Cold-active proteases from Antarctic fungi as alternatives to heat-stabilisation with bentonite



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Cold-active proteases from Antarctic fungi as alternatives to heat-stabilisation with bentonite

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Table of Contents

Abstract …….. 4

Executive Summary ……...…… 5

Background …… 6

Project Aims and Performance Targets ……. 6

Method ……... 7

Results and Discussion ……..……...…… 10

Outcome and Conclusions ……... 13

Appendix 1: References …….. 14

Appendix 2: Staff …… 15

Appendix 3: Budget reconciliation ……. 16

Abstract

White wines naturally contain heat-unstable grape proteins that must be removed before bottling to prevent unattractive hazes. Heat stabilisation with bentonite fining is a widely used but inefficient process that interferes with filtration and membrane technologies and produces hazardous waste. In this project, two hundred isolates in the Macquarie University Collection of Antarctic fungi were screened to seek an enzymatic replacement for bentonite. Cold-active proteases were found that were active at low pH; one of the proteases caused a modification to chitinase under winemaking conditions. Proteases were identified from the supernatant of a *Chrysosporium* strain that caused some degradation of chitinase and thaumatin at 60 °C.

Executive summary

The aim of this project was to seek an enzymatic replacement for bentonite suited to the low temperatures of winemaking. A cold-active protease would contribute to a new winemaking technology and, by precluding bentonite usage, could improve wine quality by reducing flavour losses and by facilitating membrane treatments for tailored wine styles. A successful cold-active protease would also reduce the environmental impact of winemaking and, as a product of biotechnology, would be far more sustainable than bentonite mining and hazardous waste removal.

Macquarie University has a collection of approximately 200 fungi from Antarctica, several of which were known to secrete cold-active proteases. In this project, the protease activities of all 200 isolates were screened using agar plate assays carried out at low temperature and low pH. The agar plates contained skim milk as a protein source, resulting in a cloudy appearance that was cleared as a result of proteases secreted by the fungal colonies. Twenty-four isolates were selected for their high protease activity ranked by the size of the clearing halo that appeared around the fungal colonies in relation to the colony size.

The 24 fungal isolates that displayed the highest protease activity in the plate assays were grown in liquid culture media. Supernatants were assessed for protease activity using liquid assays. Strains that exhibited the highest protease activity under acidic conditions at 10 ºC were *Trichoderma, Glomerella, Embellisia, Chrysosporium*, and *Phoma* species.

Protease activity against grape juice proteins was determined by incubating culture supernatants with grape juice at 10 ºC and 20 ºC and determining the extent of proteolysis by SDS-PAGE. Supernatant from a *Phialophora* stain (O1) caused some modification of chitinase, resulting in a reduction in molecular weight after two days incubation with grape juice at 10 ºC. *Chrysosporium*, *Phoma*, *Glomerella*, *Embellisia*, *Aureobasidium*, and *Phialophora* strains appeared to cause a very slight degradation of chitinase and thaumatin. The supernatant of a *Chrysosporium* strain (A6) caused significant degradation of both chitinase and thaumatin at 60 ºC. A serine protease was identified from the *Chrysosporium* A6 supernatant by mass spectrometry.

This work has been made possible thanks to funding provided by GWRDC and in-kind contribution from Macquarie University. Overall, the project has revealed proteases from Antarctic fungi that are active at cold temperatures and under acidic conditions. One of the proteases is able to degrade grape juice proteins at elevated temperatures. However, the discovery of a protease effective against haze-producing proteins under winemaking conditions still remains elusive.

Background

The vast majority of white table and sparkling wines are not protein stable and are treated with bentonite to prevent the development of unattractive haze. Bentonite fining is a crude and inefficient way to make wines acceptable to consumers due to a reliance on imported mined bentonite, interference with membrane technology, wear on equipment, and the production of hazardous waste. There are currently no viable alternatives to bentonite, but the most promising are protease additions in combination with heat treatment. However, heat treatment is undesirable because it consumes large amounts of energy and may reduce wine quality. A cold-active enzyme treatment that is effective at normal winemaking temperatures would be ideal because it could be used as easily as other enzyme additions and would not require heat treatment.

The Department of Chemistry and Biomolecular Sciences at Macquarie University has a collection of approximately 200 fungi from Antarctica that have been used successfully in the discovery of cold-active enzymes for other industries (e.g. lipases for use in detergents). During past screenings of the collection, several isolates were found to secrete large amounts of proteases active at low temperatures. The secreted proteases had not been tested for use in winemaking or for activity in the pH range of winemaking, although some isolates secreted acids in liquid media, which suggested that secreted proteases would be active under acidic conditions.

Project Aims and Performance Targets

The main objective of this project was to screen the 200 Antarctic fungal strains for cold-active proteases, active under acidic conditions that could serve as an enzymatic replacement for bentonite.

The project aims were:

1) Screen the entire Antarctic fungi collection for secreted proteolytic activity at winemaking temperature and pH.

2) Isolate cold-active proteolytic secreted enzymes from fungal liquid cultures and determine their effectiveness against haze proteins, chitinase and thaumatin, in grape juice under winemaking conditions.

Performance targets were achieved throughout the project in accordance with the original application.

Method

Agar plate assays for protease activity

The protease activities of the 200 isolates in the Macquarie University Collection of Antarctic fungi were screened using agar plate assays carried out at low temperature (10 ºC and 20 ºC) and low pH (pH 5 and pH 6.5). The agar plates contained minimal salts (Penttilä *et al*., 1987) and 1% (w/v) skim milk as a protein source, resulting in a cloudy appearance that was cleared as a result of proteases secreted by the fungal colonies. An index of "relative enzyme activity" was used to express the enzyme activities of the isolates relative to the size of the fungal colony (Bradner *et al*., 1999; Fig. 1). The relative activity was calculated by dividing the area of the halo around the colony (the area of the clearing zone minus the area of the colony) by the area of the colony. This was simplified by the equation $(D^2 - d^2)/ d^2$, in which D and d were the diameter of the halo and colony respectively (Fig. 1). The plate assays were repeated for the most promising isolates.

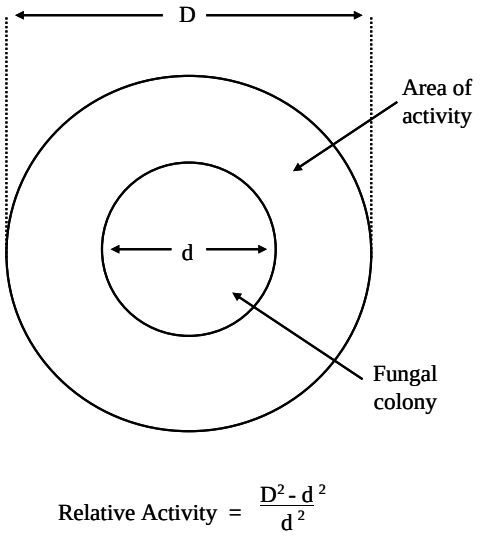


Figure 1: The method used to calculate relative enzyme activity of the fungal isolates (see text).

Note: The original application proposed screening on agar plates of pH appropriate for winemaking conditions (ideally pH 3 - 3.5). However, it became apparent that the lowest pH that could be used on the agar plates was pH 5; below that, the substrate started to precipitate. In an attempt to solve this problem, haemoglobin was trialled as a substrate, but halos were difficult to read and fungal growth was low. Therefore, pH 5 was maintained as the lowest pH used in the agar plate screening. Enzymes secreted by fungi are often found to be active at a wider pH range than that of the culture medium so the modified conditions were not considered a problem.

Liquid media

The 24 fungal isolates that displayed the highest protease activity at 10 °C, pH 5 in the plate assays were initially grown in a liquid culture medium established in the laboratory as being appropriate for inducing the secretion of hydrolytic enzymes in a wide range of fungal species. The "CLS" medium contained minimal salts (Penttilä *et al*., 1987), 1% (w/v) cellobiose, 1% (w/v) lactose and 3% (w/v) soybean flour (Lim *et al*., 2001) and inoculation was via four mycelial/ agar plugs (0.6 mm diameter) per 50 ml culture medium. Incubation was carried out in conical flasks at 28 °C for seven days, 250 rpm. After seven days the mycelia were separated from culture supernatants by centrifugation at 4,000 × *g* for 30 minutes at 4 ºC. Supernatants were used for liquid protease assays, and incubation with grape juice to determine the effectiveness of the proteases against the grape juice proteins.

An alternative culture medium containing minimal salts (without nitrogen sources) and 1% (w/v) skim milk was then utilised in an attempt to specifically encourage protease production in the Antarctic fungal strains. The medium contained (per litre) 1.5 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4$, 10 mg each of $MnCl_2$, $ZnSO_4$ and $FeSO_4$ and 1% (w/v) skim milk (Gente *et al*., 2001). The 24 top fungal isolates from the agar plate screening were incubated on a shaker (250 rpm) at 28 ºC for five days in conical flasks containing 50 ml of the skim milk medium with a starting pH of 5.5. Cultivation was then repeated in the skim milk medium with a starting pH of 4.3.

Liquid assays for protease activity

The protease activity of the CLS culture supernatants was determined by the method of Cupp-Enyard (2008) using the substrate haemoglobin (2.5% w/v) in 0.1 M sodium acetate buffer, pH 3 and pH 4. Incubation was at 10 ºC for 30 minutes. Absorbance was measured at 620 nm and compared to standards of micromoles of tyrosine. One unit of protease activity was defined as the amount which catalysed the release of one micromole of tyrosine per minute under the conditions described.

Incubation of fungal supernatants with grape juice and analysis by SDS-PAGE

The supernatants from the liquid cultures were incubated with Hunter Valley Sémillon (2:1) for two to five days at 10 ºC and 20 ºC to determine the ability of the proteases secreted by the isolates to degrade the grape juice proteins. Controls contained supernatant that had been heat denatured at 85 °C for five minutes prior to incubation with the juice. The extent of proteolysis was determined by SDS-PAGE, with particular attention to a reduction in the intensity of the protein bands

representing chitinase (approx. 22 kDa) and thaumatin (approx. 26 kDa). Samples were prepared for SDS-PAGE using methanol/ chloroform precipitation (Wessel and Flugge, 1984).

Some supernatants were also tested for protease activity against the grape juice proteins at elevated temperatures by incubating supernatant and juice (2:1) at 45 °C, 55 °C and 60 °C for ten minutes, followed by methanol/ chloroform precipitation and SDS-PAGE.

Identification of proteases by mass spectrometry

Biological triplicates were obtained from three cultures of A6 *Chrysosporium* grown in the skim milk medium (starting pH 4.3). Supernatant from each culture was concentrated 60 fold using ultrafiltration devices (Millipore; 10 kDa cut-off). The concentrated samples were precipitated with methanol/ chloroform as above and subjected to mass spectrometry (nanoLC- MS/MS). The Global Proteome Machine (GPM) was used to search spectra against three databases: Merops (merops.sanger.ac.uk), the *Geomyces destructans* predicted proteins, and *Sclerotinia sclerotiorum* predicted proteins (http://www.broadinstitute.org).

Results and Discussion

The 200 isolates in the Macquarie University Collection of Antarctic fungi were screened using agar plate assays for protease activity at 10 ºC and 20 ºC, pH 5 and pH 6.5. Twenty-four isolates were selected for their high protease activity (relative activity; Bradner *et al*., 1999), with a particular focus on activity at 10 ºC and pH 5 (column 6, Table 1).

Table 1: Antarctic fungal strains with the highest protease activity in the agar plate assays. Relative activity was calculated by the size of the halo around the colony in relation to the colony size.

		Relative Activity			
Strain code	**Strain name**	**pH 6.5 20°C**	**pH 6.5 10°C**	**pH 5.0 20°C**	**pH 5.0 10°C**
J8	*Phoma*	3.6/48.0	4.4	2.17	**379.25**
H3	*Embellisia* sp2 (t)	1.64/0	8	5.48	**59.44**
K8	*Phoma*	1.04	5.25	0.09	**48**
G3	*Embellisia* sp2	0	0	2.67	**30.36**
I6	*Aureobasidium* (2)	1.25/13.3	8	6.94	**25.69**
I1	*T.harzianum* A3091	0.36/2.36	0	0.33	**25.45**
M6	*Glomerella*	19.7/48.0	0	20.5	**19.9**
L3	*Phoma* (1)	0	0	0	**17.49**
B8	*Aureobasidium*	0	0	1.78	**17.37**
N3	*Phoma*	6.43/10.1	10.1	22.1	**17.36**
C7	*Aureobasidium*	0.69	0	7.16	**17.06**
A6	*Chrysosporium*	3.31	0.33/1.76	0	**15.3**
C8	*Chrysosporium*	2.0	0	1.78	**12.22**
C1	*T. harzianum* RG3	0.17	11.25	0	**11.64**
H1	*T. viride* A3092	0.33	0	0.07/0.52	**10.46/5.25**
F5	*Phoma*	5	0	1.56	**10.11**
O1	*Phialophora*	1.19	0	1.78	**9.38**
B5	*Geomyces pannorum*	0	0	1.42	**8.68**
K2	*Aureobasidium* (1) *white*	1.04	15	0.74	**8**
G8	*Basidomycete* 24_/1	7.35	15	1.25	**4.06**
D2	*Ulocladium charanum*	0.49/3.84	0.78	0.21	**3.94**
A3	*Geomyces pannorum*	0	15	0	**3.84**
L7	*Chrysosporium*	0	20.8	1.47	**3.84**
M3	*Chrysosporium*	3.41	12.2	0.72	**3**

Note: Figures shown are averages from duplicate plate assays. Two numbers are listed in cases where the two results were substantially different.

The 24 fungal isolates that displayed the highest protease activity at 10 °C, pH 5 in the plate assays were grown in CLS medium (Lim *et al*., 2001). Liquid protease assays at pH 4 and pH 5, 10 °C, revealed the highest protease activity, from the supernatants of I1 *Trichoderma harzianum* A3091, H1 *Trichoerma viride A3092*, M6 *Glomerella*, H3 *Embellisia* sp2 (t), C8 *Chrysosporium*, J8 *Phoma* and N3 *Phoma*.

The supernatants from the CLS cultures were incubated with grape juice (2:1) for two to five days at 10 ºC and 20 ºC and proteolytic activity was assessed by SDS-PAGE. Isolates that appeared to cause a slight degradation of the chitinase and thaumatin bands after five days incubation at 20 ºC were C8 *Chrysosporium*, N3 *Phoma*, M6 *Glomerella*, H3 *Embellisia*, O1 *Phialophora*, and B8 *Aureobasidium*. Degradation of chitinase only was apparent in B8 *Aureobasidium*, C7 *Aureobasidium* and K2 *Aureobasidium* (Fig. 2).

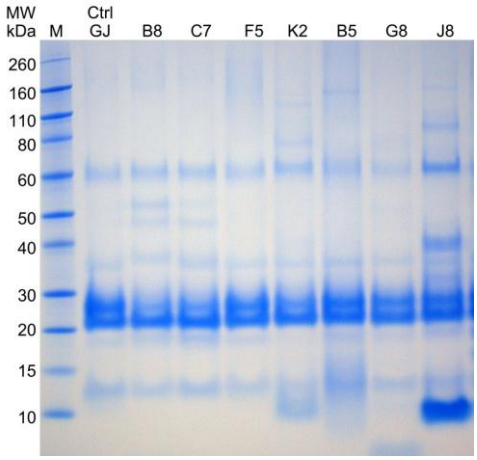


Figure 2: SDS-PAGE of protein samples resulting from the incubation of grape juice with a selection of the supernatants from Antarctic fungi grown in CLS medium. Supernatants and juice (2:1) were incubated at 20 ºC for 5 days. M: Molecular weight marker. Ctrl GJ: Control, water and juice (2:1). Fungal strains: B8 *Aureobasidium*, C7 *Aureobasidium*, F5 *Phoma*, K2 *Aureobasidium*, B5 *Geomyces pannorum*, G8 *Basidomycete*, J8 *Phoma*. 4-12% Bis-Tris gel, Invitrogen.

The top 24 isolates from the plate assays were then cultivated in skim milk medium. Whereas the CLS medium contained diverse nutrient sources and is known to induce the secretion of a wide range of hydrolytic enzymes, the skim milk medium limited the carbon and nitrogen sources to protein, thereby requiring the isolates to secrete proteases to obtain nutrition. In the first round of cultivation the culture medium was initially set at pH 5.5, a pH commonly used for the cultivation of wide range of filamentous fungi. The second round of cultivation took place using a starting pH of 4.3. The increase in acidity was intended to encourage the secretion of acidic proteases, which are characteristically secreted by fungi in acidic environments in favour of neutral or alkaline proteases (Jarai and Buxton, 1994; Gente *et al*., 1997; Gente *et al*., 2001).

The supernatants from the skim milk cultures were incubated with grape juice (2:1) at 10 °C and 20 °C for two to five days. Degradation of the grape juice proteins was investigated by SDS-PAGE as previously described. As shown in Figure 3, after two days at 10 °C, supernatant from skim milk culture (pH 4.3) of O1 *Phialophora* caused some modification of chitinase, appearing to result in the reduction of the molecular weight to approximately equal that of the thaumatin; supernatant from K8 *Phoma* caused a very slight degradation of both thaumatin and chitinase; and supernatant from N3 *Phoma* caused a slight degradation or modification of thaumatin (resulting in a slight lightening and widening of the 22 kDa band representing thaumatin).

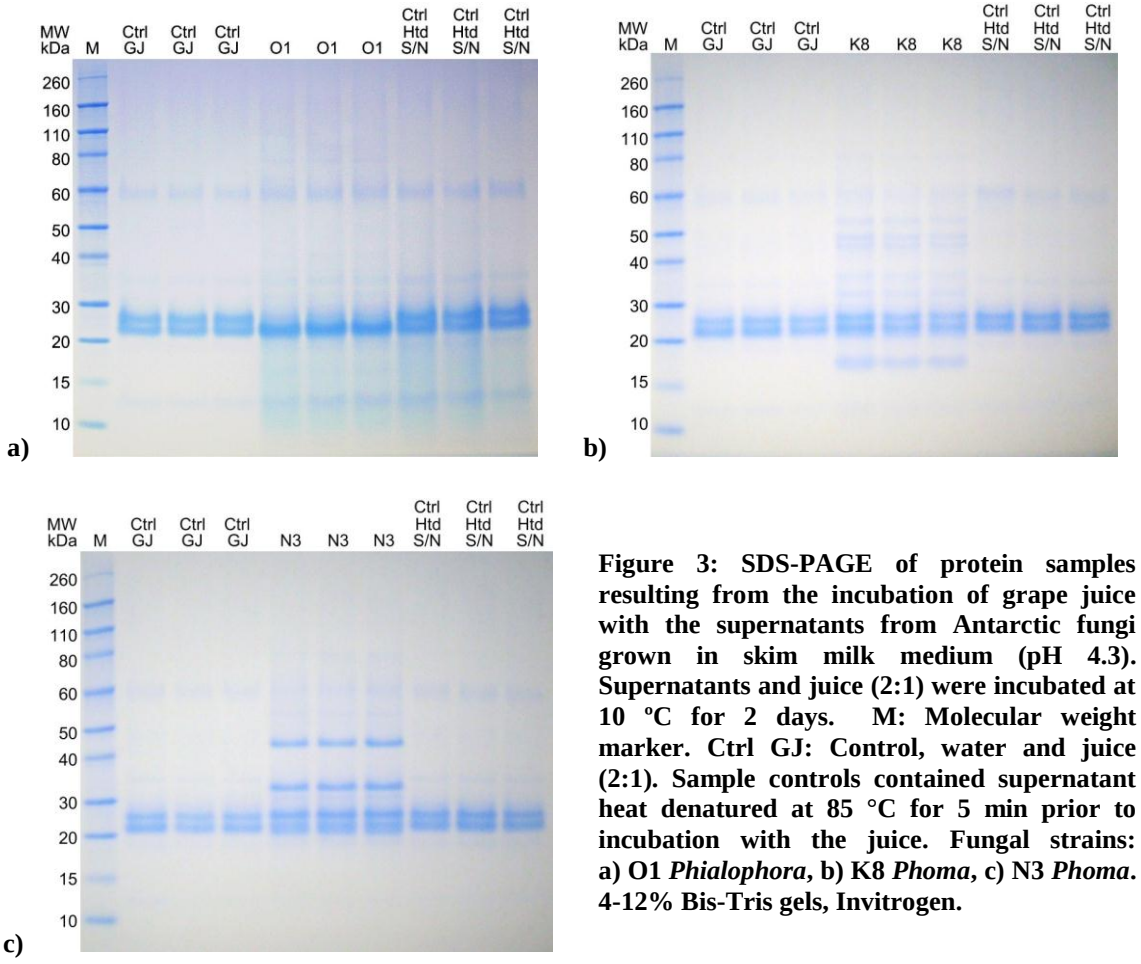


Figure 3: SDS-PAGE of protein samples resulting from the incubation of grape juice with the supernatants from Antarctic fungi grown in skim milk medium (pH 4.3). Supernatants and juice (2:1) were incubated at 10 ºC for 2 days. M: Molecular weight marker. Ctrl GJ: Control, water and juice (2:1). Sample controls contained supernatant heat denatured at 85 °C for 5 min prior to incubation with the juice. Fungal strains: a) O1 *Phialophora*, b) K8 *Phoma*, c) N3 *Phoma*. 4-12% Bis-Tris gels, Invitrogen.

The supernatant of one strain, A6 *Chrysosporium* grown in skim milk medium (pH 4.3), was found to degrade the grape juice proteins at elevated temperatures. When the A6 supernatant was incubated for ten minutes with grape juice at 60 °C, a reduction in the chitinase and thaumatin resulted (Fig. 4). A very slight reduction in the grape juice proteins was evident after ten minutes incubation at 50 °C. No proteolysis was evident at 45 C.

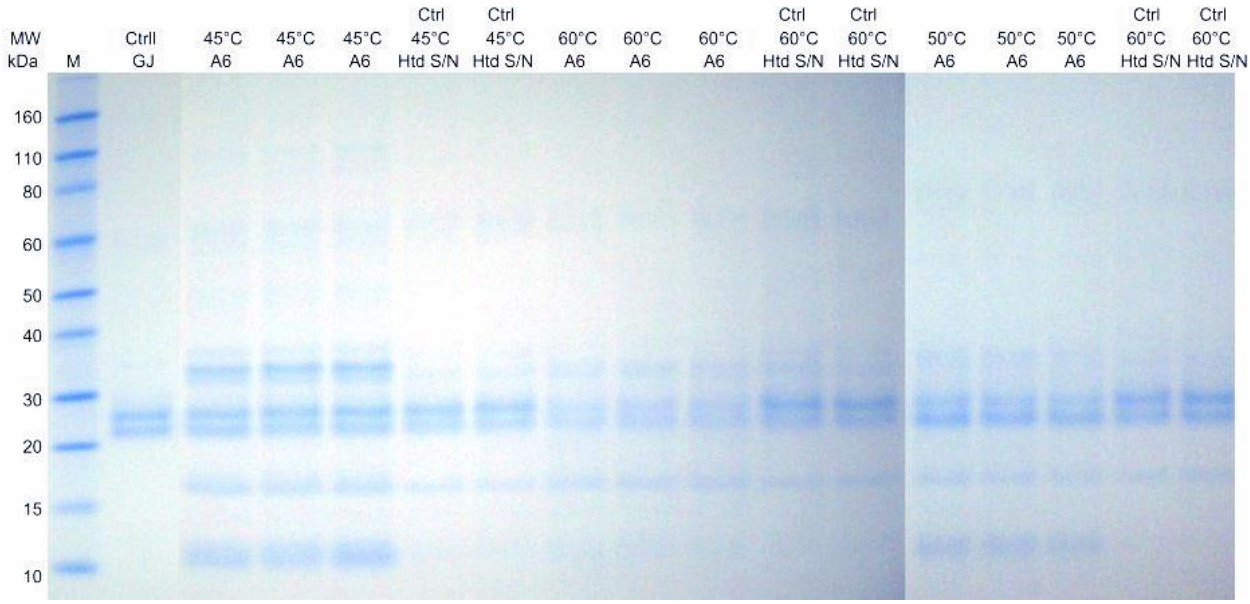


Figure 4: SDS-PAGE of proteins resulting from the 10 min incubation of A6 *Chrysosporium* supernatant (skim milk medium, pH 4.5) with grape juice (2:1) at 45 °C, 60 °C and 50°C as indicated. M: Molecular weight marker. Ctrl GJ: Control, water and juice (2:1). Sample controls contained supernatant heat denatured at 85 °C for 5 min prior to incubation with the juice. 4-12% Bis-Tris gels, Invitrogen.

Tentative identifications of the A6 proteases were achieved by mass spectrometry. There was a dominant serine protease in the A6 supernatant, with many good peptide matches to a "subtilisin-like protease" (Uniprot A6XA83 from *Verticillium lecanii*) and an "alkaline protease" (Uniprot Q02291) from *Fusarium* sp. S-19-5. There were also hits to a carboxypeptidase (E9E5P3) from *Metarhizium acridum*.

Outcomes and Conclusions

Project objectives have been achieved. The entire collection of 200 fungal isolates from Antarctica were screened for protease activity using plate assays carried out at low temperatures and under acidic conditions. Isolates producing proteases of interest were revealed. Cultivation in liquid media was carried out to produce fungal supernatants containing the secreted proteases, appropriate for incubation with grape juice. After two days incubation with grape juice at 10 ºC, supernatant from a *Phialophora* stain (O1) grown in an acidic skim milk medium caused some modification of chitinase, resulting in a reduction in molecular weight. The supernatant of a *Chrysosporium* strain (A6) caused significant degradation of both chitinase and thaumatin at 60 ºC, probably through the action of a serine protease that was identified by mass spectrometry. The *Chrysosporium* protease could be of interest for accelerating protein degradation in juice in combination with heat treatment. The search for a protease effective against haze-producing proteins under winemaking conditions remains a task for the future.

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Appendix 2: Staff

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