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Use of novel bacteria from winery waste sites to reduce the organic content of lees and to produce enzyme extracts that will alleviate fermenter biofouling

Introduction

Winery wastes comprise a rich cocktail of tannins, complex carbohydrates and protein in an aqueous mixture. The National Water Quality Management strategy (NWQMS) Effluent Management Guidelines for Wineries and Distilleries stress use of cleaner production strategies for minimising water waster and pollution load, recovery of by-products and use of liquid effluent for irrigation. Industry use of minimal storage and pre-treatment before land discharge to minimise odours is the major limitation to adoption of the above policy, as the system does not cater for effective recovery of by-products and reuse of wastewater for irrigation of vineyards. Extensive use of caustic soda for washing equipment results in the wastewater being moderately saline and highly sodic. High use of water for cleaning also results in many land based discharge systems not being able to cope with amounts of waste water produced post vintage during cold wet months – companies have to tanker the excess to alternate sites at great cost. Current R&D on development of alternat cleaning products has enabled some reduction in the use of caustic soda, but no reduction in wastewater; alternative products have a higher organic loading (which may benefit treatment systems by proving a more continuous supply of substrate carbon). Although marc and lees are sent off for recovery of by-products this practise is based on wine industry reliance on 2 major processing companies.
This research project proposed to continue the development of alternat cleaning products to strong alkali and acids which are currently needed for removal of "brownskin" polyphenol protein complexes that line the walls of tanks, creating an intractable biofilm. The hypothesis was that we could take advantage of bacterial species that thrived on the components on the waste. Some of the microorganisms present in marc dumps and in lees are likely top transform complex compounds into simpler molecules that are more water soluble – effectively the role of strong alkali and acid washing. The bacteria will be isolated and transformation products and key enzymes will be identified and rated for potential use in proprietary cleaning products. Enzyme extracts that can degrade tannin-protein complexes and metabolise tannins will therefore be of enormous value to the wine industry.
The potential of promoting growth of these bacterial species using product wastes to maintain substrate supply in fermenters within the winery was also proposed. Better use of product that has a very high oxygen requirement for microbial degradation will lessen the malodour problem and make pre-treatment systems less expensive. Pretreatment will in turn, allow storage of the wastewater, and realise its reuse for irrigation of vineyards – the best statement that the wine industry could make about the quality of its wastewater.

The project was therefore to harvest bacteria from waste collection sites, and isolate novel species of bacteria that can reduce the organic content of wine lees, improve water quality and yield enzyme extracts to reduce fermenter biofouling. This response

should represent an environmentally responsible and cost effective strategy for waste minimisation in the wine industry.

Overall aim of project

To develop a microbiological method of removing contamination from winery waste and from fermenters.

Experimental approach

The experimental approach involved screening marc and lees dumps, as well as other possible sites for the presence of bacterial species that could be used in wastewater remediation. The test would be whether these bacteria could degrade or reduce the level of tannin-protein complexes in a laboratory test system.
If this aspect of the project was successful, then further studies would be carried out with individual or mixed species to identify enzyme extracts that could be used commercially.

Experimental plan

1. Samples were taken at various times from different winery sites including grape marc dumps, liquid effluent streams, and soil. These samples were used as inocula, together with other crude bacterial samples from local soils, into basal medium containing tryptone, yeast extract, salt, 1% BSA and 1% condensed tannin, water-extracted from grapemarc and dried.
Initial screening of bacteria was in Medium 1, containing 10% Bacto-tryptone, and 5% yeast extract. This was followed by enrichment in Medium 2 contained 2% Bacto-tryptone and 0.5% yeast extract. All media contained 1% condensed tannin, 1% BSA, and were prepared over the pH range of 5-8. The aim was to enrich for bacterial species that could utilise some or all components of the condensed tannin extract, or the tannin-protein precipitate, as major carbon and nitrogen sources for growth.

Cultures were grown in batch culture and in continuous fermentation either aerobically or anaerobically, and changes in colour intensity and precipitation were measured.

2. Mixed bacterial cultures isolated above, that achieved effective clearing and removal of the tannin-protein precipitate were stored as glycerol stocks at –80C. These were labelled as TPC1 – TPC5. The description of these mixed cultures was subjective, and grading of their effectiveness was by eye. No colour change was scored as 1, and total colour change and clearing was scored as 9. Table 1 describes the groupings.

To test for activity against brownskin, stainless steel fragments (5 x 30 cm) were obtained from a local (Barossa) winery and were inserted into the fermenter for the duration of the commercial wine fermentation. The resulting film of yellow/brown coloured material was subjected to microbial degradation.
A 2 l beaker was filled with medium and the microbe mixtures was incubated along with the steel fragments and the brownskin film was examined daily. Medium was

replaced every 24 hr to allow for continuous bacterial growth and the experiment was continued for 7 days.
Since it was not possible to obtain sufficient quantities of actual brownskin from fermenters, we also prepared a model precipitate by extraction of grapemarc tannins. 2 gm of grapemarc tannin were added to 2 gm of BSA and 100 ml of water, and the precipitate was collected and dried. 1 gm of this precipitate was added to minimal medium and inoculated with mixed cultures TPD1 – TPD5. Cultures were tested on nutrient agar plates and in liquid medium.

3. Growth of bacteria in tannin/protein minimal medium.
A tannin/protein precipitate was prepared by mixing 2 ml of TARAC grape seed tannin preparation with 2.0 g of BSA in 100 ml of water.
Bacterial growth medium was prepared containing the following:

0.5%, 1.0%, 1.5% or 2.0% of tannin/protein preparation
mineral mix comprising $KHPO_4$, NaCl, $MgSO_4$, and $CaCl_2$.

Mixed bacteria from TPD1-TPD5 were tested for growth in this medium, under both aerobic and anaerobic conditions, at 25 ^oC. Experiments were carried out in triplicate.

4. Single culture studies.
Single colonies were isolated from mixtures TPD1-TPD5 and grown in enrichment medium 2, at various pHs. Cultures were tested for their ability to decolourise tannin/protein solutions

5. Total phenolics were assayed in culture medium, before and after incubation, using the butanol-HCl method, and purified grape seed tannin as an assay standard.

6. Preparation of cell extracts
Cell extracts were prepared in phosphate-buffered saline (PBS) from mixed and pure cultures using a French Press, and each was tested for decolourising activity *in vitro*.

Results.

1. Mixed culture tests
The clearing activity of mixed cultures TPD1—TPD5 are shown in Table 1. Mixtures 1,2,4 and 5 all showed clearing activity in vitro. Mixture 3 was not so active. These mixtures appear to have the potential to be used for the purpose of decontaminating waste. Cultures were evaluated by subjective assessment of clearing activity (1=none, 9= cleared)

Table 1. Mixed microbial populations that discolour tannin/protein medium

Sample	Anaerobic in tube	Aerobic in tube	Continuous fermenter	Plate anaerobic	Plate aerobic	Anti-brownskin activity
TPD-1	8	9	9	7	9	None
TPD-2	8	8	8	8	8	None
TPD-3	5	5	6	5	5	None
TPD-4	8	7	9	8	8	None
TPD-5	9	9	9	9	9	none

2. Testing for brownskin removal.

Using mixtures TPD1-TPD5 against actual brownskin on stainless steel pieces, no removal of the layer was observed. When lab 'brownskin' was used either on plates or in liquid medium, some clearing activity was observed around come colonies, and some reduction in the colouration of the medium occurred. These mixtures were therefore spread on plates and single colonies were picked. When tested as single colonies, no reproducible clearing of tannin/protein complexes was obtained.

3. Growth of mixed bacterial populations in tannin/protein containing minimal medium

The results of these growth studies are shown in Tables 2 and 3.

Table 2. Aerobic conditions
Cell counts are means of triplicate cultures

Time(hr)	TPD1	TPD2	TPD3	TPD4	TPD5
	Cell no.	Cell no.	Cell no.	Cell no.	Cell no.
0	$2x10^4$	$2x10^4$	$2x10^4$	$2x10^4$	$2x10^4$
6	$1.5x10^5$	$6x10^4$	$4x10^4$	$6x10^4$	$1.5x10^5$
12	$3x10^5$	$1x10^5$	$6x10^4$	$2x10^5$	$6x10^5$
18	$2x10^6$	$2x10^5$	$1.5x10^5$	$6x10^5$	$1.2x10^6$
24	$4x10^6$	$2.6x10^5$	$1.5x10^5$	$1.6x10^6$	$4x10^6$
30	$2x10^7$	$2.6x10^5$	$1.5x10^5$	$6x10^6$	$1.5x10^7$
36	$2x10^7$	$2.6x10^5$	$1.5x10^5$	$6x10^6$	$1.5x10^7$

Table 3. Anaerobic conditions
Cell counts are means of triplicate cultures

Time(hr)	TPD1	TPD2	TPD3	TPD4	TPD5
	Cell no.	Cell no.	Cell no.	Cell no.	Cell no.
0	$2x10^4$	$2x10^4$	$2x10^4$	$2x10^4$	$2x10^4$

6	$4x10^4$	$2x10^4$	$2x10^4$	$4x10^4$	$5x10^4$
12	$1x10^5$	$6x10^4$	$4x10^4$	$1.5x10^5$	$2x10^5$
18	$1x10^5$	$7x10^4$	$4x10^4$	$4x10^5$	$1.5x10^6$
24	$1x10^5$	$7x10^4$	$4x10^4$	$1x10^6$	$6x10^6$
30	$1x10^5$	$7x10^4$	$4x10^4$	$1x10^6$	$2x10^7$
36	$1x10^5$	$7x10^4$	$4x10^4$	$1x10^6$	$2x10^7$

The results show that under aerobic conditions, TPD1 and TPD5 mixtures grew well in the tannin supplemented minimal medium. TPD4 also grew but not so strongly. Since the medium only contained protein and tannin in a complex, the bacteria must have been obtaining their energy from this source. TPD2 and TPD3 did grow, but only increased in numbers 10 fold (3 doublings) compared with 1000 fold increases for TPD1 and 5.
Under anaerobic conditions, only TPD5 showed strong growth, with TPD4 about 1 log lower in value.

4. Single culture studies
The pure cultures, isolated from TPD1-5 mixtures were grown in tubes, on plates and in a fermenter, and all were assessed subjectively for clearing activity. Only 3 cultures showed positive activity and the results are shown in Table 4.

Table 4. Pure culture activity in tannin/protein medium
Subjective assessment of clearing activity (1=none, 9= cleared).
ND = none detected

Culture	Anaerobic tube	Aerobic tube	Fermenter	Anaerobic plate	Aerobic plate	Brownskin activity
M1	4	5	4	5	4	ND
M2	3	3	3	3	3	ND
M3	6	4	6	6	3	ND

The results show that in pure culture, clearing activity was greatly reduced from that seen in mixed cultures. Only M3 showed reasonable activity in both liquid culture or on plates, under anaerobic conditions.
Pure cultures were also tested in various mixed combinations. No increased clearing was observed.

5. Phenolic assays.
The aim was to quantify the loss of phenolics at the end of the bacterial incubations. However, data were inconsistent and inconclusive. There was no evidence of significant degradation of the tannins.

6. Cell extracts
Cell extracts from either mixed or pure cultures were tested for decolourising activity. No significant changes in colour or precipitation were observed in any of the assays.

Discussion

Most mixed bacterial cultures isolated from various waste dump sites showed strong decolourising activity (although the activity was not quantified), both aerobically and anaerobically, and at first glance appeared to offer great potential to remediate waste sites and reduce organic loading of wastes. Microscopic examination of the cultures showed that each was a mixture of rods and cocci, but none stood out as being in obvious abundance. It was not possible to distinguish between the mixtures on a microscopic basis.
The mixtures were tested under aerobic and anaerobic conditions and there were obvious differences in their abilities to utilise tannin-protein complexes, since this was the only source of carbon and nitrogen available to the bacteria. TPD1 and TPD5 were the only mixtures that grew well under these conditions, and only TPD1 grew both aerobically and anaerobically. This suggested that TPD5 or a mixture of TPD1 and TPD5 may have been suitable candidates for a microbiological cleansing agent. Further purification of the cultures to individual bacterial species resulted in a large reduction in clearing activity, and no single culture was able to achieve what the mixtures achieved. Moreover, when the pure cultures were mixed together in various combinations, there was still no recovery of the original clearing activity.

This suggests that as purification of the mixed cultures progressed, either some essential species were lost because they were not able to grow in isolation, or that the bacteria adapted to growth in culture and ceased to express the clearing function. It was also found not to be possible to maintain the mixed cultures for prolonged periods, and over time, they lost their viability and clearing activity. Glycerol stocks of the mixtures were also prepared, but on resurrection of these cultures, it was found that they had also lost much of the clearing activity that was originally observed. Attempts to quantify the assays by measuring changes in the concentration of phenolics between the beginning and end of the incubation period were not successful, possibly due to interference from other components in the mixtures, or because the total level of phenolics had not really changed. The latter is quite possible because bacterial action may have been focussed on utilisation of the protein component of the complexes, from which the bacteria would have been able to obtain both nitrogen and carbon, in which case, total phenolics would have remained the same.

This may suggest that bacterial action may reduce the nitrogen component of the waste, but not change the total organic loading or the phenolic content. This suggestion is supported by the tests against “brownskin”, in which the mixed cultures were tested against brownskin on stainless steel plating - no activity was observed. This could be for varying reasons, including the possibility that the bacteria predominantly acted against protein but not the phenolics, which could have remained bound to the stainless steel. Alternatively, the association of the brownskin with the surface may have been sufficiently thick that only the outer layers were removed but not the material directly bound to the surface. The ionic/electrostatic conditions on the steel may also have prevented close association of the bacteria, and so prevented dissociation and degradation of the complexes. Because the steel had been exposed to air after being removed from the fermenter, it is also possible that the surface was oxidised and therefore not amenable to bacterial action. Perhaps the stronger action of acid or alkali is needed to overcome this.

In conclusion therefore, the results of this study were disappointing in that there appeared to be a potential for bacterial action to be effective in reducing winery waste, however, we were not able to maintain this activity, nor to isolate it in a purer and more stable form. Despite considerable work using laboratory fermenters for continuous cultures, as well as batch cultures of mixed and pure species, we were not able to isolate or maintain a viable bacterial preparation that retained its clearing activity. Notwithstanding this negative result, it was nevertheless apparent from our studies that waste dump sites did contain microbial communities that were able to very effectively dissociate and utilise the components of tannins and tannin-protein complexes. To make effective use of these is more problematic. It may be possible to isolate enzyme extracts from large batches of mixed microorganisms and demonstrate clearing activity *in vitro* (although we were not able to show this in our small scale experiments), or to test a wider range of culture conditions in order to determine the optimum conditions for survival. Possibly other dump sites could be evaluated. Because of the somewhat open ended nature of these studies, with no obvious focus on any particular bacterial species, we did not progress in that direction in this project.

Outcomes

1. The demonstration that dumpsites do contain mixtures of microorganisms that can remediate tannin-protein precipitates
2. The demonstration that the remediation activity can occur both aerobically and anaerobically
3. The demonstration that it was not a simple task to isolate pure cultures that maintained remediation activity, and in fact was not achieved by us.
4. The finding that perhaps remediation activity was more focussed on protein degradation rather than the phenolic molecule itself.