Screening *Botrytis cinerea* for proteases suitable for removing haze-forming proteins in winemaking

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Declaration

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All other research described in this report is my own original work and has not been submitted for a higher degree to any other university or institution.

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ABSTRACT

Protein haze in wine is caused by plant pathogenesis related (PR) proteins that precipitate when heated, cause the wine to look cloudy and render it unsaleable. Advancements have been made to reduce the economic, environmental and wine quality impacts that come with traditional bentonite stabilisation. Enzymatic treatment with recombinant proteins has shown some promise, however genetically modified organisms cannot be used in Australian commercial winemaking. In order to find a non-genetically modified alternative for protein haze removal, 99 isolates of *Botrytis cinerea* were initially screened for strong growth and sporulation, with 55 of these isolates selected and screened for protease production by skim milk plate clearing assay. These results coupled with guaiacol plate assays to assess laccase production from these isolates, which would oxidize the finished wine, resulted in 6 *B. cinerea* isolates that were assessed for protease secretion in liquid culture. Analyses of the protease and laccase production of these isolates by fluorescence microplate and syringaldazine assays identified 6 *B. cinerea* isolates that secrete high levels of protease, to be used in continuing research.

Keywords: *Botrytis cinerea*, winemaking, protease, heat stabilisation, wine protein haze

INTRODUCTION

Winemaking is a $4.2 billion dollar industry, contributing approximately $48 billion to the Australian economy [1]. Winemakers aim to make the winemaking process more efficient, less costly and more environmentally friendly by incorporating technological advances. However, one winemaking process which has undergone little change over time is that of heat stabilizing wine to prevent protein haze. Wine protein haze is caused by the aggregation of grape pathogenesis related (PR) proteins, which makes wine look cloudy and unsaleable. Typically, bentonite is used to remove these proteins, though this process has associated economic and environmental impacts and can also damage the quality of the finished wine. *B. cinerea* has been shown to secrete a protease that has direct action on PR proteins [2]. The gene for this protease was successfully expressed in *Pichia pastoris*, resulting in a significant reduction in PR proteins and protein haze compared to untreated wine [3]. The current study aimed to find a method of replicating these results without recombinant genetic modification, in order to harness this process for commercial winemaking in Australia.

This paper commences with a brief literature review covering wine haze, bentonite use and alternatives, and an overview on the properties of *B. cinerea*. The screening and evaluation tools developed during this study were successfully used to identify six isolates of *B. cinerea* with strong protease production to be used for continuing research. The isolates are candidates for use in production of proteases for preventing wine haze.

Wine Haze is caused by Grape Pathogenesis-Related Proteins

Wine protein haze occurs when a wine is heated for a period of time, either during transport or under regular storage conditions over long periods of time. Proteins in the wine slowly unfold and aggregate, causing a fluffy precipitate, making the wine look cloudy and unappealing to consumers [4]. Protein haze can also be mistaken for microbial spoilage [5].

Several studies have analyzed the proteins that occur in wine haze, with the overall aim of identifying and targeting these proteins for degradation and removal. These proteolytically resistant proteins were identified as pathogenesis-related (PR) proteins, namely chitinases and

thaumatin-like proteins (TLPs)[6]. Further studies examined the behavior of these proteins during wine-haze formation and identified chitinase as the major cause of protein haze [7], while only TLP isoforms that denature under winemaking conditions contribute to protein haze [8]. Exo-β-1, 3-glucanases [9] have also been identified in the protein fractions of white wine haze.

Bentonite and Alternative Methods of Wine Fining

To prevent protein haze in wine, bentonite is added at varying stages; either before, during or following fermentation [10, 11]. Bentonite is a silica-based clay with a large surface area, owing to the flat sheets of its structure. At the pH of wine (generally 3.0 to 3.5) bentonite has a negative charge and wine proteins a positive charge. These mutually attract, and aggregate by cation exchange [4]. The bentonite adsorbs the proteins and settles out of solution to the bottom of the tank, forming bentonite lees.

While this method has been used for over a century to prevent wine-haze, there are many disadvantages to its use. At a chemical level, studies have observed that bentonite can remove some of the finer, varietal characteristics of a wine, such as the volatile compounds that incorporate the distinct mouthfeel or aroma of a wine [12, 13]. The environmental impact of bentonite use occurs from mining and transporting the clay and from the disposal of the used bentonite after wine fining, usually requiring specialised transport and storage and increased manual labour [4, 14]. However, the most costly factor of bentonite treatment is the volume of wine which is trapped following treatment, with up to 10% of total wine volume sequestered in the bentonite lees[14]. Most of the wine can be recovered by rotary drum vacuum filtration or centrifugation, but recovery can cause loss of quality through oxidation and subsequent decrease in value of the wine [4, 14]. This is the greatest cause of monetary loss in the bentonite fining process, costing the Australian wine industry approximately $45 million in 2005 [14].

As such, researchers have been examining alternatives to bentonite for removing haze-forming proteins from wine. Alternative adsorptive methods were examined as a replacement for bentonite, such as ion-exchange resins, low-swelling clays and other adsorbents of proteins such as silica gel and alumina [15]. Although some compounds had good potential as alternatives to bentonite, none of these methods has been adopted by the commercial winemaking industry. Ultrafiltration of wine to collect haze forming proteins has also been researched [16], though the

high cost of set up and unreliable heat-stability results have made this option unattractive to commercial winemakers.

As early as the 1960's, researchers were using enzymes to attempt to remove proteins from fermented beverages [17]. An immobilized acid protease has been tested to treat wine protein haze [18] however the treatment did not successfully prevent protein haze formation. Later studies examined heat treating wine at 90 °C for 1 minute, with and without the addition of an enzyme to cleave the haze-forming proteins [19]. This method showed promising results, though more efficient proteolysis was required. A mix of Aspergillopepsins I and II (AGP) coupled with flash pasteurisation was shown to successfully heat-stabilise wine [20]. Ideally though, an enzymatic treatment that does not require heat activation would provide a viable, low cost alternative to bentonite, and remove the unnecessary labour and environmental impact associated with bentonite use. Such an enzyme has recently been identified from the fungus *Botrytis cinerea* [3]. The activity of this protease was first identified in plants infected with *B. cinerea* which showed lower levels of PR proteins than those infected with different species of phytopathogenic fungi, though the cause could not be identified [21]. Later studies showed that *B. cinerea* secreted proteases have a direct effect on haze-forming PR proteins [2], with the most abundantly secreted of these proteases identified as BcAP8 [22]. In a 2013 study, Van Sluyter et al. inserted the *Bcap8* gene into *Pichia pastoris* and the addition of the recombinant protease to wine produced a marked reduction in protein haze compared to the control wine. Unfortunately, the use of recombinant DNA excludes this protease preparation from use in the Australian wine industry. Hence, the aim of the current study is; to isolate strains of *B. cinerea* that have the highest natural secretion of proteases for cost-effective use in commercial winemaking.

Botrytis cinerea

1. Host species

Botrytis cinerea is one of the best-studied of all pathogenic fungi [23], possibly due to its extensive host range, adaptable growth requirements and the economic impact it has on crops worldwide. *B. cinerea* is a phytopathogenic fungus with a host range spanning over 200 species of agriculturally significant crops [24]. While researchers have studied the fungus on hosts including beans, tomatoes, cucumbers and roses, the most commonly associated host, and name

sake (from Greek Botrys meaning grape bunch)[25] is *Vitis vinifera* or the common grape vine. *B. cinerea* causes grey rot or 'bunch rot' in infected host plants, causing damage to the exterior and leaving the plant susceptible to secondary infection with environmental bacteria or yeasts. Infection typically occurs in host plants after late-season rain, or following times of high humidity [26].

Under the right conditions, *B. cinerea* can also be utilised to make sweet and desirable dessert wines. When a period of high humidity and rainfall is followed by a stretch of low humidity, the fungus permeates the walls of the grape, allowing the fruit to dry out and concentrating the sugars and other compounds in the berries [27]. This phenomenon is known as noble rot [28].

Researchers have suggested that *B. cinerea*'s adaptability to many host species and opportunistic tendencies are largely due to the great variation within the species, both morphologically and physiologically [29]. *B. cinerea* is well known for its genetic variability, even within strains, due to its multinucleate cells [30]. Studies have found significant variation both within the species and within strain types, making the isolation and maintenance of stable cultures difficult [31-33].

2. In vitro Growth and Analysis of *B. cinerea*

The most commonly used growth media for growth of *B. cinerea* are potato-dextrose agar (PDA)[34], malt-extract agar (MEA) and yeast-extract agar (YEA)[35]. Researchers have also created specific media for enumerating and identifying isolates of *B. cinerea* from environmental samples, such as *Botrytis* spore trap medium [35], though these methods are not necessary for general laboratory use.

Differentiation among *B. cinerea* isolates has recently been assessed using a combination of morphological, genetic and biochemical approaches [36]. Following publication of the entire genome sequence of *B. cinerea* [37], methods for its identification and comparison have become more DNA based. However, in this study, the focus will be on the morphological differences and biochemical processes of *B. cinerea* before progressing to DNA based methods.

3. *B. cinerea* Secretome

Studies on the secretome of *B. cinerea* are integral to the current study, as proteases, laccases and extra-cellular glucan production are all part of the *B. cinerea* secretome. Much of the recent research efforts on *B. cinerea* involve the identification, comparison and utilisation of the many gene products that the fungus produces. Researchers have identified enzymatic activity such as pectinase, xylanase and acid proteases [38], ascorbic and glutathione peroxidases [39] and acid phosphatase [40] amongst many others in the secretome of *B. cinerea*.

Recently, researchers have focused more on analyzing the composition, role and timing of the proteins and other components secreted by *B. cinerea*. 2D electrophoresis followed by mass spectrometric (MS) analysis of the protein profile of *B. cinerea* identified 64 major spots visible for all replicates, varying from 14 to 85 kDa in size and spanning pH 5.4 to 7.7 [41]. These proteins were identified as mostly malate dehydrogenase, glyceraldehyde-3-phosphate and hypothetical proteins. 2D electrophoresis and MS were subsequently used to identify differences in secreted proteins between two *B. cinerea* strains with differing virulence [42], showing both quantitative and qualitative differences in protein expression patterns between the two isolates.

High-throughput liquid chromatography–mass spectrometry (LC-MS/MS)[43] has also been used to identify and compare proteins secreted by *B. cinerea* during interaction with different plant hosts, finding that sixty of eighty-nine proteins contained a motif that implied their extracellular nature, with seven proteins observed in all growth conditions, including two peptidases and an aspartic protease. A similar method was used to look at the compounds secreted during germination in a simulated plant environment [44], identifying aspartic acid protease BcAP8 as the most abundant protein secreted in the first 16 hours of culturing.

More recent studies have used 2-D electrophoretic techniques coupled with the introduction of label-free liquid chromatography–mass spectrometry to identify variable proteins between the mycelia and secretome [45] and 2-D electrophoresis combined with qPCR to identify proteins secreted during conidial germination [46]. These studies identified the secretion of many compounds related to *B. cinerea* pathogenicity and virulence, along with other proteins involved in the biological functions of the fungus, including those proteins mentioned previously, and again, aspartic protease.

4. *B. cinerea* Protease as a Potential Alternative to Bentonite

Early studies into protease production in *B. cinerea* [38] showed that more virulent strains of *B. cinerea* secreted an acid protease at higher levels compared to less virulent strains. Further purification and characterization of the protease found that the enzyme had a mass of 38 to 39 kDa, was inhibited by pepstatin, was present in ungerminated spores and was also produced during germination [47]. Additionally, it was discovered that the activity of aspartic protease secreted by *B. cinerea* could only be seen in synthetic media with a pH of between 3 and 4 [48]. Concurrently, researchers noted the degradation of proteins in champagne musts from grapes infected with *B. cinerea* in comparison with those of healthy grapes [49], which decreased the desirable foaming properties of the champagne. Subsequent studies [2] identified the specific proteolytic activity of proteins from *B. cinerea* at wine pH, using SDS-PAGE and the Bradford protein assay.

Investigations into the expression of proteases by *B. cinerea* coupled with phylogenetic analyses of the protease genes and RNA, identified a family of aspartic proteases designated as BcAP1 to 5 [50]. Further studies [22] identified an additional nine BcAP proteases in *B. cinerea*, designated BcAP6 to 14. Of these aspartic acid proteases, BcAP8 alone comprised up to 23% of the total protein secreted by *B. cinerea*. To establish a link between the abundance of secreted BcAP8 from *B. cinerea* and the ability of *B. cinerea* proteins to break down wine proteins, a 2013 study by Van Sluyter et al.[3] used genetic manipulation techniques to insert the *Bcap8* gene into *Pichia pastoris*. When expressed in *P. pastoris*, this enzyme successfully removed grape chitinases under normal winemaking conditions and had some activity on TLPs. Overall, this method resulted in wine that was substantially more heat stable than the control wines under normal winemaking conditions.

5. Additional Considerations - Glucan and Laccase

In addition to many proteins and other compounds, *B. cinerea* secretes an extracellular β-(1, 3)(1, 6)-D-glucan sheath which is believed to regulate the activity of secreted enzymes, help spores adhere to host plants and assists in the virulence of the fungus [51]. This extra-cellular sheath is mostly comprised of carbohydrates, proteins and lipids [52], and also displays polygalacturonase and laccase activity. The presence of this glucan in culture results in a greatly

increased viscosity of culture media [53] which can interfere with subsequent assays. The glucan is also undesirable to the winemaking process as it can clog filters and increase the viscosity of the wine. As such, reduction or inhibition of glucan production in *B. cinerea* isolates would be advantageous to prevent downstream interference in the winemaking process.

Laccases secreted by *B. cinerea* are another area of interest to this study, as the addition of laccase to wine can cause serious oxidative damage [54] and it is difficult to remove or degrade due to its stability at acidic pH (3.5 to 4.6) [54, 55]. Several assays have been developed to identify and quantify laccase activity from fungi, including *B. cinerea*. Depending on the target organism, 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), polymeric dyes like remazol brilliant blue-R (RBB-R), guaiacol or syringaldazine [56] have been used in different combinations by researchers as indicators of laccase activity. Of these, syringaldazine is commonly used for the detection of laccase activity in *B. cinerea,* and has even been used for localizing laccase activity cytochemically [57]. A specific test for the detection of *B. cinerea* laccase activity in musts and wines has also been developed [58]. Laccases are a highly useful product in many industrial material processing operations such as textile dye decolourization, delignification of pulp and effluent detoxification [59] and have even been tested for use in heat stabilization of wine through pre-fermentative treatment [60].

4. Experimental Approach

Although beyond the scope of the current study, the overall aim of this research program is to grow *B. cinerea* on an industrial scale in order to produce high amounts of proteases that can be utilised for protein haze removal during commercial winemaking. Ultimately, the large scale growth will need to be coupled with the elimination or inhibition of laccase and glucan production to prevent the degradation of wine quality. Additionally, the use of recombinant genetic modification on the organism must be avoided so that the protease can be used in Australian commercial winemaking. With this in mind, the current study aims to screen *B. cinerea* isolates from the wild in order to identify strains with the greatest natural protease, and low laccase production. The particular study aims were as follows:

1. Isolate and identify *B. cinerea* isolates that grow and sporulate well and quickly enough to facilitate large scale growth

2. Identify *B. cinerea* isolates with the highest natural secretion of proteases and lowest levels of laccase as expressed on agar medium

3. Test culture media and techniques that provide the best level of protease secretion from *B. cinerea* isolates, while minimizing interfering glycan production

4. Compare relative levels of protease and laccase production between *B. cinerea* isolates by fluorescence protease assay and syringaldazine assay, respectively

METHODS

Botrytis cinerea Isolates

Sixteen isolates of *B. cinerea* were provided by Treasury Wine Estates (Nuriootpa, South Australia), collected from grape vines in Robin Vale, VIC, Sellicks Beach, Robe, and Riverland, SA and Coomella, NSW. Five stock isolates previously collected from SA and Fernhill, VIC were purchased from AgPath (Vervale, Victoria), and 65 isolates were provided by the Primary Industries and Regions South Australian division of the SA Research and Development Institute (PIRSA-SARDI; Adelaide, South Australia), collected from various regions in the Adelaide Hills, SA. Treasury Wine isolates were designated a number prefixed by 'TWE', Agpath isolates prefixed by 'AP' and PIRSA-SARDI isolates by 'S' (Table 1).

Growth and Sporulation of *B. cinerea* Isolates

Isolates were grown on potato dextrose agar (PDA) plates, prepared as per manufacturer's instructions (Sigma-Aldrich, NSW) by adding 39 g/L of potato-dextrose agar to deionized water (dH_2O), for initial observation of morphology and to check for contamination. In the case of contaminated isolates, spore solutions were serially diluted to 10^{-5} mg /mL and the lowest three dilutions plated onto PDA plates containing 0.1% Triton X-100 to inhibit the spread of colonies. Isolated *B. cinerea* colonies were then collected from these plates, re-grown on fresh PDA plates and newly generated spores collected and stored as described below. Induction of sporulation was also tested on V8 tomato-juice agar, containing 600 mL V8 vegetable juice, 300 mL tomato juice, 100 mL water, 15g sucrose and 20g agar in 1L, with pH adjusted to 6.5 using malic acid [61].

Collection and Storage of Spores from Isolates

Spores were harvested from *B. cinerea* isolates by the addition of 8 to 10 mL of 0.01% Tween80/ 0.9% NaCl solution to the agar plates which was gently spread with a microbiological spreader to loosen spores. The spore solution was then removed from the plate using a serological pipette and collected into a 15 mL Falcon tube. Samples were centrifuged on low speed and the supernatants were discarded. Spores were suspended in Milli Q water for storage

Table 1. *B. cinerea* isolates used in this study, including identification number, location of collection and host plant from which the isolate was collected. For brevity, consecutive samples with the same details have been grouped.

Sample Name & ID no	**Species**	**Collected / Provided By**	**Location Collected**	**Host**
TWE02	*B. cinerea*	TWE	Sellicks Beach, SA	Chardonnay
TWE03	*B. cinerea*	TWE	Robin Vale, VIC	Chardonnay
TWE05	*B. cinerea*	TWE	Robin Vale, VIC	Pinot gris grape
TWE06	*B. cinerea*	TWE	Coomella, NSW	Pinot gris grape
TWE07	*B. cinerea*	TWE	Riverland, SA	Chardonnay grape
TWE08 to 18	*B. cinerea*	TWE	Robe, SA	Shiraz grape
AP1 (AgL67)	*B. cinerea* B400	AgPath	SA	Grape Leaf
AP2 (AgL72)	*B. cinerea* B406	AgPath	Fernhill, VIC	Chardonnay Berry
AP3 (AgL73)	*B. cinerea* B407	AgPath	Fernhill, VIC	Chardonnay Berry
AP4 (AgL76)	*B. cinerea* B419	AgPath	Fernhill, VIC	Chardonnay Berry
AP5 (AgL75)	*B. cinerea* B418	AgPath	Fernhill, VIC	Chardonnay Berry
S1to S77	*B. cinerea*	SARDI	Adelaide Hills, SA	Grape bunch

at 4 ^{o}C, with an additional 1 mL of each isolate placed in a solution of 12.4% glycerol/ 0.04% Tween 80 for long term storage at -80 ^{o}C. Prior to storage, 10 µL of each isolate was used to measure spore density with a haemocytometer. In the case of spore densities lower than 10^4 cells/ mL, isolates were replated onto PDA for additional collection at a later date.

Plate Assays of *B. cinerea* Protease and Laccase Production

Skim milk agar was used to test for protease activity of isolates by proteolysis of casein in the medium, producing a zone of clearing around the growing colony. Skim milk agar plates were adapted from a previous study [62] and contained 2% skim milk powder (Coles, Australia), 0.5% yeast extract, 1.5% agar and 0.1% Triton X-100, dissolved in a minimal salts solution [63] containing 1 g KH_2PO_4, 0.5 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl and 0.01g $FeSO_4$ per litre. Skim milk mixture was autoclaved at 121^{o}C for 15 minutes, and then approximately 20 mL was poured into plastic petri plates. Guaiacol agar was used to assess laccase production in *B. cinerea* isolates, indicated by a red/brown colouration of the agar from oxidation of guaiacol in the medium [59]. Plates contained PDA, prepared as mentioned previously, with 0.02% guaiacol added before autoclaving at 121^{o}C for 15 minutes, with approximately 20 mL poured into plastic petri plates.

Inoculation methods for these media were tested first by placing a 10 µL drop of spore suspension onto a divided plate and allowing the droplets to absorb into the agar before being placed into a 22 ^{o}C incubator for daily observation. The second method involved collecting and placing a 1 µL loop full of mycelium onto each of the agar types. Mycelia were slightly pushed into the agar so that any secreted proteases/laccases could be absorbed by the depth of the agar then plates were incubated at 22 ^{o}C for daily observation. All plate assays were performed in duplicate and the results averaged.

Shake Flask Cultures of *B. cinerea*

Shake flasks were prepared using a variety of liquid media to find a solution that provided adequate nutrients for the growth of the *B. cinerea* isolates while providing a low pH environment to simulate the pH of wine making. The skim milk medium contained 2% skim milk powder and 0.5% yeast extract dissolved in a minimal salts solution. Minimal salts based medium was comprised of 1 g KH_2PO_4, 0.5 g K_2HPO_4, 0.5 g $MgSO_4.7H2O$, 0.5 g KCl and 0.01g

$FeSO_4$ per litre [63] in dH_2O. 50 mL volumes were used in all flasks, with incubation temperature at 22°C and shaking at 124 OPM for 7 days. As this particular skim milk medium had not been used to grow *B. cinerea* in previous studies, several runs were attempted in order to optimise the formulation, autoclaving conditions and pH of this medium.

A yeast extract- lactose medium was also trialled, comprised of 3% yeast extract 1% lactose and minimal salts media as above. The pH was adjusted to 3.5 using malic acid or H_2SO_4. Shake flask experiments were typically run with a single flask for each *B. cinerea* isolate and one negative control with no *B. cinerea*. A final experiment was conducted in triplicate using the yeast extract-lactose medium, to test the reproducibility of protease production and compare *B. cinerea* isolates. A 20 µL drop of supernatant from each flask was also spotted onto skim milk and guaiacol agar plates as a preliminary test of protease and laccase production from the *B. cinerea* isolates, prior to further testing with fluorescence protease assays.

Fluorescence Assays for Assessment of Protease Production

In order to quantify and more accurately compare protease production between *B. cinerea* isolates, a fluorescence protease assay was conducted using supernatant collected from culture media in the shake flasks. This assay was performed using an EnzChek® Protease Assay Kit with green fluorescence (Life Technologies, VIC) prepared using citrate-phosphate buffer at pH 3.5 (14.1 mL 0.2M Na_2HPO_4 plus 35.9 mL 0.1M citric acid in dH_2O) and tested with 50 µL each of substrate and *B. cinerea* supernatant sample. Pepsin from porcine gastric mucosa (Sigma-Aldrich, NSW) was used as a reference protease at 8 dilutions from 2.5 to 0.02 µg/mL, to produce standard curves for comparison of *B. cinerea* isolate protease activity in each assay. The protease activity units were expressed as mg/L pepsin equivalents. Pepsin standards, citrate-phosphate buffer blank and *B. cinerea* supernatant samples were loaded in triplicate onto black, flat bottomed 96 well plates (Greiner, Sigma-Aldrich), incubated for 20 minutes at room temperature and fluorescence was read with filters at 485 excitation and 530 emission using OPTIMA Software (BMG Labtech, VIC) and a FLUOstar OPTIMA microplate reader (BMG Labtech). To calculate protease activity in the *B. cinerea* sample supernatant, a 2nd order polynomial standard curve was created with the pepsin dilutions, then protease activity in mg/L was calculated as pepsin equivalents using the quadratic equation and corrected for dilution factors. From those values, the top daily value for each isolate was chosen, and then the mean of

the triplicates for each isolate was plotted in a bar chart with error bars showing the standard deviation. A single-factor ANOVA was performed on the results using the Data Analysis Tools add-in for Microsoft Excel 2010.

Syringaldazine Assays for Assessment of Laccase Production

To quantify the amount of laccase produced by the isolates, a laccase assay was performed using syringaldazine (Sigma-Aldrich) with laccase from *Rhus vernicifera* (≥50 units/mg; Sigma-Aldrich) as a positive control. Syringaldazine solution was prepared at a concentration of 0.216 mM by dissolving syringaldazine powder in absolute methanol (0.0077 g in 100 mL) and placed on a magnetic stirrer until fully dissolved (approximately one to two hours). Laccase enzyme solution standard was prepared immediately before use by adding 1 mg of laccase powder to 1 mL of cold dH_2O. Assays were conducted using 100 mM potassium phosphate buffer at pH 6.5, prepared by adding 1.36 g of KH_2PO_4 to 100 mL of dH_2O and adjusting the pH to 6.5 by addition of 1M KOH. Assays were performed at room temperature (approximately 22°C)[64]. Assays were read in a spectrophotometer at 530nm in 30 second intervals to 10 minutes total assay time, using kinetics over time setting.

One unit of laccase activity is described as the quantity of enzyme required to oxidize 1 nanomole of syringaldazine[58]. Laccase units /mL of enzyme were determined by the ΔA_{530nm} of a sample, using the formula below:

$$\frac{\Delta A_{530nm}\ \text{Sample} = A_{530nm}/\text{min Test} - A_{530nm}/\text{min Blank})}{(0.001)(0.5)}$$

0.001 = the change in A530nm/min. per unit of laccase at pH 6.5 in a 3 mL reaction mix
0.5 = volume (in milliliters) of enzyme used.

RESULTS

Growth and Sporulation of *B. cinerea* Isolates

B. cinerea isolates showed great variation in colony morphology, ranging in colour from off white to greyish-brown (Figure 1). Isolates typically grew in diameter between 2 to 20 mm a day, with an average of 5 days to reach the edges of an agar plate. Some isolates, for example TWE13, grew slowly and did not produce much aerial hyphae, but produced spores within three to four days of being plated. Most other isolates had more fluffy growth and produced abundant aerial hyphae with a thick mat of hyphal growth when the edge of the plate was reached. *B. cinerea* isolates were typically grown on PDA plates. Most isolates sporulated well on PDA so were maintained on this medium. The V8 tomato-juice sporulation medium was also tested, but was not preferred for the optimal growth and sporulation of *B. cinerea* isolates, as growth on this medium was often coupled with greater extra-cellular glucan production from the isolates, visible as an orange slime covering the mycelial growth. Due to the large number of isolates screened and time constraints of this study, single spore isolation of the *B. cinerea* isolates was not performed.

Collection and Storage of Spores from Isolates

Sporulation of *B. cinerea* cultures typically occurred when an isolate had covered the plate and the hyphal growth had darkened. If sporulation was not evident within 14 days of growth on PDA, the *B. cinerea* isolate was plated onto V8-tomato juice agar to encourage sporulation. If sporulation was still not evident on either the PDA or V8-tomato juice agar an isolate was deemed ‘non-sporulating’ (Table 2).

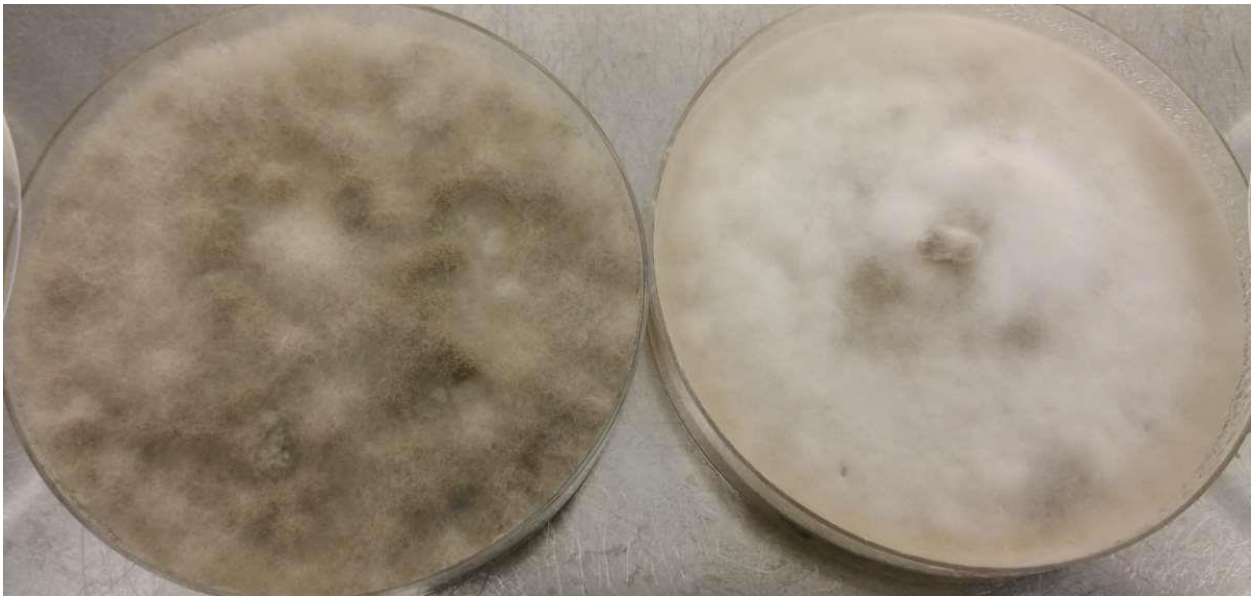

Figure 1. Example of differing colony appearance of 2 *B. cinerea* isolates (AP3 and AP4) after 10 days on PDA at 22 °C in 12-hour alternating light and dark cycles.

Table 2. Total rankings for 55 *B. cinerea* isolates following protease and laccase plate assays, including decisions based on adequate growth and purity of cultures. 44 isolates that did not provide adequate growth or sporulation for further testing were not used for plate assays. Favourable protease ratings were 2 and above, while favourable laccase ratings were 8 and above. Shaded cells indicate the final 6 *B. cinerea* isolates used in shake flask cultures. Sporulation rating from - for poor sporulation to +++ for strong sporulation. N.d (not determined) indicates that no further assessment was required for that isolate to be excluded from further experimentation.

Isolate	**Average Protease score**	**Days to Laccase 3 score**	**Sporulation**	**Comment**
TWE02	2	7	++	Contaminated
TWE03	1.8	3	++	
TWE05	1.9	6	++	Contaminated
TWE08	1	6	++	
TWE10	0.8	3	++	
TWE12	2	10	-	
TWE13	2.1	4	+++	Contaminated
TWE17	0.8	4	+	
TWE18	0.4	4	+	
AP4	2.5	4	+	
AP5	2.1	4	++	
S7	1.5	6	+	
S8	1.5	6	+	
S14	2.6	4	++	Contaminated
S15	2.3	3	++	Clean sample
S16	0	10	n.d	
S17	1.1	4	n.d	
S19	2.8	3	++	Clean sample
S21	2.8	5	n.d	Contaminated
S23	1	5	n.d	
S27	1.6	10	-	
S32	1.8	4	n.d	
S37	2	3	n.d	
S38	1.4	10	++	Clean sample

S39	0	8	n.d	
S40	1.8	4	+	
S41	1.6	5	+	
S42	2.5	3	-	
S43	2.1	3	++	Contaminated
S45	0.9	6	n.d	
S46	2.3	10	-	
S47	1.3	3	n.d	
S48	1.3	7	n.d	
S50	1.4	3	n.d	
S51	1.1	10	n.d	
S52	2.1	8	++	
S53	1.4	5	n.d	
S54	2	7	+	Contaminated
S55	1.6	10	+	
S56	2.1	3	-	
S58	0.1	10	n.d	
S59	1.3	5	n.d	
S60	1.4	8	+	
S61	1.3	10	+	
S63	1	10	++	
S64	1.3	4	n.d	
S65	1.9	10	++	Clean sample
S68	1.1	8	n.d	
S69	2.5	4	++	Clean sample
S70	2.3	10	-	
S71	0	10	n.d	
S72	1.8	10	++	Clean sample
S73	0.9	10	n.d	
S74	1.1	10	++	
S77	1.6	5	-	

Protease and Laccase Plate Assays

Plate assays were used to test for protease and laccase production in the *B. cinerea* isolates. Assessment of protease activity was performed by observing the zone of clearing around the growing colony each day. The optimal plating method for protease and laccase testing was to collect a 1 µL disposable loop of mycelia and lightly press the growth into the agar, as opposed to plating a drop of spore solution, which resulted in more uncontrolled growth that quickly overtook the plates. A numeric rating system was used to rate the protease production of the isolates, based on the rating of protease activity used in a similar study[65]. A rating of 0 was given if there was no visible clearing of the medium upon which the *B. cinerea* colony was growing; a rating of 1 was given if only the area of agar below the growing colony showed clearing; a rating of 2 was given if the area of clearing around the colony was approximately 2mm or less and a rating of 3 was given for any clearing greater than this (Figure 2).

The assessment of laccase production from *B. cinerea* isolates was rated using a similar numeric system. A rating of 0 was given if there was no colour change to the guaiacol agar on which the *B. cinerea* isolate was growing; a rating of 1 was given if there was a slight or uneven colour change to the medium, typically localised around the centre of the colony; a rating of 2 was given if there was a more noticeable colour change in the agar, typically spread across the entirety of the colony, and a rating of 3 was given for any strong colour change in the agar (Figure 3).

After 8 days of observation the protease and laccase plate results were tabulated. Days 5 to 8 represented the most significant period of growth for the isolates on the skim milk/ protease plates, so the total score a *B. cinerea* isolate earned over those days was averaged to give an overall rating. For the guaiacol agar/ laccase plates, a rating was given for how many days it took the growing colony to reach a rating of '3' for laccase production. A rating of 10 (days) was given if the laccase production had not reached a rating of '3' by the last day of observations. This scoring system provided quantifiable data to differentiate high and low protease producing strains, while also taking into consideration strains that have lower laccase production.

Preliminary selection of *B. cinerea* isolates for further testing included any isolate with a protease rating of 2 or more regardless of laccase production, a protease score of 1.8 and above

with a laccase score of 5 or more, and then any isolate with a protease score greater than 1.4 and a laccase score of 8 to 10 (Table 2).

While the ratings for the protease and laccase activity alone provided 11 strains for further testing, there were secondary considerations for the final choice of *B. cinerea* isolates to be taken through to further testing. For example, some isolates provided good protease and laccase results, but took a long time to sporulate or would produce insufficient spores for inoculating shake flask medium. Some *B. cinerea* isolates also had contaminating fungal species growing when the isolates were subcultured, due to the collection of the original isolates from the wild. These latter types were excluded from further experimentation.

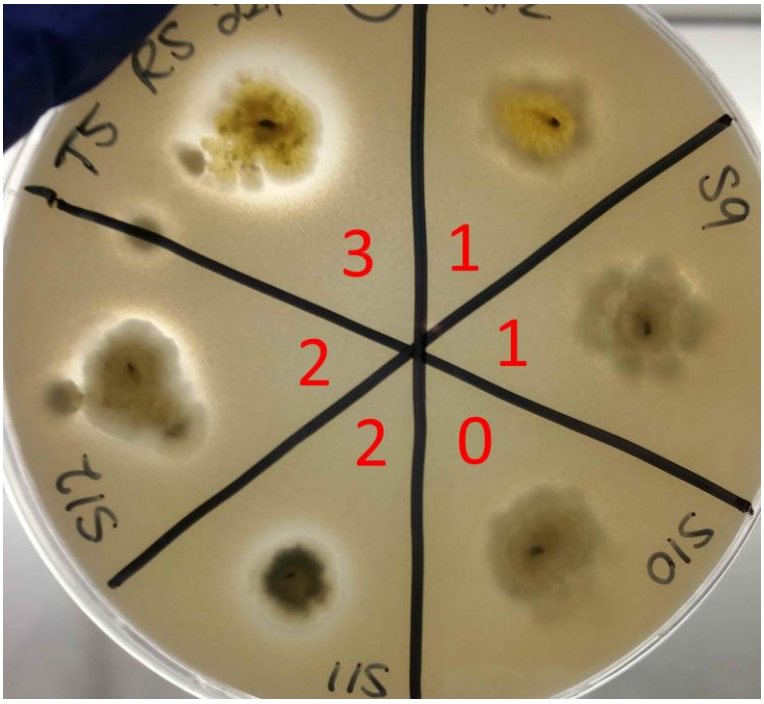

Figure 2. Example of clearing around a *B. cinerea* colony on a skim milk agar plate indicative of protease production and secretion. This plate represents 5 days of growth, with numbers indicating the denoted rating of the clearing (0 = no clearing, 1 = clearing under colony, 2 = <2mm clearing, 3 = >2mm clearing).

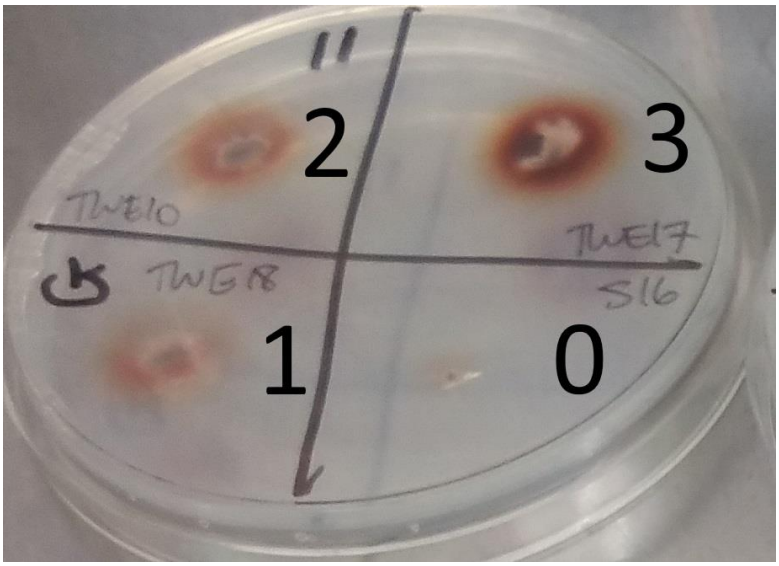

Figure 3. Example of colour change to a guaiacol agar plate indicative of laccase production and secretion from different *B. cinerea* isolates. Numbers denote the associated numeric rating of the colour change (0 = no change, 1 = feint change, 2 = stronger and more even change, 3 = dramatic colour change).

Shake Flask Cultures of *B. cinerea*

The aim of using skim milk medium in shake flasks was to emulate the protease production shown by *B. cinerea* isolates on the skim milk agar plates. The culture supernatant was subsequently utilised to compare the levels of protease and laccase production across the isolates in order to identify the *B. cinerea* isolates that had the highest extracellular protease production, ideally also with low laccase production. Many different shake flask methods were tested with varying organic carbon and nitrogen sources added to the minimal salt base, with varying autoclave conditions, in order to determine the formulation that provided the best protease production while eliminating any background interference from microorganisms inherent to the medium constituents (e.g. yeast from yeast extract or bacilli in skim milk powder). The propensity of skim milk to curdle and caramelise at pH below 4 and at autoclave conditions longer than 10 minutes at 121°C was a cause for much adjustment of culture medium. Ease of collecting supernatant isolates was also a focus when assessing the suitability of a particular medium for culturing the *B. cinerea* isolates, along with monitoring of the pH to assess the change *B. cinerea* isolates made to the medium (Table 3).

The optimum conditions for preparation of the skim milk medium was to add 1 g (2%) skim milk powder and 0.25 g (0.5%) yeast extract to each flask, topped up with 48 mL dH_2O and shaken at 60 RPM to dissolve powders. A separate 25x solution of the minimal salts based medium was prepared with 0.5 g/L KH_2PO_4 substituted for 2g/L K_2HPO_4 (up from 1.5 g/L) to provide a more buffered solution at pH 3.5, and all autoclaved at 121°C for 30 minutes. 2 mL of the minimal salts based medium was then added to each flask in a laminar flow hood at the same time as inoculating flasks with the *B. cinerea* isolates. This method provided the desired pH of 3.5 and prevented the milk proteins from curdling and adhering to the base of the flasks during autoclaving, as previous trials with pH adjusted prior to autoclaving had demonstrated.

Protease production by the *B. cinerea* isolates in this medium was preliminarily tested on skim milk agar and laccase production on guaiacol agar (data not shown). Protease production was evident by day 3 for many of the *B. cinerea* isolates, while by day 7 protease activity could be noticed for all of the isolates. Laccase production in the isolates was first noticed on day 3 in several isolates, and was evident in all isolates by day 5. When grown in skim milk medium the *B. cinerea* isolates produced high levels of extracellular glucan, which made the medium viscous

Table 3. Comparison of methods trialled for shake flask preparation. Minimal salts solution as per Cotoras et al, (2009) contained 1 g KH_2PO_4, 0.5 g K_2HPO_4, 0.5 g $MgSO_4$.7H2O, 0.5 g KCl and 0.01g $FeSO_4$ per litre, all pH adjusted with malic acid unless stated.

Media Composition (all prepared in minimal salts base unless stated)	**Autoclave Conditions**	**Preparation of Flasks**	**Comments**
2% skim milk at pH 3.6 separately to; 0.5% yeast extract, pH 3.1	100°C for 10 min 121°C for 15 min	Individually autoclaved at 121°C for 15 min then poured in laminar flow hood	Found bacilli and yeast growing by day 3 of sampling. Possibly from media components
1.5% soybean flour and 2% sucrose compared to 2% skim milk and 0.5% yeast extract	121°C for 30 min	Media added before autoclaving	Soybean flour increased glucan production/ viscosity of media. Skim milk preferable. Autoclaving in flasks made powders adhere to base
2% skim milk media in dH_2O, heated to 65°C for 30 mins, added to 0.5% yeast extract at pH 3.5	121°C for 30 min	Individually autoclaved at 121°C for 30 min then poured in laminar flow hood	Milk coagulated under these conditions and samples became very viscous by day 3 of growth. No bacilli or yeast visible
2% skim milk media in dH_2O, heated to 65°C for 30 mins, added to 0.5% yeast extract. pH adjusted to 3.5 post autoclaving	121°C for 30 min	Individually autoclaved at 121°C for 30 min then poured in laminar flow hood	2 samples not heated prior - none showed bacilli growth so pre-heating milk stopped. Milk still coagulated with pH adjustment post autoclaving
2% skim milk powder and 1.5% yeast extract, pH adjusted to 3.5 prior to autoclaving	121°C for 30 min	Media added to each flask and mixed for 5 mins before autoclaving	Milk adhered to base of flask and oil droplets from small amount of fat in milk were visible. Media very viscous by day 3 of sampling
2% skim milk powder added to 1.5% yeast extract, pH adjusted to 3.0 prior to autoclaving	121°C for 30 min	Milk media added to each flask and mixed for 5 mins before autoclaving, salts added in laminar flow	pH of media when salts added was 5.2, too high to be desirable. Milk still coagulated when pH dropped and media very viscous by day 3 of sampling
2% milk and 0.5% yeast extract with 25x concentrated minimal salts at pH 3.5 added after autoclaving	121°C for 30 min	Milk media autoclaved in flasks, with 2mL minimal salts added in laminar flow hood	Very little milk curdling, but flasks still very viscous by day 3 of sampling. Trialed alternative nitrogen source
1, 2 and 3% yeast extract with 1% lactose compared to 2% skim milk and 0.5% yeast extract, all pH 3.4	121°C for 30 min	Media added to each flask and mixed for 5 mins before autoclaving	3% yeast extract showed best protease production and media was not viscous from glucan production
3% yeast extract and 1% lactose at pH 3.4	121°C for 30 min	Media added to each flask and mixed for 5 mins before autoclaving	Media remained non-viscous and easy to sample from, with good protease production
3% yeast extract and 1% lactose at pH 3.4 adjusted with H_2SO_4, in triplicate for each isolate	121°C for 30 min	Media added to each flask and mixed for 5 mins before autoclaving	See detailed results

and difficult to sample. This observation was noticeable for several *B. cinerea* isolates from day 3 of incubation, but particularly for isolate S55 (Figure 4; subsequently removed from testing due to low sporulation), which was also overgrown and almost impossible to collect from by day 7. The pH of the skim milk medium also changed drastically over the 7 days of incubation, with most isolates peaking at or above pH 6 (Figure 5).

As the collection of culture supernatant was difficult when the *B. cinerea* isolates were grown in skim milk medium, an alternative media formulation was tested with a fixed carbon (lactose) to varying nitrogen (yeast extract) concentrations. Varying concentrations of yeast extract at 1, 2 and 3% were preliminarily tested with *B. cinerea* isolate S55 to find the amount that would produce the optimal protease production compared to the previous formulation of skim milk medium. The yeast extract medium flasks were supplemented with 1% lactose as a standard amount of carbon source as used in other fungal cultivations [66] along with the standard minimal salts based medium adjusted to pH 3.5 with malic acid. Yeast extract at 3% was found to generate the highest protease production for *B. cinerea* isolate S55 when compared to the other 3 formulations (1 and 2% yeast extract and skim milk media; data not shown), so this media formulation was tested further.

B. cinerea isolates were each inoculated into 6 flasks with 1 medium only control and incubated for 7 days with sampling occurring immediately post inoculation, then at days 1, 3, 5 and 7. Subsequent testing of the supernatant using the EnzChek® Protease Assay Kit for comparison of protease levels showed the highest maximum protease levels for all isolates on day 3 of sampling, however this assay was not optimised to allow for background fluorescence from the yeast medium used, so these results were inconclusive (data not shown).

Laccase production in these flasks was also preliminarily tested by syringaldazine assay. Calculation of the laccase activity in each flask was not possible as a ‘no inoculum control’ was not measured as a baseline comparison; however over 10 minutes of assaying a difference in the linear increase in laccase activity was observable between isolates. Four of the five isolates that showed the greatest protease activity at day 3 of sampling, namely S38, S72, S15 and S19, all showed the highest maximum readings for laccase by syringaldazine assay. However, *B. cinerea*

Figure 4. Progression of growth of *B. cinerea* isolate S55 in skim milk medium from the initial day of inoculation (far left) through days 1, 3 and 5 to day 7 (far right), noting the change in viscosity of the media by day 3 and overgrowth by day 7.

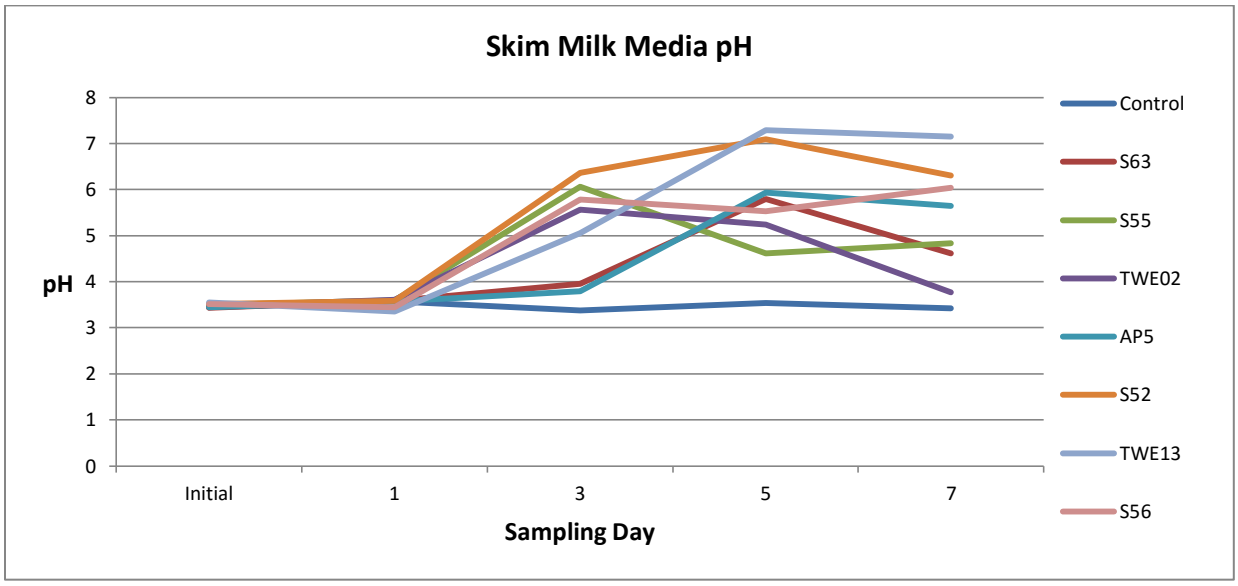

Figure 5. Change in pH of skim milk media flasks over 7 days of incubation with *B. cinerea* isolates.

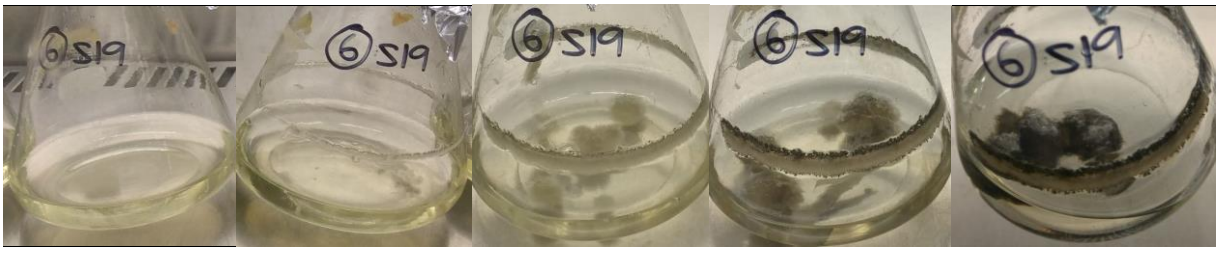

Figure 6. Progression of growth of *B. cinerea* isolate S19 in yeast extract-lactose medium from the initial day of inoculation (far left) through days 1, 3 and 5 to day 7 (far right), noting the change in morphology from growth in skim milk media and the maintained low viscosity of the medium.

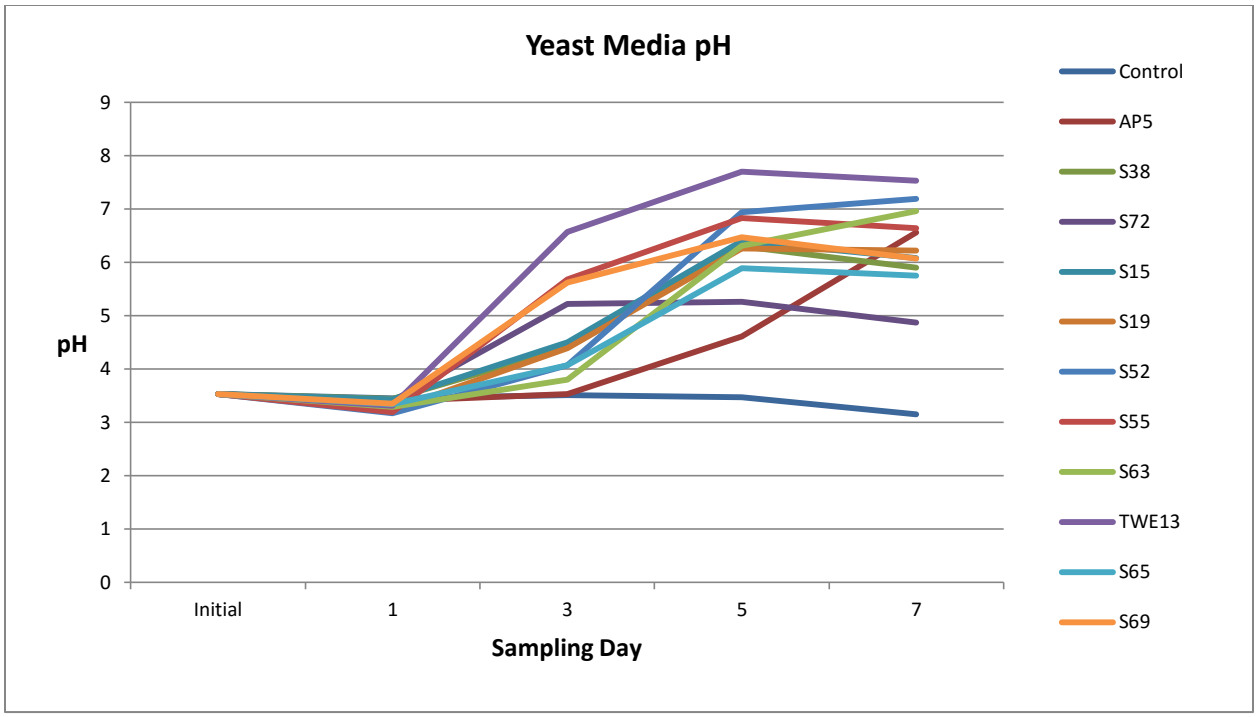

Figure 7. Change in pH of yeast extract-lactose medium flasks over 7 days of incubation with *B. cinerea* isolates.

isolate S65 showed negligible laccase activity while producing a higher level of protease (data not shown).

The morphology of growth of the *B. cinerea* isolates in the yeast extract-lactose media was another noticeable difference from the skim milk media. Instead of displaying dispersed growth with increasing glucan production which resulted in increasingly viscous media, the *B. cinerea* isolates grew in clusters and did not noticeably produce glucan at levels high enough to affect the viscosity of the media (Figure 6). The pH of this media did however show a dramatic increase over the 7 days, with most isolates peaking around pH 6 (Figure 7).

With the success of the yeast extract-lactose medium over the skim milk media for protease production and ease of sampling taken into account, an additional experiment with the yeast extract-lactose medium composition was run with triplicate flasks for each *B. cinerea* isolate. Included in this experiment were the isolates that showed strong protease production in the previous flask experiment (*B. cinerea* isolates S38, S72, S15, S19 and S65) with one lower protease producing strain for comparison (S69). Triplicates of each isolate were used in order to analyse the reproducibility of protease and laccase results for each *B. cinerea* isolate and to increase the reliability of comparisons between isolates.

Based on previous observations that sampling day 3 showed the highest protease activity, sampling was also conducted on day 2 to see if protease activity in fact peaked on day 2. Isolates showed great variation in morphology of growth across 7 days, both within and between groups of triplicate isolates. For example, isolate S38 showed similar morphology across all 3 flasks on day 3, but by days 5 and 7 the media in flask 5 was notably less viscous than that in flasks 4 and 6. The pH profile for this run was much less variable than previously observed, with the pH adjustment being made with dilute H_2SO_4 instead of malic acid. No isolate had a pH higher than 7, with the pH only passing 6 in all 3 replicates of *B. cinerea* S15 by day 7 (Figure 8).

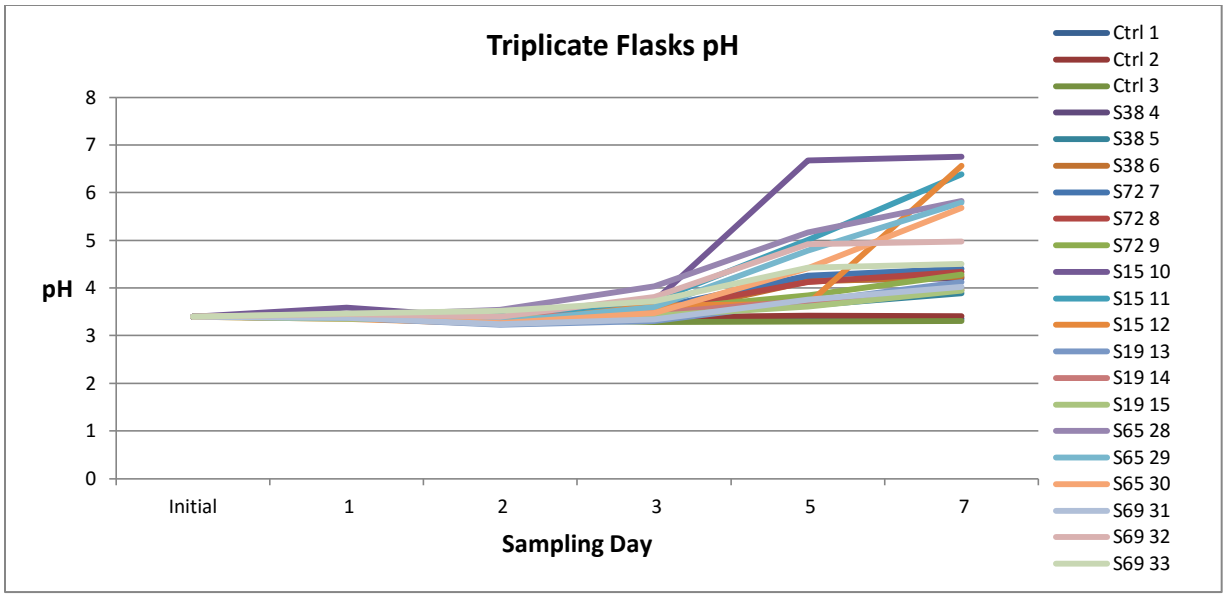

Figure 8. Change in pH of triplicate flasks over 7 days of incubation with *B. cinerea* isolates

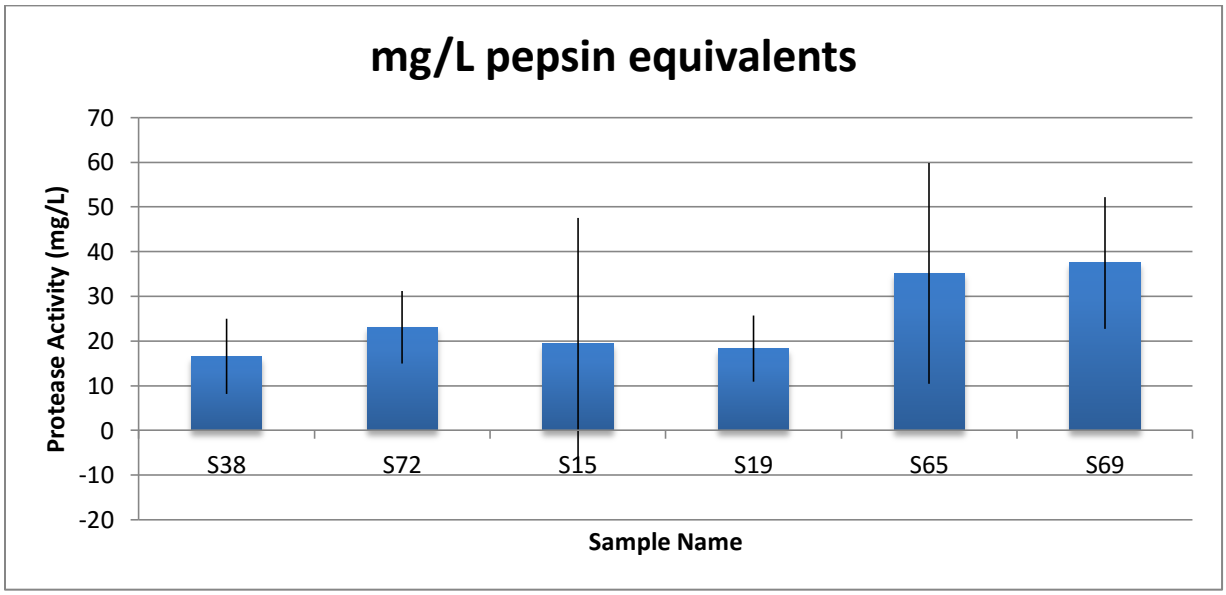

Figure 9. Maximum protease activity of *B. cinerea* isolates calculated as equivalent to known pepsin concentrations. Error bars indicate the standard deviation of each sample.

Quantification of Protease Production by Fluorescence Microplate Assay

Protease activity for the final run of triplicate flasks was quantified by EnzChek® Protease Assay, with pepsin used as a reference protease to create a standard curve to calculate pepsin equivalents for the protease production of *B. cinerea* isolates. A single-factor ANOVA was performed for these results, with a *p* value of 0.56 indicating no significant differences across the isolates. *B. cinerea* isolate S69 showed an overall high level of protease production, followed closely by *B. cinerea* S65, while isolate S15 showed a great level of variability across the triplicate flasks as evidenced by the large error bars for this isolate (Figure 9).

Quantification of Laccase Production by Syringaldazine Assay

For the final triplicate flask run laccase activity was quantified using syringaldazine assay as per Sigma-Aldrich's enzymatic assay of laccase protocol. Laccase activity for the *B. cinerea* isolates was calculated against a 'no inoculum control' from the same sampling day. Results were again variable between triplicates of each *B. cinerea* isolate, with isolate S15 showing high overall laccase activity on day 5 in one replicate, reaching similarly high levels of activity on day 7 for the further two replicates. A *p* value of 0.83 indicated that there were no significant differences within the dataset.

Table 4. Laccase activity from triplicate flasks of *B. cinerea* isolates. Numbers are expressed as Units/mL enzyme, with 0 denoting that laccase activity was not detected in sample.

***B. cinerea* isolate**	**Day**	**Replicate Flask**			**Mean**	**+/- St Dev**
		1	**2**	**3**		
S38	3	2	1.6	0.8	1.47	0.5
	5	140.8	2.4	114	85.7	59.9
	7	518.5	25.5	345.5	296.5	204.2
S72	3	11.5	5.5	9.5	8.8	2.5
	5	162	207.6	43.2	137.6	69.3
	7	247.5	464	693	468.2	181.9
S15	3	3.2	2	0	1.7	1.3
	5	696	277.2	1021.3	664.8	304.6
	7	935.5	756	58.5	583.3	378.3
S19	3	0	0	4.6	1.5	2.2
	5	6.8	3.2	2.4	4.1	1.9
	7	173.5	6	27	68.8	74.5
S65	3	8.8	12.8	13.6	11.7	2.1
	5	319.2	72.8	49.7	147.2	122.0
	7	230	133.5	100.5	154.7	54.9
S69	3	12	6.8	1.6	6.8	4.2
	5	17.6	8	10	11.9	4.1
	7	43	37.5	42.5	41	2.5

DISCUSSION

Wine protein haze is an important issue to the winemaking community, with the typical bentonite treatment having a negative impact on labour time, wine quality and the environment. As such, an alternative method to bentonite treatment for protein haze removal is paramount. The greater aim of this research program is to find an alternative method for protein haze removal, in the form of an enzymatic treatment using proteases secreted by the necrotrophic fungus *Botrytis cinerea*. This stage of the study focused on targeting wild strains of *B. cinerea* with the greatest natural protease secretion.

Growth experiments with *B. cinerea* presented a simple and logical place to start identifying more desirable candidates for ongoing research. Observing and documenting the growth and sporulation of a large number of isolates provided easy identification of isolates that would grow quickly enough and sporulate well enough to allow for ongoing experiments. Here it was observed that *B. cinerea* isolates grow at differing rates and with different morphology. This has been previously noted in studies on *B. cinerea*, describing white colonies that aged to grey with hyphal morphology described as either "cottony or matty" [36]. Robust growth of *B. cinerea* on PDA has also been noted previously, showing stronger growth and faster conidiation compared to a minimal media [67], with differing rates and morphologies of growth also observed. Observations of growth and morphology were subsequently utilised to filter the collection of 99 *B. cinerea* isolates to a smaller sample size of 55 for protease and laccase plate assays, and also facilitated the identification of any contaminating pathogens present with the *B. cinerea* isolate.

Other pathogens associated with grapevines were the cause of repetition and frustration in the early stages of this study. The contaminating species were presumptively identified as Aspergillus, Penicillium and Rhizopus species, which are all known pathogens of grapes identified in Australia and abroad [68, 69]. Due to the large number of *B. cinerea* isolates tested in this study, single spore isolation was not performed at this time, however will be carried out prior to any further research to ensure there is no contamination carried over with any isolate which could interfere with results.

Preliminary testing for protease production was performed by growing a small sample of the *B. cinerea* isolates on skim milk agar. The clearing assays provided an easy and quantifiable method for identifying *B. cinerea* isolates with strong protease production. Proteolytic activity secreted from the isolates was distinguished as zones of clearing where the milk protein, casein, had been degraded in the media. Similar plate clearing assays have been used previously to detect protease production from fungi [62, 70], showing similarly variable results among isolates tested. Rating systems such as the one used in this study have also been used to differentiate protease production [65], whereby the activity of fibrinolytic protease activity from the fungus *Ganoderma lucidum* was rated between nil (-) and 3 (+++) for weak to strong activity. The *B. cinerea* isolates tested with this method displayed variation in protease activity, with several isolates such as AP5 and S14 showing clearing around the growing colony within the first 24 hours of testing, while many others showed little to no protease activity over the entire testing period, such as S39 and S71. This observation could be linked to the virulence of the isolates, as previous studies have identified that acid protease levels were up to five times lower in a *B. cinerea* isolate that was identified as being less virulent than its comparison [38].

Assessment of the *B. cinerea* isolates' laccase production on guaiacol agar also presented a simple and reliable method for discerning isolates with more desirable properties over others. Laccase production has been well studied in *B. cinerea* [54, 71, 72], though for the aims of this study, laccase production was not desirable due to the negative affect the oxidation that laccase causes would have on the finished wine. Laccase production in the *B. cinerea* isolates was observable through plate assays, with guaiacol medium showing a red-brown colour in the presence of laccase, due to the oxidation of the guaiacol substrate [56]. This medium has been used previously to identify laccase activity from other fungal and bacterial species [56, 59] and provided a simple detection method for varying levels of laccase secretion. The *B. cinerea* isolates tested with this assay displayed great variation in the time that it took to reach a rating of 3, with many isolates showing laccase production in the first day of testing, while several isolates, namely S38 and S74, did not reach a strong laccase rating within the 8 day testing period. The combination of protease and laccase results identified 6 isolates with a varying range of strong protease and lower laccase secretion to be used for quantifiable assays in shake flask cultures.

Optimising the media for use in shake flask cultures of *B. cinerea* presented a challenge to this study. While the skim milk media presented a good solution for plate assays of protease production, liquid cultures were much more difficult to maintain. *B. cinerea* is well known for its extra-cellular glucan production [73, 74], causing an increase in the viscosity of culture medium. Skim milk media was exhaustively tested in order to simulate the protease production observed in the protease plate assays and also due to the observation that *B. cinerea* grown on lactose did not produce the extra-cellular glucan that was present when *B. cinerea* was grown on other carbon sources such as glucose, sucrose or maltose [75]. When the lactose was added to a simple nitrogen source such as yeast extract, a reduction in extracellular glucan was observed. In comparison to the growth morphology observed in the skim milk medium, with hyphae dispersed throughout the medium and great viscosity by day 7 of sampling, the yeast extract-lactose medium produced *B. cinerea* isolates that grew in clumps with no noticeable change to the viscosity of the culture medium.

Production of extracellular glucan by *B. cinerea* isolates has been reported as occurring above pH 4.0 [48]. This observation is supported by this study, as the viscosity in culture media from the secretion of extra-cellular glucan by the *B. cinerea* isolates was regularly noticeable by day 3 of sample collection; also the point at which the culture media pH had increased to pH 4.0 or above. This pH associated increase in glucan secretion will be another point for further investigation in ongoing experiments, as glucan inhibition will be necessary to avoid interference with assays, damage to and clogging of wine filters, and even just for basic ease of collection of supernatant samples. Removal of glucan from *B. cinerea* cultures has been successful in the past, by addition of Glucanex which degrades the polysaccharide [74] however this glucan reduction was coupled with an observable increase in laccase production, which will not be acceptable for this study.

The use of malic acid to reduce the pH of most flask trials could also have contributed to the abundant growth of the *B. cinerea* isolates, as it has been previously observed that the addition of malic or citric acid to media resulted in greater mycelial production than in media without the acid [76]. When sulphuric acid (H_2SO_4) was used to reduce the pH of the yeast extract and lactose medium a slower increase in pH over testing days was observed, along with a reduced maximum pH. For example, *B. cinerea* isolates S69 and S72 both reached a pH above 5

by day 3 of sampling in the malic acid adjusted flasks, but neither reached pH 5 in any replicate when the pH was adjusted with sulphuric acid. As such, sulphuric acid will be utilised for pH reduction and maintenance in ongoing experiments.

The maintenance of pH in flask cultures will be of importance to further studies, as it has been identified that aspartic acid protease production was only observed between pH 3.0 and 4.0 [48]. We would therefore expect lower secreted protease levels beyond pH 4, which was not observed in this study. In fact, the highest protease activity was observed in isolate S69 with 54.2 mg/mL of protease detected at pH 4.92, and 53.2 mg/mL of protease measured at pH 5.79 in isolate S65. The protease activity detected at higher pH could possibly be from other proteases secreted by *B. cinerea*, though these would have to be secreted at high levels, as previous studies have shown that aspartic acid BcAP8 is the most abundantly expressed protease by *B. cinerea*, comprising up to 70% of total secreted proteases [22]. This brings to light the necessity for further analysis of proteases in the culture supernatants, with methods such as SDS-PAGE and mass spectrometry for identifying the specific enzymes produced by the *B. cinerea* isolates on differing sampling days and at varying pH, which can then be quantified by fluorescence plate assay.

Protease activity analysis by the cleaving of quenched fluorescent labelled substrates is a powerful tool in quantifying the protease production of *B. cinerea* isolates. Protease activity in general was variable for all of the final *B. cinerea* isolates tested, with production ranging between 2.1 mg/mL in one replicate of *B. cinerea* S15, to 54.2 mg/mL in one replicate of *B. cinerea* S69. Sampling on day 2 of incubation did not show an increase in protease levels as suspected, however protease levels could have reached a maximum on days 4 or 6, which will also be included for sampling in future experiments. Protease production in these *B. cinerea* isolates was expressed at the highest levels between pH 3.72 (isolate S15, day 3) and pH 5.79 (isolate S65, day 7). An outlier was also observed for isolate S15 on day 7, with maximum protease activity displayed at pH 6.56. This delineates the need for more focused observation of the protease production over each day of incubation, while also testing media compositions that aim to increase the levels of protease secreted.

The boron-dipyrromethene (BODIPY) substrate used in this study has been harnessed successfully in several previous studies involving microbial proteases [77, 78] though a variation on the calculation of activity was used in this study. Typically, protease activity is quantified directly, with one unit of activity calculated as "the amount of protease required to liberate 1 mmol of the fluorescent dye from substrate-dye conjugates in 1 min" [78]. However, owing to the use of yeast extract in the shake flask media, which is known to express a yellow autofluorescence under ultraviolet or blue light [79] an alternative method for quantifying the protease activity from the *B. cinerea* isolates was adopted. Protease activity was instead measured as the increase in fluorescence in a sample over time, with the protease activity of an isolate extrapolated from the fluorescence increase in a known concentration of pepsin over the same time frame. The BODIPY substrate used can also detect the activity of several proteases, including serine, sulfhydryl, acid and metalloproteases [80] most of which have been identified in the *B. cinerea* secretome [44]. This, in combination with the autofluorescence of yeast extract, delineates the need to isolate and purify the proteases secreted by the isolates before performing further fluorescence assays, in order to most accurately quantify *B. cinerea* protease and laccase activity.

Calculation of laccase activity by syringaldazine assay also provided a quantifiable method for quantifying laccase secretion in the *B. cinerea* isolates. Laccase production was highest for most isolates on day 7 of sampling, with isolate S15 showing maximum activity of 935.5 and 756.0 Units/mL enzyme in two replicates, and one day 5 maximum of 1021.3 Units/mL enzyme. The pH profile of the flasks was also variable with relation to laccase secretion. Maximum secretion of laccase for most isolates was seen on day 7, ranging from 6.0 Units/mL enzyme in isolate S19 to 935.5 Units/mL in isolate S15, observing a 2 to 16 fold increase in secretion from days 5 to 7. The pH range for the isolates on day 7 ranged from 4.02 for isolate S69 to 6.75 for isolate S15. This data is somewhat supported by previous research, which found two pH maxima for laccase; at approximate pH 3.1 and 6.0 [48]. Laccase activity was observed at the overall highest levels in isolate S15, which also reached a pH of over 6.3 in each replicate.

Inhibition of laccase production by *B. cinerea* has been identified, showing that EDTA[81] was able to repress laccase formation, while sodium azide was found to totally inhibit laccase

production in *Trametes versicolor* [82], however this compound is highly toxic and would not be feasible for use in the winemaking process. Researchers have found that substances in cucumbers, later identified as curcurbitacins I and D [83, 84] have shown to inhibit the production of laccase in *B. cinerea* at certain concentrations. It was also identified that the cucurbitacins do not have activity against major enzymes, including acid proteinases [85], which presents an additional method for testing in future research.

Overall, the findings of this study highlight the stochastic nature of *B. cinerea* isolates. Additionally, due to the lack of statistical difference between the quantified protease and laccase production of the final 6 *B. cinerea* isolates, no significant differences could be identified to reduce the sample size any further. Even so, the screening methods used in this study successfully identified a set of *B. cinerea* isolates with varied but quantifiable levels of protease and laccase production. With further research focused on global mutagenesis of isolates by UV exposure and an aim to more industrial scale protease production, these methods will provide an ongoing tool for use in quantifying the increasing levels of protease production and to monitor laccase production. As the culturing and protease production of these isolates is optimized, additional experiments will be carried out to identify and differentiate the secreted proteases, such as SDS-PAGE and mass spectrometry. The isolates will also be identified by PCR and phylogenetics, coupled with identification of the protease and laccase genes of each isolate. Finally, the identified and purified proteases will be tested on heat unstable wine to evaluate the suitability of these proteases for removing haze-forming proteins under normal winemaking conditions.

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ACKNOWLEDGMENT

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1. Brown, J.; Jones, M.; Green, D. Article title. *J. Agric. Food Chem.* **1980**, *28*, 1–4. (Issue number must be used if each issue of the periodical begins with page 1.)

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2. Smith, L; Caldwell, A. Chapter title. In *Book Title*, edition no.; Keys, F., Park, G., Eds.; Publisher: City, State (or Country if non-U.S.), Year; Vol. no., pp.

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