CFPA	SAAPPA / SASPA / SAT	DFTS	Winetech
Canning Fruit Producers' Assoc. Submit to: Wiehahn Victor Tel: +27 (0)21 872 1501 inmaak@mweb.co.za	Fruitgro Science Submit to: Louise Liebenberg Tel: +27 (0)21 882 8470/1 louise@fruitgro.co.za	Dried Fruit Technical Services Submit to: Dappie Smit Tel: +27 (0)21 870 2900 dappies@dtd.co.za	Submit to: Jan Booysen Tel: +27 (0)21 807 3324 booysenj@winetech.co.za

			X

Indicate (X) client(s) to whom this final report is submitted.
Replace any of these with other relevant clients if required.

FINAL REPORT
FOR 2011
PROGRAMME & PROJECT LEADER INFORMATION

	Programme leader	Project leader
Title, initials, surname	FF Bauer	Prof FF Bauer / Dr John Moore
Present position	Professor	Researcher
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PROJECT INFORMATION

Project number	IWBT-Y 08/04
Project title	Mannoproteins: Analysis, identification and improved release
Project Keywords	Mannoproteins, Yeast, Haze Reduction.

Industry programme	**CFPA**	
	Deciduous	
	DFTS	
	Winetech	X
	Other	

Fruit kind(s)	Wine Grapes
Start date (dd/mm/yyyy)	01-01-2008
End date (dd/mm/yyyy)	31-12-2011

(Note: adjust footer – insert the project number no, researcher and research institution)

FINAL REPORT

(Completion of points 1-5 is compulsory)

1. Executive summary

Give an executive summary of the *total* project in no more than 250 words

Methods based on protein estimation and carbohydrate content were established and used to accurately determine mannoprotein content of the yeast strains evaluated. A spectral database (Near Infrared and Mid Infrared Spectroscopy) was evaluated using multivariate methods and compared to reference protocols. These methods offered a means to determine relative mannoprotein to glucan ratios in yeast preparations but absolute quantitative results are not possible. The methods show that yeast strains can be clustered based on genus and species level, a correlation also appears to exist between the relative mannose/glucose ratio and the relative mannoprotein to glucan content. However, discerning differences below the strain level proved to be difficult, hence these methods have limitations based on the degree of variation in wall mannoprotein composition.The use of deletion mutants and transgenic yeast strains constructed to be deficient in mannoprotein levels, i.e. high release strains, showed no significant differences between wild type parental strains under fermentative conditions. It was noted that the high levels of Hpf1' present in a haze protective strain that was identified appeared to correlate with protection compared to wild type controls. The mechanism(s) responsible for haze protection identified in the sixteen yeast strains appears to link to the chitinase content present in must/wine solutions which is derived from grape components during maceration. Further studies are currently underway in order to test the applicability of these findings regarding haze protection in real wine fermentations. A Saccharomyces paradoxus strains with high chtinase content w3as identified, and the method was patented by SU (Inventors: Florian Franz Bauer (80%) and Thulile Ndlovu (20%). 2012. Patent application number: ZA2011/09304. Title: Protein haze prevention in wine).
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2. Problem identification and objectives

State the problem being addressed and the ultimate aim of the project.

Wine yeast contribute significantly to the aroma, the flavour, the mouth feel and the visual appearance of wine. Several of these wine attributes have been shown to be positively affected by the presence of a group of compounds collectively referred to as mannoproteins. In particular, several studies have shown that the release of such proteins by yeast during fermentation has a significant influence on the organoleptic quality of wine, in particular the mouth feel, and can protect wines against haze formation and "pinking", defects that may in particular affect white wines and lead to significant economic losses. However, most yeast strains appear to be unable to release significant levels of such proteins.

Along with polysaccharides, mannoproteins form an important part of the yeast cell wall and directly affect cell wall related behaviour or phenotypes. Mannoproteins have been shown to influence the hydrophobicity of the cell wall and to directly control cell to cell adhesion phenotypes such as flocculation ("floc" forming cell populations with increased sedimentation efficiency) and cell to substrate adhesion. Mannoproteins are typically released by wine yeast into fermenting must during alcoholic fermentation, as well as into wine upon contact with yeast lees. Thus, cell wall mannoproteins could positively affect wine clarification and filterability.

While some information regarding the nature of haze-protecting mannoproteins has become available in recent years, very little is known about the underlying molecular processes and about how cell wall composition and cell wall regulatory processes influence the release of

such proteins. Understanding such processes is a prerequisite for the future selection of new yeast strains that would secrete optimised levels of specific haze-protection proteins.
Our data in 2011 have revealed that, more so than mannoproteins, cell wall chitin plays a major role in the ability of wine yeast strains to reduce wine haze. We were also able to show that this ability was linked directly to the binding of grape chitinases to the cell wall of yeast cells. This is a novel finding with significant potential for future commercial exploitation in the wine industry.

Project 1: Establishment of a rapid and cost-effective method for analysis of mannoproteins in wine

The accurate measurement of mannoprotein levels in wine, fermenting must and intact yeast cells is essential in determining the relationship between mannoprotein content in the yeast cell wall, the yeast's ability to release these mannoproteins into the extra cellular environment and the actual level of mannoproteins in wine. Established analytical techniques for mannoprotein quantification are often destructive, laborious, time consuming and nevertheless inaccurate. In this project we propose an approach based on the use of Fourier Transform (FT) Mid or Near Infrared (MIR or NIR) spectroscopy to determine mannoprotein levels in samples. The ultimate aim will be to analyse mannoprotein levels in wine and yeast in a fast, efficient and non-destructive manner. We also propose to develop an easy testing system to measure the potential of yeast and isolated samples to inhibit the formation of protein "haze".

Project 2: The regulation of mannoprotein release and its relation to cell wall properties

In yeast, the mannoprotein composition of the cell wall is ultimately regulated by intracellular signal transduction pathways that modulate the expression of mannoprotein encoding genes. Thus genetic regulation of mannoprotein expression directly affects all of the above mentioned phenotypes and processes. Yeast strains differ in the way that the various mannoprotein encoding genes are regulated. This is dependent on the specific genetic makeup (genotype; genetic background) of the yeast, and the nature of environmental stimuli (oxygen/nitrogen/carbon availability, temperature/osmotic stress) that ultimately controls gene expression via signal transduction pathways. Previous research has shed substantial light on the mechanisms controlling expression of the mannoprotein Flo11p. At the IWBT, a large number of strains that carry mutations in genes that affect the production of mannoproteins have been generated in this process.
Genetic control of most mannoprotein encoding genes in general is still poorly understood. A good understanding of the various specific and overlapping mechanisms of gene expression could open new possibilities for the controlled expression of specific mannoprotein encoding genes. Modulating the levels of specific mannoproteins in the cell wall may well lead to yeast performing a more desirable role in the process of wine production. In this study we propose to investigate the expression and regulation of cell-wall encoding mannoproteins in general through transcriptome analysis. Genes of interest will be followed in more detail, in particular the expression of mannoprotein encoding genes of the flocculation (*FLO*) gene family, as well as two members of the *TIR* (*TIP1* related proteins) family of genes. In addition, several genes encoding transcription factors whose deletion affect cell wall properties will be screened for mannoprotein release.
Once the impact of these genes has been established, the information will be used to develop new strains with improved mannoprotein release through genetic modification or marker-assisted breeding.

Project 3: . Cell wall chitin and haze protection. This project was added as part of the amended workplan in 2011, based on findings during the PhD of Dr Ndlovu. These findings have led to a patent. Some of the results are reported below.

3. Workplan (materials & methods)

List trial sites, treatments, experimental layout and statistical detail, sampling detail, cold storage and examination stages and parameters.

Project 1: Establishment of a rapid and cost-effective method for analysis of mannoproteins in wine

Milestone 1:

Development of a quantitative analysis method for the determination of mannoprotein levels in wine and synthetic media

Milestone 2:

Development of an analysis method for the determination of mannoprotein levels in whole yeast cell preparations

Milestone 3:

Assessing if methods from milestones 1 and 2 will suffice as screening methods for the analysis of mannoprotein content and -release by various yeast strains

Milestone 4:

Establishment of an assay system to measure protein haze formation in a model wine system

Project 2: The regulation of mannoprotein release and its relation to cell wall properties

Milestone 1: Assessment of the impact of mutations in transcription factors and cell-wall protein encoding genes on mannoprotein production and release.

Task 1:

Analyse all cell-wall affecting deletion mutants and overexpression strains in the collection of the IWBT for their ability to release mannoproteins. Identify genes that lead to increased mannoprotein production and release.

Task 2:

Assess the physiology of such strains to evaluate their continued suitability to wine fermentation (fermentative ability, stress resistance)

Task 3:

Assess the haze protection capacity of the proteins released by such strains.

Task 4:

Analyse specific regulation of each identified target gene through RT-PCR.

Milestone 2: Analysis of the molecular regulation of genes that lead to increased mannoprotein release: Transcription and protein levels

Task 1: Transcriptome (DNA microarray) analysis and proteome (i-TRAQ) analysis to identify significant targets

Task 2: Assess transcriptional regulation of such targets in wine yeast strains and in improved mutants.

Milestone 3: Development of new yeast strains with improved mannoprotein release

Task 1: From information obtained in Milestone 1, design strategy for breeding of new mannoprotein-releasing yeast strains and for genetic modification.

Task 2: Implement strategies in an industrial wine yeast strain.

Milestone 4: (Amended workplan for 2011): Investigating the link between cell wall chitin and haze protection.

Figure 1 and 2 below demonstrate the main finding: The chitinase binding ability of individual yeast strains (Figure 1), itself linked to the chitin concentration in the cell wall (data not shown), correlate with the ability of these strains to reduce wine haze potential (Figure 2).

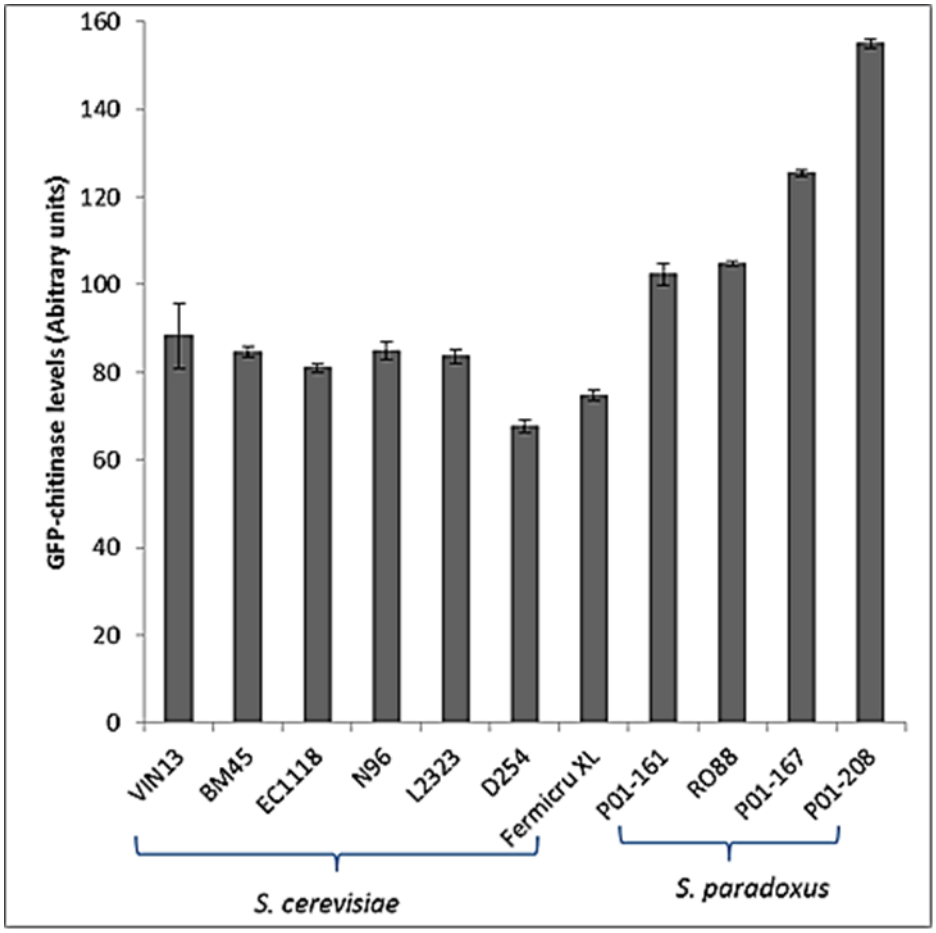

Figure 1. GFP-chitinase levels bound to different yeast strains, quantified using BD FACS Aria flow cytometer. Cells were grown in YPD as described by de Groot *et al.* (2001) and equal amount of cells based on OD measurement at 600nm were washed and re-suspended in PBS buffer before adding GFP-tagged chitinase. Cells were further incubated for 2 hours at room temperature with shaking before washing and re-suspending in PBS buffer in preparation for quantification using a flow cytometer.

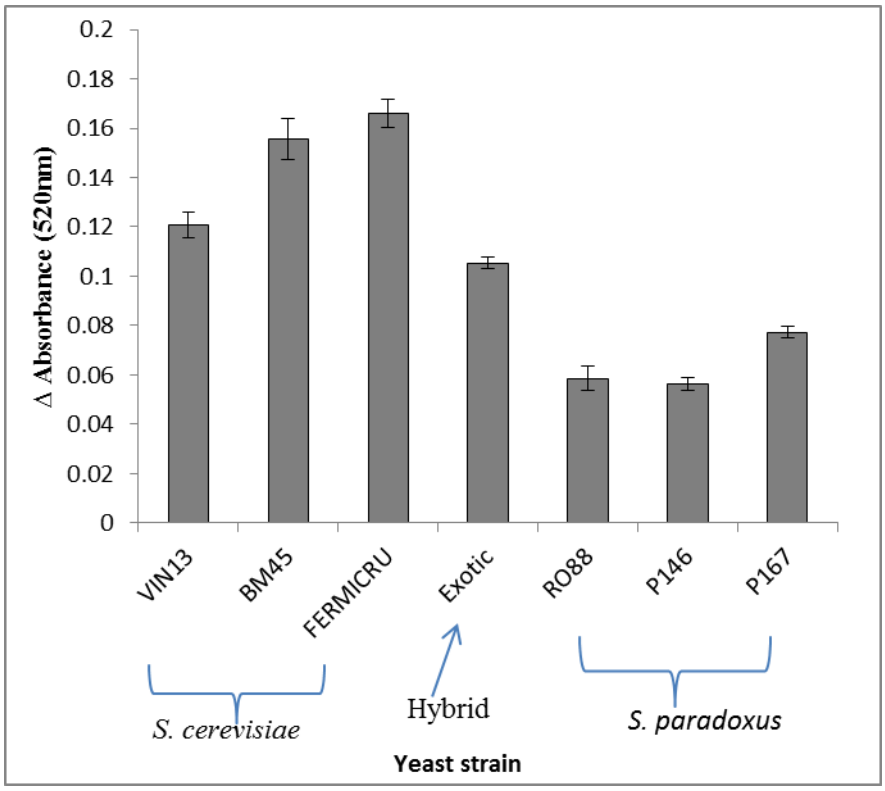

Figure 2: Wine haze levels in fermented Chardonnay must using *S. cerevisiae* and *S. paradoxus* wine yeast strains. Differences in haze levels (mean difference in absorbance before and after heating ± standard deviation of triplicate measurements) between *S. cerevisiae, S. cerevisiae*/*S. paradoxus* hybrid and *S. paradoxus* yeast strains formed in fermented chardonnay grape must juice at the end of fermentation. No potassium sulphate and protein were added during the haze assay

4. Results and discussion

State results obtained and list any benefits to the industry. Include a short discussion if applicable to your results.
This final discussion must cover ALL accumulated results from the start of the project, but please limit it to *essential* information.

Project 1: Establishment of a rapid and cost-effective method for analysis of mannoproteins in wine

Milestone	Achievement
1. Development of a quantitative analysis method for the determination of mannoprotein levels in wine and synthetic media	Methods based on protein estimation and carbohydrate content were used to accurately determine mannoprotein content in wine and synthetic media. Accurate estimates of carbohydrate polymer

	component of mannoproteins present in wine and synthetic media were achieved using hydrolysis and quantification of mannose levels using gas chromatography.
2. Development of an analysis method for the determination of mannoprotein levels in whole yeast cell preparations	Estimates of mannoprotein quantification in whole yeast preparations were performed using a near infrared spectroscopy (NIR) and attenuated total reflectance (ATR) mid-infrared (MIR) spectroscopy. Yeast preparations were evaluated as wet biomass, freeze-dried cells and alcohol insoluble residues. These preparations were made from colonies, yeast-peptome dextrose (YPD) media and fermentations (synthetic and must). The datasets obtained were evaluated using multivariate methods and used to compare against reference methods. These methods offer a means to determine relative mannoprotein to glucan ratios in yeast preparations – not absolute quantitative results are obtainable.
3. Assessing if methods from milestones 1 and 2 will suffice as screening methods for the analysis of mannoprotein content and -release by various yeast strains	Multivariate methods were used to evaluate spectroscopic datasets (NIR and MIR) collected from a variety of yeast preparations (colonies, YPD, synthetic media and must). These same preparations were used to assess for mannose content using hydrolysis, coupled to gas chromatography quantification. The methods show that yeast strains can be clustered based on genus and species level, a correlation also appears to exist between the relative mannose/glucose ratio and the relative mannoprotein to glucan content. However, discerning differences below the strain level proved to be difficult, hence these methods have limitations based

	on the degree of variation in wall composition (measuring wall content directly acts as a proxy for mannoprotein release (i.e. in *absentia*) a deficiency should be indicated by an alteration in the ratio).
4. Establishment of an assay system to measure protein haze formation in a model wine system	A spectrophotometric method based on turbidity of a yeast grown media solution (i.e. spent media and must/wine) was developed to determine haze produced from heating under defined conditions. This method proved useful to evaluate the relative differences between strains (in protecting against haze) under defined conditions (i.e. media or wine).

Project 2: The regulation of mannoprotein release and its relation to cell wall properties

Milestone	**Achievement**
1. Assessment of the impact of mutations in transcription factors and cell-wall protein encoding genes on mannoprotein production and release	In order to aid in designing strategies for the genetic improvement of industrial wine yeasts strains for haze protective material production, we have studied the impact of *S. cerevisiae* laboratory strains deleted for specific cell wall genes. These genes were chosen based on their impact on cell wall related phenotype. However, no significant differences in wine haze protection were observed between the different mutant strains with exception of BY4742Δygp1. *KNR4, GPI7* and *GAS1* genes were deleted in the Flo proteins overexpressing strains in VIN13 background, a commercial wine yeast strain. It was observed that Vin13-F1HΔ*gas1*, Vin13-F11HΔ*knr4*, Vin13-F5AΔ*gpi7*, Vin13-F5HΔgpi7 and Vin13-F1AΔknr4 transgenic

	strains resulted in significant wine haze reduction when compared to VIN 13 parental strain. No significant differences were also observed in total protein secreted by the transgenic strains during fermentation. In addition, starins with modified expression levels of the mannoproteins encoded by the FLO genes were assessed, but no difference in haze protection was observed. However, the strains showed other prominent phenotypes of relevance to wine making (See Govender et al. 2011, Bester et al. 2012)
2. Analysis of the molecular regulation of genes that lead to increased mannoprotein release: Transcription and protein levels	Sixteen yeast strains were screened for their haze protective ability and mannoprotein release. No differences were observed in total mannoproteins released at the end of alcoholic fermentation and also during wine aging on yeast lees. However, differences were observed in haze protective capacities with some strains demonstrating markedly increased wine haze protection capacity. A hybrid linear quadrupole ion trap/FT-ICR mass spectrometry **(**LTQ-FT-ICR) was used for the identification of extracellular protein identities between different strains. Comparison was made between a haze protecting yeast and strain and a non-haze protecting yeast strain using isobaric tags for relative and absolute quantitation (iTRAQ) analysis for the identification and quantification of secreted global exoproteome released by the strains during wine making. The haze protective strain released more cell wall related proteins when compared to the non-haze protecting strain

	which released proteins involved in metabolic processes. In particular, high levels of Hpf1' were quantified in the culture supernatant of the haze protective strain (11 times more than in that of the non-haze protective strain). Although the function of this protein has not been unravelled yet, it has been previously identified to be involved in wine haze protection.
3. Development of new yeast strains with improved mannoprotein release Based on the identification of strains with high haze protecting abilities, this milestone was changed into the further characterization of these strains rather than the development of a new strain.	Considering the observation that there were significant differences in total protein released by the haze protecting strains and the non-haze protecting strains, further cell wall analysis was carried out with the aim of understanding the haze protecting capacity of some strains. It was observed using flow cytometry that the haze protecting strains have high cell wall chitin levels. The gene encoding the main grape chitinase was fused to a GFP fluorescent tag and overexpressed in *E. coli*. High concentrations of this recombinant protein were collected and used in yeast cell wall-chitinase binding assays. The amount of chitinase bound to the cell walls was determined by flow cytometry. The highest levels were found for the haze protecting strains. Similar results were found when commercial chitinase was used. It was therefore concluded that the yeast cell wall chitin is responsible for selectively removing grape chitinase from white wines during racking resulting in improved protein stability. Further studies are currently underway in order to test the applicability of this finding on haze protection in real wine.

4. Investigating the link between cell wall chitin and haze protection.	Additional mutants with modified cell wall chitin were selected, and a clear correlation between the concentration of cell wall chitin, the binding of GFP-Chitinase and reduction in the levels of haze were established.

5. Accumulated outputs

List ALL the outputs from the start of the project.
The year of each output must also be indicated.

Technology development, products and patents

Indicate the commercial potential of this project (intellectual property rights or a commercial product(s)).

1. Patent: Inventors: Florian Franz Bauer (80%) and Thulile Ndlovu (20%). **2012.** Patent application number: ZA2011/09304. Title: Protein haze prevention in wine.
2. Technical methods for evaluating mannoprotein content of wine yeast strains in laboratory and industrial settings.
3. A spectroscopic (NIR and MIR) method, coupled to multivariate methods, protocol is now established (with an associated spectral database of yeast strains) which could be utilised for industry to select/screen for new strains of commercial potential.
4. Yeast strains were identified as haze protective agents. This property was further investigated.
5. A product derived from the results of this study is currently evaluated for use by the winemaking industry. In this context a patent has been submitted.

Human resources development/training

Indicate the number and level (e.g. MSc, PhD, post doc) of students/support personnel that were trained as well as their cost to industry through this project. Add in more lines if necessary.

	Student level (BSc, MSc, PhD, Post doc)	Cost to project (R)
1.	Ms T Ndlovu (PhD student) graduated in 2012	170,310
2.	Mr Jason Zhang (technical assistant)	10,360
3.	Dr Eric Nguema-Ona (post-doctoral fellow)	28,250
4.	Mr E. Duckitt (honours)	1,500
5.	Ms G. du Plessis (honours)	1,500
6.	Ms M. Strydom (honours)	1,500

Publications (popular, press releases, semi-scientific, scientific)

(1) **Bauer F.F.**, M.C. Bester & Govender, P. **2010.** Yeast flocculation and its biotechnological relevance (on invitation). *Applied Microbiology and Biotechnology* **88:**31-39.

(2) Govender, P., M.C. Bester & **F.F. Bauer. 2010.** *FLO* gene dependent phenotypes in industrial wine yeast strains. *Applied Microbiology and Biotechnology* **86:**931-945.
(3) Govender, P., S. Kroppenstedt & **F.F. Bauer. 2011.** Novel wine-mediated *FLO11* flocculation phenotype of commercial *Saccharomyces cerevisiae* wine yeast strains with modified *FLO* gene expression. *FEMS Microbiology Letters*. **317:**117-126.
(4) Bester, M., D. Jacobsen & **F.F. Bauer. 2012.** Many cell wall protein encoding genes are regulated by Mss11p, but adhesion phenotypes solely depend on *FLO* gene expression. *G3 (Genes, Genomes, Genetics)* **2**:131-141.
(5) Thulile Ndlovu, Benoit Divol & Florian F. Bauer. **2013.** A new mechanism of haze protection: S. paradoxus strains reduce wine haze formation in part through higher cell wall chitin. Submitted to Food Microbiology

Presentations/papers delivered

1. Duckitt E., du Plessis G., Strydom M., Ndlovu T., Govender P., Bauer F.F., Divol B. and Moore J.P. (2009) Functional analysis of *FLO* gene expression in yeast (*Saccharomyces cerevisiae*) and developing a rapid screen for mannoprotein modified wine yeast strains. Fourth international viticultural and oenology conference, Cape Town, South Africa.
2. Ndlovu T., Divol B., Moore J.P. and Bauer F.F. (2010) Yeast protein release and haze formation during aging in model wine. Poster at the Cape Biotechnology Forum, Somerset West, Cape Town, South Africa
3. Ndlovu T., Divol B., Moore J.P., and Bauer F.F. (2010) Wine haze and protein release during wine ageing. 32nd congress of the South African Society of Enology and Viticulture, Cape Town, South Africa.
4. Ndlovu, T., B. Divol, B.A. Prior & F.F. Bauer. 2011. Selected yeast strain can reduce protein haze formation in wine. 2011 SASM, Southern Sun Cape Sun Hotel, Cape Town.
5. Ndlovu, T., B. Divol & F.F. Bauer. 2011. Influence of yeast strain on wine haze: Exoproteomic differences between *S. cerevisiae* and *S. paradoxus* yeast strains. Second Wine Sciences Research Day. DVO-IWBT, Stellenbosch University.
6. Ndlovu, T., B. Divol & F.F. Bauer. 2011. The quest to reduce wine haze formation: Novel protective activities of wine yeast strains. Faculty of AgriSciences Research Day, Stellenbosch University.
7. Ndlovu, T., B. Divol & F.F. Bauer. 2011. Hazy wine – using yeast to make it crystal clear. New voices in Science. Stellenbosch University.
8. John P. Moore, Song-lei Zhang, Helene Nieuwoudt, Benoit Divol, and Florian F. Bauer. 2011. Monitoring mannoprotein and ß-glucan content in a variety of laboratory and industrial wine yeast strains under fermentative conditions using ATR-FT-MIR spectroscopy and multivariate data analysis. Journal of Agricultural and Food Chemistry (in preparation)
9. John P. Moore, Song-lei Zhang, Helene Nieuwoudt, Benoit Divol, and Florian F. Bauer. 2011. Using NIR spectroscopy and PLS calibration to model the mannoprotein and ß-glucan content in a variety of laboratory and industrial wine yeast strains under fermentative conditions. Journal of Agricultural and Food Chemistry (in preparation)

4. Total cost summary of project

	Year	CFPA	Decidious	DFTS	Winetech	THRIP	Other	TOTAL
Total cost in real terms for year 1	2008				R 195,000	R 92,625		**R 287,625**
Total cost in real terms for year 2	2009				R 210,600	R 105,300		**R 315,900**
Total cost in real terms for year 3	2010				R 231,660	R 115,830		**R 347,490**
Total cost in real terms for year 4	2011				R 254,500	R 127,250		**R 381,750**
Total cost in real terms for year 5	2012				R 0	R 0		**R 0**
TOTAL					**R 891,760**	**R 441,005**		**R 1,332,765**