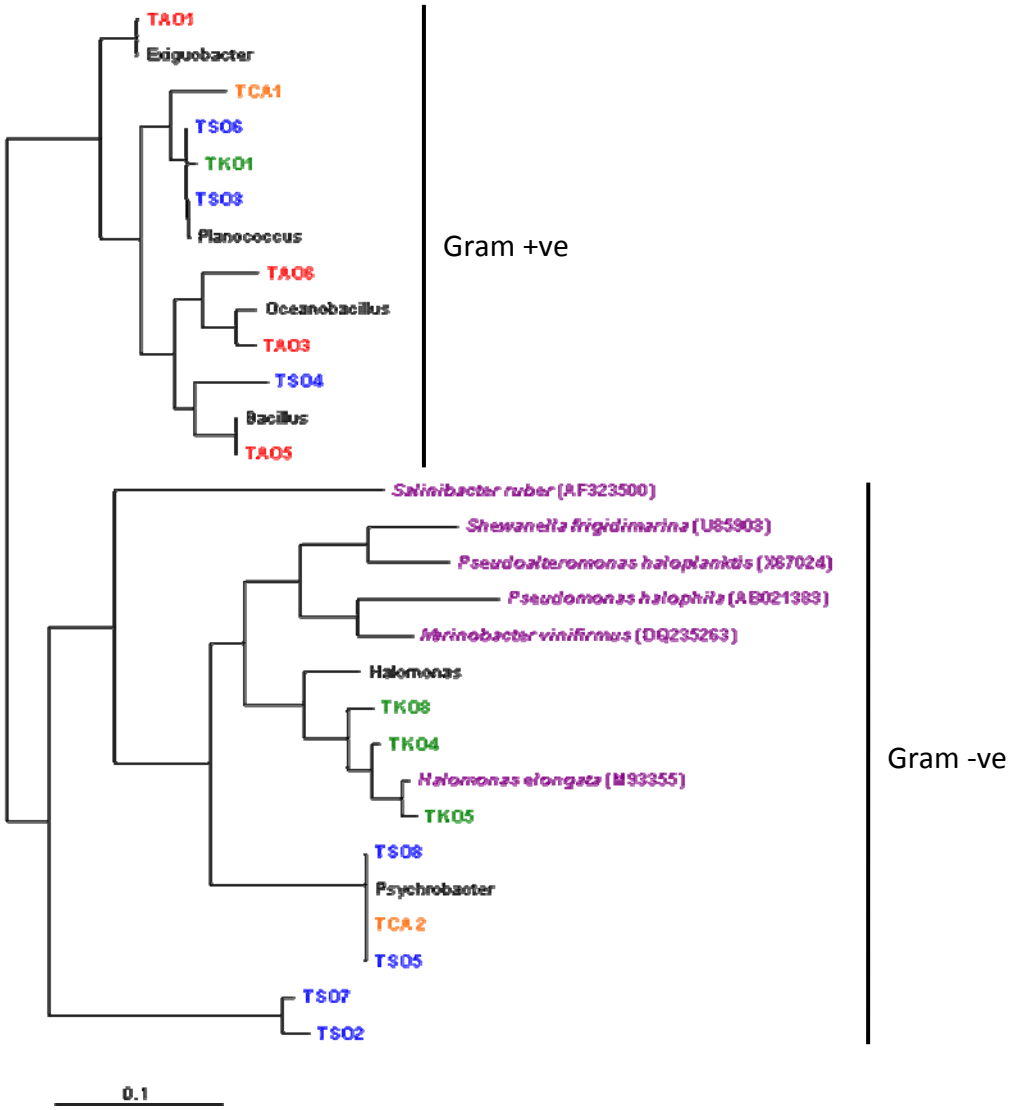


Figure 3: A 16S rRNA tree showing the phylogentic affiliation of the WWW isolates. Isolates obtained from the same treatment plant share the same colour, with purchased halophiles depicted in purple. The tree was calculated using the maximum-likelihood method.

Table 3. Identity of 40 pure culture isolates based on comparative 16S rRNA gene sequence analysis**.**

Organism	Identity	Family	Class
TK01	*Planococcus* sp.	*Planococcscaea*	*Bacilli*
TK02	*Burkholderia fungorum*	*Burkholderia*	*Betaproteobacteria*
TK03	*Burkholderia fungorum*	*Burkholderia*	*Betaproteobacteria*
TK04	*Halomonas virabilis*	*Halomonadaceae*	*Gammaproteobacteria*
TK05	*Halomonas virabilis*	*Halomonadaceae*	*Gammaproteobacteria*
TK06	*Halomonas* sp.	*Halomonadaceae*	*Gammaproteobacteria*
TK07	*Planococcus* sp.	*Planococcscaea*	*Bacilli*
TK08	*Halomonas* sp.	*Halomonadaceae*	*Gammaproteobacteria*
TS01	*Bacillales bacterium* NR100	*Bacillaceae*	*Bacilli*
TS02	*Arthrobacter*	*Micrococcaceae*	*Actinobacteria*
TS03	*Planococcus rifitiensis*	*Planococcscaea*	*Bacilli*
TS04	*Staphylococcus succinus*	*Staphylococcaceae*	*Bacilli*
TS05	*Psychrobacter faecalis*	*Moraxellaceae*	*Gammaproteobacteria*
TS06	*Planococcus rifitiensis*	*Planococcscaea*	*Bacilli*
TS07	*Arthrobacter*	*Micrococcaceae*	*Actinobacteria*
TS08	*Psychrobacter pulmonis*	*Moraxellaceae*	*Gammaproteobacteria*
TA01	*Exiguobacterium sibiricum*	*Bacillaceae*	*Bacilli*
TA02	*Bacillus* sp	*Bacillaceae*	*Bacilli*
TA03	*Oceanobacillus sp*	*Bacillaceae*	*Bacilli*
TA04	*Serratia* sp.	*Enterobacteriaceae*	*Gammaproteobacteria*
TA05	*Bacillus pumilus*	*Bacillaceae*	*Bacilli*
TA06	*Bacillus gibsonii*	*Bacillaceae*	*Bacilli*
TA07	*Serratia grimesii*	*Enterobacteriaceae*	*Gammaproteobacteria*
TA08	*Betaproteobacteria* sp.	*Betaproteobacteria*	*Betaproteobacteria*
TCA1	*Dietzia maris*	*Dietziaceae*	*Actinobacteria*
TCA2	*Nocardia* sp.	*Nocardiaceae*	*Actinobacteria*
TCA3	*Kocuria carniphila*	*Micrococcaceae*	*Actinobacteria*
TCA4	*Rhodococcus erythropolis*	*Nocardiaceae*	*Actinobacteria*
TCA5	*Rhodococcus erythropolis*	*Nocardiaceae*	*Actinobacteria*
TCA6	*Dietzia maris*	*Dietziaceae*	*Actinobacteria*
TCA7	*Nocardia* sp.	*Nocardiaceae*	*Actinobacteria*
TCA8	*Actinomycetes* sp.	*Actinomycetes*	*Actinobacteria*
TSA1	*Rhodococcus sp.*	*Nocardiaceae*	*Actinobacteria*
TSA2	*Bacillus sp.*	*Bacillaceae*	*Bacilli*
TSA3	*Bacillus sp.*	*Bacillaceae*	*Bacilli*
TSA4	*Betaproteobacteria clone*	*Betaproteobacteria*	*Betaproteobacteria*
TSA5	*Bacillus gibsonii*	*Bacillaceae*	*Bacilli*
TSA6	*Pseudomonas sp.*	*Pseudomonadaceae*	*Gammaproteobacteria*
TSA7	*Rhodococcus erythropolis*	*Nocardiaceae*	*Actinobacteria*
TSA8	*Rhodococcus sp.*	*Nocardiaceae*	*Actinobacteria*

As it is estimated that only 1% of any microbial population can be cultivated in pure culture (Bull et al. 2000) a molecular/non-culture technique was employed to increase the likelihood of isolating candidate bacteria for further evaluation. For this FACS in conjunction with PBFI and PI was used. Controls were used to set the gating parameters to ensure that only viable cells with high intracellular potassium were collected (data not shown). Figure 4 shows a typical plot of PBFI *vs* PI intensities of WWW cells passing through the flow cytometer. Cells with high viability and low intracellular potassium are represented as green events (below intensities of 10^3). Cells that were viable and high in intracellular potassium are represented by the blue events. The instrument was gated such that it would only sort and collect cells in this region.

Of all clones (100) recovered by FACS (Fig. 5), 42% were identified to be *Burkholderia phytofirmans*, with a further 19% being *Burkholderia* species. This genus is not well resolved using 16S rRNA sequencing alone (Garrity et al. 2005). Although it is not listed as a halophile, pure culture studies have shown that some strains of this species are able to grow at 4.5% w/v NaCl (Sessitsch et al. 2005).

Burkholderia phytofirmans has been used in agriculture to improve crop performance as it is a highly effective plant-beneficial bacterium, and is able to establish rhizosphere and endophytic populations associated with various plants (Garrity et al. 2005). More recently, it has been used in viticulture with its application to Chardonnay plantlets to enhance chilling resistance in grapes, stimulate grapevine growth and protect grapevines against *Botrytis cinerea* (Ait Barka et al. 2000; 2002; 2007).

Most of the other isolates from the high potassium sorted samples were related to chloroplasts from *Chlorella vulgaris* (15%, an algae) and *Cyanobacteria* (11%, MiJ43 clone). However, the similarity of these isolates to these organisms was only 93% based on 16S rRNA gene sequencing and therefore suggests that these may be novel species. The presence of *Cyanobacteria* in the high potassium FACS samples is not surprising as many halophilic phototrophic bacteria are members of this phylum (Castenholz 2001). Interestingly, *C. vulgaris* is the algae predominantly used for the production of biodiesel (Scragg et al. 2003; Francisco et al. 2010).

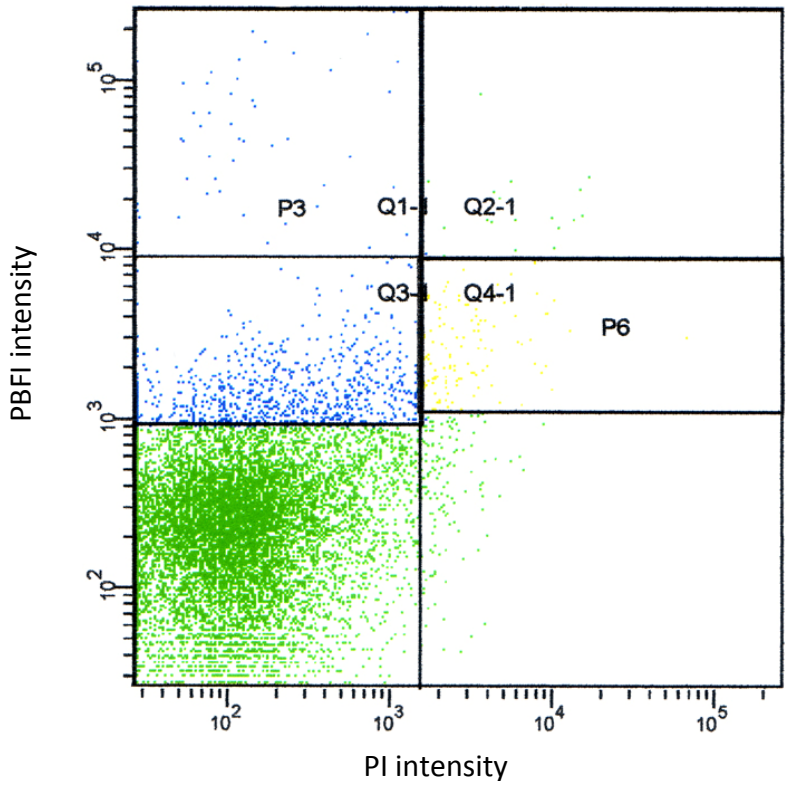


Figure 4. Flow cytometer intensity plot showing the number of events correlating to non-viable cells with low intracellular potassium (green events), and viable cells with high intracellular potassium (blue events). Propidium iodide (PI) was used to distinguish between viable and non-viable cells, and PBFI was used to stain for intracellular potassium.

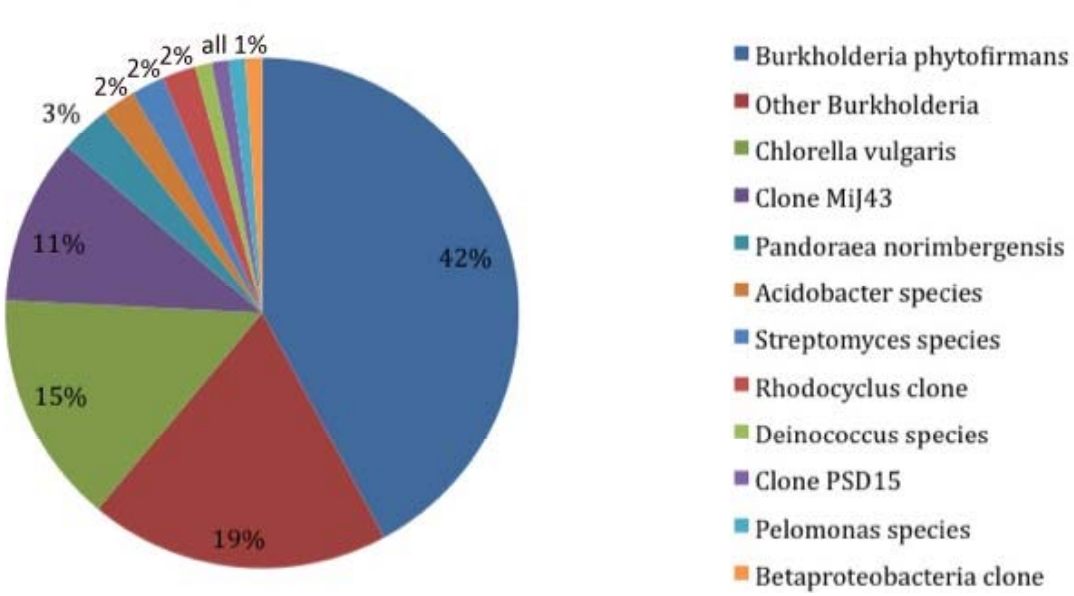


Figure 5: Diversity of clones generated from FACS of WWW samples.

Output 3: Effect of dosing winery wastewater with halophiles

The effect of dosing synthetic WWW with halophiles was assessed by changes in pH, electrical conductivity, colour, COD removal and salts. Of the purchased halophiles only *Pseudoalteromonas haloplanktis, Marinobacter vinifirmis* and S*hewanella frigidimarina* were able to grow in the synthetic WWW media. The synthetic WWW was inoculated with 0.5 OD_{550} of culture and grown for 4 days.

Each organisms ability to accumulate intracellular potassium was assessed and showed notable differences (subset illustrated in Fig. 6). Isolates TKO5, TKO6, TSO1, TSO2, TSO5, TSO7, TSO8 accumulated potassium concentrations 7 to 20 times greater than others and were chosen for further study. These organisms were identified as members of *Halomonas*, *Arthrobacter* and *Psychrobacter* (Table 3). TKO2 and TKO3 were also included in further studies as they were identified as *Burkholderia* species from 16S rRNA gene sequencing and *Burkholderia* sp. in the previous FACS analysis and thus appeared important.

In addition to measuring intracellular potassium; sodium, calcium and magnesium were measured in the supernatant so sodicity could be measured. The expectation was that there would be little change in the concentration of each of these ions other than the trace amounts required for microbial growth, unlike potassium which may be accumulated in the cytoplasm for osmotic regulation in halophiles (Oren 2002). SAR was used as it is the standard measure of sodicity and the required EPA parameter. The SAR values reported in Figure 7 are higher than that of typical WWW, due to the high sodium concentration of the synthetic WWW medium. However, SAR results indicate that the ratio of sodium to magnesium and calcium is largely unchanged by these organisms (Fig. 7). TSO2 and TSO5 did decrease the ratio of sodium ions to magnesium and calcium, indicating these organisms can potentially accumulate some sodium. No significant change was observed for pH, electrical conductivity or COD (Figs. 8 and 9).

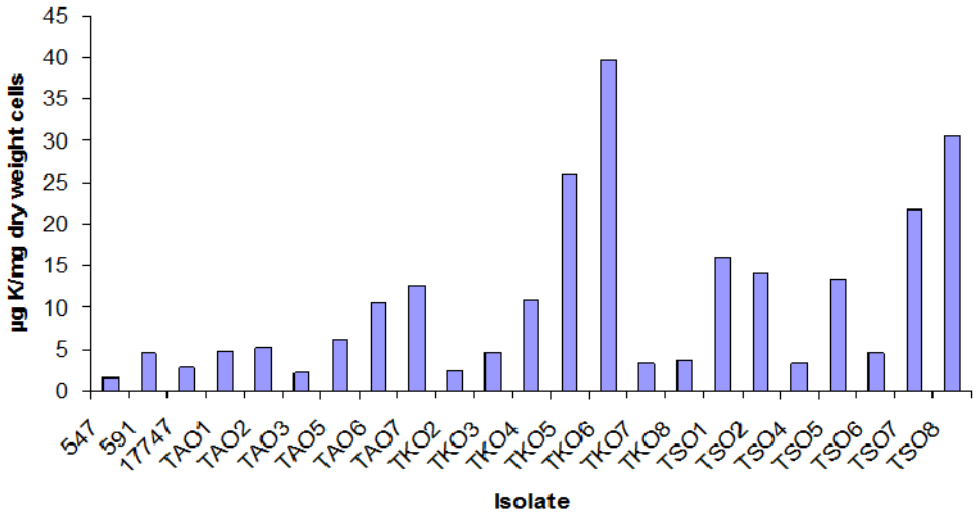


Figure 6. Intracellular potassium concentrations of isolates.

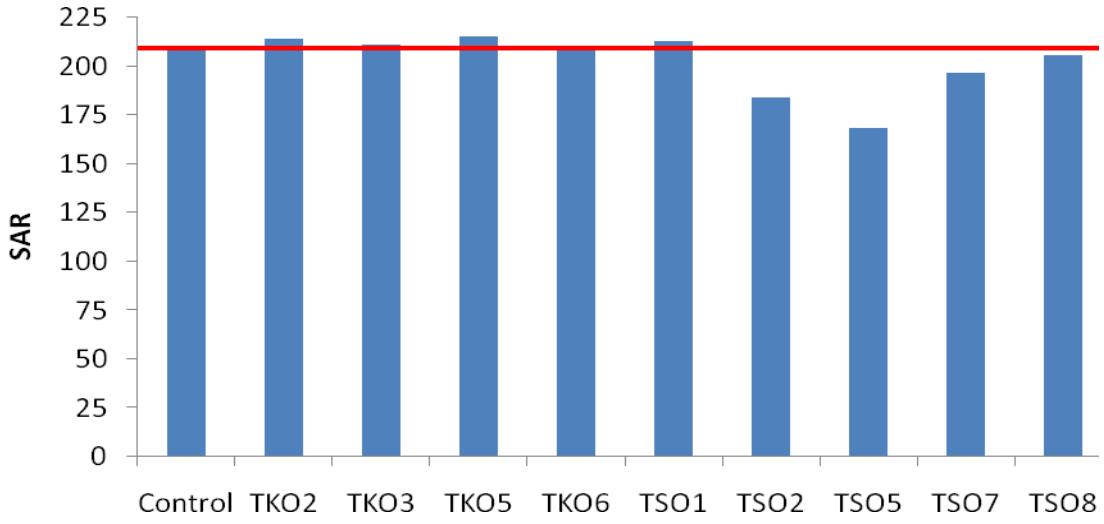


Figure 7. SAR values of selected isolates. Red line indicates the value of the control.

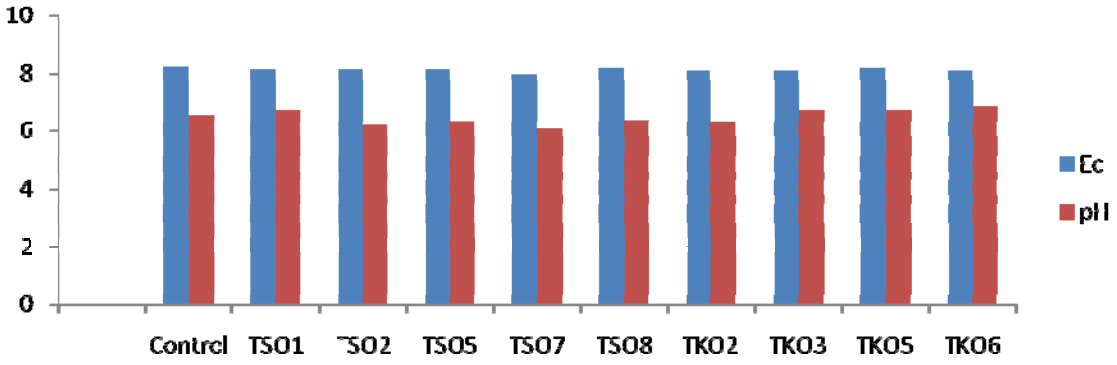


Figure 8. Changes in synthetic WWW pH and Ec after culturing with high potassium accumulating isolates.

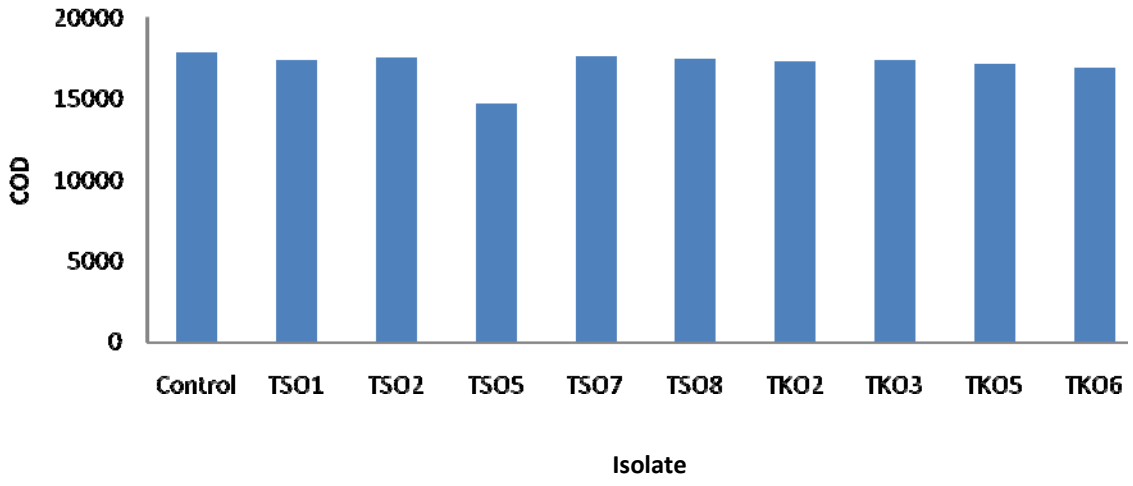


Figure 9. COD (ppm) of the supernatant after 4 days culturing.

In addition to the isolates ability to accumulate potassium, the colour change of the medium was also measured. After 4 days incubation, the control medium was a pale purple/orange (Fig. 10). Of all the isolates only TS02, TS05 and TS08 showed significant decreases in b values, chroma and hue. Visually this slightly de-saturated the colour and shifted the hue towards orange. What was not evident with these isolates, but was in isolating pure cultures (Fig. 11), was the lack of colouration in the medium of some cultures. Although these isolates had the ability to accumulate potassium, their ability to reduce the red wine colour was poor.

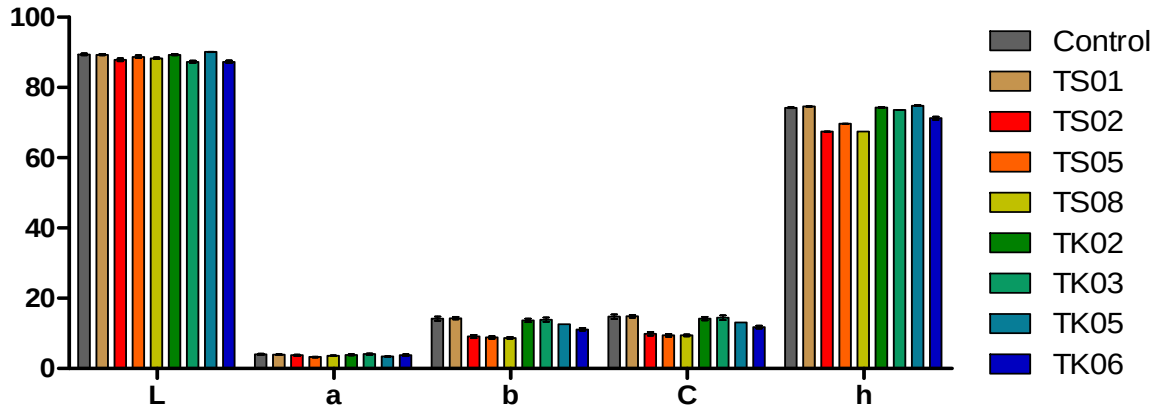


Figure 10. Colour changes of supernatants after culturing with the high potassium accumulating isolates. L^* = lightness, a^* = red to green, b^* = blue to yellow, C = colour, h= hue. Error bars indicate standard deviation of duplicate analyses.

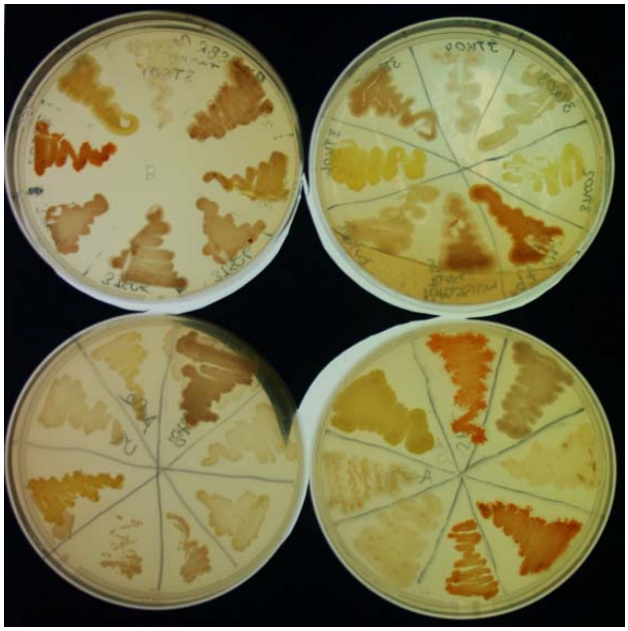


Figure 11. Examples of pure culture isolates plated on synthetic halophilic WWW medium. Note that colony colours vary from cream, brown, yellow, red and orange.

After 4 days of growth, there were significant differences in the amount of biomass produced by each organism. Biomass production was in-turn related to the concentration of intracellular potassium. A further observation was made, the more biomass, the greater the impact on residual ion concentrations and COD removal. Further experiments need to be carried out to confirm these observations and determine their full effect.

It is likely that organisms exist in WWW samples that do remove colour and would change pH, however these were not targeted in this study.

There are 7 possible candidates from this preliminary study for further investigation. While this number is relatively small it has been found in other treatment systems that one candidate organism can provide a successful solution, for example in enhanced biological phosphorus removal EBPR plants worldwide the majority of phosphorus is taken up by a single organism, *Candidatus* 'Accumulibacter phosphatis' (Seviour and Blackall 1999).

The synthetic WWW designed in this study attempted to represent typical WWW components with elevated salt levels to make it selective for halophylic organisms. Current conditions are probably not favourable for optimised uptake of potassium, so further studies are required to investigate these organisms and ways to optimise their uptake capabilities.

Outcomes and Conclusions:

1. Protocol for applying fluorescent dyes to control organisms and winery wastewater biomass

A protocol to identify organisms with high intracellular potassium was successfully developed using the intracellular potassium specific fluorescent stain PBFI. WWW samples stained with this dye showed cells with variable levels of intracellular potassium. The method was then applied to WWW samples and used in combination with FACS to sort for viable cells with levels of intracellular potassium.

2. Identification of organisms with high intracellular salt levels

Two approaches were used to identify organisms with the ability to accumulate potassium. The first was to isolate pure cultures from WWW using media specifically designed for medium halophiles. Identification of these isolates showed a phylogenetically diverse population. The second approach was to use FACS to specifically target and sort viable cells with high levels of intracellular potassium. This also showed a diverse population, which was dominated mainly by the genus *Burkholderia*.

3. Effect of dosing winery wastewater with known species of halophiles

Isolates related to *Arthrobacter*, *Psychrobacter*, and *Halomonas* accumulated high concentrations of intracellular potassium. They had little effect on pH, Ec, COD or color removal.

4. Dissemination of project findings

Findings from this project were disseminated via:

- The GWRDC's Recycled Water Workshop, National Wine Centre, Adelaide, 4 May, 2009 (K. Eales)
- Winery Wastewater Workshop (W28) held at the Australian Wine Industry Technical Conference (AWTIC), Adelaide, 5 July, 2010 (K. Eales).
- Wine microbiology – Application of technology. April 2010, UC Davis, California (F. Schmid).
- A poster presented at the AWTIC, July 2010 (Appendix 1).

While this project was designed to be a preliminary study into the potential of improving WWW treatment through the application of salt accumulating organisms to remove salt, throughout the year, it became evident that there was a general lack of knowledge in relation to the microbiology of WWW. This prompted us to publish an article in the January/February 2010 edition of the Australian and New Zealand Wine Industry Journal entitled 'The microbiology of winery wastewater treatment plants', and further we presented a poster at the AWTIC titled

‘Microscopic identification of the microbial populations associated with solid separation problems in winery wastewater treatment plants’.

Recommendations:

This study has clearly demonstrated the potential for microorganisms to accumulate potassium. This suggests that the biological removal of potassium from WWW warrants further investigation. Optimising the conditions for organisms to accumulate large intracellular potassium concentrations need to be explored

The conditions these isolates were grown under do not represent a typical WWW. This approach was taken to provide more selective conditions under which the ability to accumulate potassium would be more likely be demonstrated. Therefore, the capability of these isolates to grow and accumulate potassium in true WWW samples needs to be investigated. It is clear that these organisms can survive in WWW, however, further work is required to determine if they can thrive in treatment facilities.

The natural occurrence of *Burkholderia phytofirmans* strain PsJN in WWW also warrants further investigation. It was dominant in the FACS samples indicating that it has the ability to accumulate very high intracellular potassium concentrations. This could be beneficial not only for the removal of potassium from WWW but could add value to current waste product, the biomass or biosolids. Biomass from biological treatment facilities is routinely sold as fertiliser and PsJN has also been applied as a biofertiliser.

The work reported here was carried out using samples from only three WWW treatment facilities; two in the Barossa Valley and one in the McLaren Vale. A more in-depth study, investigating a greater number of facilities at various locations would be appropriate, as this would provide a greater pool of candidate organisms to characterise for salt accumulation. Further it could be targeted at the different stages of the wine production cycle, enhancing the chance of further success.

Appendix 1: Communication

- Presentations detailed above.
- Poster presented at the Australian Wine Industry Technical Conference, July 2010

Identification of salt accumulating organisms from winery wastewater

Kathryn L Eales, Frank Schmid, Vladimir Jiranek, Paul R Grbin

[1]School of Agriculture, Food and Wine, University of Adelaide, PMB 1, Glen Osmond, SA, 5064 and Wine Innovation Cluster, Adelaide, SA, Australia

The problems of salts in winery wastewater (WWW)

- High salt content often persists throughout the biological treatment of WWW.
- This limits the application of WWW to land via irrigation.
- Biological removal of organic nutrients and salt (phosphorus) has been studied in domestic wastewater treatment systems (Seviour et al. 2003).
- Some bacteria accumulate high levels of potassium and sodium (Grant et al. 2001) but no research has been carried out on WWW to investigate the presence and use of such potentially beneficial bacteria.
- The identification of such organisms could lead to the development of a novel treatment process and allow the sustainable reuse of a key water resource.

Research Objectives

Determine if normal WWW microflora contains microorganism that can accumulate salts

- Isolate
- Identify
- Characterise

Assess the ability of commercially available halophiles to accumulate potassium

Can biological treatment remove salts from WWW?

Experimental Outline

- Pure culture based and fluorescence activated cell sorting (FACS) were used to isolate halophilic and potassium accumulating microbes from WWW.
- Microorganisms from environmental samples were isolated using a halophilic WWW medium (Figure 1) and identified by sequencing 16 sRNA gene (Figure 2).
- Using collected WWW samples FACS was used to separate those cells that could accumulate higher concentrations of intracellular potassium.
- FACS samples were collected, DNA isolated and clone library generated for identification by sequencing (Figure 4).
- 40 WWW isolates and 8 halophiles from culture collections were assessed for their ability to accumulate intracellular potassium (Figure 5), remove colour, reduce chemical oxygen demand, change pH and lower the electrical conductivity of synthetic wastewater.

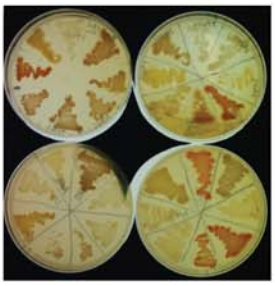


Figure 1. WWW pure culture isolates grown on synthetic halophilic winery wastewater media

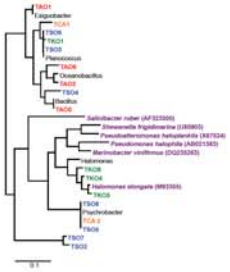


Figure 2. A 16S rRNA gene tree showing the phylogentic affiliation of the WWW isolates (different samples are different colours) with purchased halophiles (purple).

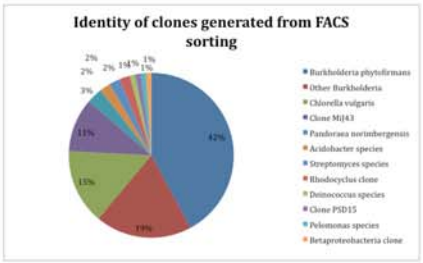


Figure 3. Diversity of the clones generated from FACS sorting of WWW samples

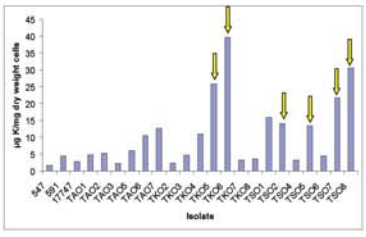


Figure 4. Accumulation of intracellular potassium by WWW isolates

Conclusions

- Bacteria where isolated from WWW with the ability to accumulate intracellular potassium
- The outcomes of this study indicate that the many halophiles naturally occurring in WWW exhibit large phylogenetic diversity, and some accumulated large concentrations of intracellular potassium.
- Potential for the biological accumulation of potassium from WWW requires further investigation.

www.adelaide.edu.au

contact: Paul Grbin
phone: +61 8 8303 7302
email: paul.grbin@adelaide.edu.au
web: www.sciences.adelaide.edu.au/winemicro

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References: Grant WD. et al. (2001) Boone, DR. Castenholz RW. (eds.) Manual of Systematic Bacteriology Volume 1, 2nd ed., New York: Springer Verlag.
Seviour RJ. et al. (2003) FEMS Microbiol. Rev. 27: 99-127.

Appendix 2: Intellectual Property

All intellectual property arrangements are detailed in original research agreement.

Appendix 3: References

Ait Barka E., Belarbi A., Hachet C., Nowak J. and Audran, J-C. (2000) Enhancement of *in-vitro* growth and resistance to gray mould of *Vitis vinifera* co-cultured with plant growth-promoting Rhizobacteria. FEMS Microbiol Lett **186**, 91–95.

Ait Barka E., Gognies S., Nowak J., Audran J-C. and Belarbi A. (2002) Inhibitory effect of endophyte bacteria on *Botrytis cinerea* and its influence to promote the grapevine growth. Biol Control **24**, 135–142.

Ait Barka E., Nowak J. and Clément C. (2007) Enhancement of chilling resistance of inoculated grapevine plantlets with a plant growth-promoting rhizobacterium, *Burkholderia phytofirmans* strain PsJN. Appl Environ Microbiol **72**, 7246–7252.

Australian Grape and Wine Industry Report (2008) ABS Publication cat. no. 13290.2008

Benson D.A., Karsch-Mizrachi I., Lipman D.J., Ostell J. And Wheeler D.L. (2004) GenBank: update. Nucleic Acids Res **32**, Database issue D23-D26.

Bull A.T., Ward A.C. and Goodfellow M. (2000) Search and discovery strategies for biotechnology: the paradigm shift. Microbiol Mol Biol Rev **64**, 573-606.

Castenholz R.W. (2001) "Phylum BX. Cyanobacteria.", in D.R. Boone and R.W. Castenholz, eds.: *Bergey's Manual of Systematic Bacteriology Volume 1: The Archaea and the deeply branching and phototrophic Bacteria*, 2nd ed., New York: Springer Verlag.

Felsenstein J. (1981) Evolutionary trees from DNA sequences: A maximum likelihood approach. J Mol Evol **17**, 368-376.

Francisco E.C., Neves D.B., Jacob-Lopes E., Franco T.T. (2010) Microalgae as feedstock for biodiesel production: Carbon dioxide sequestration, lipid production and biofuel quality. J Chem Tech Biotech **85**, 395-403.

Garrity G.M., Bell J.A. and Lilburn T. (2005) "Order I. Burkholderiales ord. nov.", in G.M. Garrity, D.J. Brenner, N.R. Krieg, Noel and J.R. Staley, eds.: *Bergey's Manual of Systematic Bacteriology, Volume Two: The Proteobacteria, Parts A - C,* 2nd ed., Springer US.

Grant W.D., Kamekura M., McGenity TJ. and Ventosa A. (2001) "Class III. Halobacteria class. nov.", in D.R. Boone and R.W. Castenholz, eds.: *Bergey's Manual of Systematic Bacteriology*

Volume 1: The Archaea and the deeply branching and phototrophic Bacteria, 2nd ed., New York: Springer Verlag.

Jiranek V., Langridge P. and Henschke P.A. (1995) Amino acid and ammonium utilisation by *Saccharomyces cerevisiae* wine yeasts from a chemically defined medium. Am J Enol Vitic **46**, 75-83.

Kumar A., Saison C., Grocke S., Doan H., Correll R. and Kookana R. (2006) Impact of winery wastewater on ecosystem health – an introductory assessment. Adelaide: CSIRO. GWRDC (CSL02/03).

Lindrea K.C., Seviour E.M., Seviour R.J., Blackall L.L. and Soddell J.A. (1999) Practical methods for the examination and characterization of activated sludge. In: R.J. Seviour and L.L. Blackall, Eds, The Microbiology of Activated Sludge, Kluwer Academic Publishing, Boston.

Mpelasoka B.S, Schachtman D.P., Treeby M.T. and Thomas M.R. (2003) A review of potassium nutrition in grapevines with special emphasis on berry accumulation. Aust J Grape Wine Res **9**, 154-168.

Oren, A. (2002) Halophilic microorganisms and their environments. Kluwer Academic Publishing, Dordrecht.

Scragg A.H., Morrison J. and Shales S.W. (2003) The use of a fuel containing *Chlorella vulgaris* in a diesel engine. Enzyme Microb Tech **33**, 884-889.

Sessitsch A., Coenye T., Sturz A.V., Vandamme P., Ait Barka E., Salles J.F., van Elsas J.D., Faure D., Reiter B., Glick B.R., Wang-Pruski G. and Nowak J. (2005) *Burkholderia phytofirmans* sp. nov., a novel plant-associated bacterium with plant-beneficial properties. Int J Syst Evol Microbiol **55**, 1187-1192.

Seviour R.J. and Blackall L.L. (1999) *The Microbiology of Activated Sludge*. Kluwer Academic Publishing, Boston.

Seviour RJ, Mino T. and Onuki M. (2003) The microbiology of biological phosphorus removal in activated sludge systems. FEMS Microbiol Rev **27**, 99-127.

Thompson J.D., Higgins D.G. and Gibson T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res **22**, 4673-4680.

Xavier B.M., Houlihan A.J. and James B. Russell J.B. (2008) The activity and stability of cell-associated activity of bovicin HC5, a bacteriocin from *Streptococcus bovis* HC5. FEMS Microbiol Lett **283**, 162 – 166.

Appendix 4: Staff

The following staff contributed to this project:

Dr Paul Grbin

Dr Kathryn Eales

Dr Frank Schmid

Assoc. Prof. Vladimir Jiranek

Appendix 5: Acknowledgements

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