PICFPA	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 2012

PROGRAMME & PROJECT LEADER INFORMATION

	Programme leader	Project leader
Title, initials, surname	Prof FF Bauer	Dr B Divol / Dr E Setati
Present position	Professor	Researcher / Snr Researcher
Address	IWBT – SU	IWBT – SU
Tel. / Cell no.	021 808 4346	021 808 3141 / 072 325 5428
Fax	021 808 3771	021 808 3771
E-mail	fb2@sun.ac.za	divol@sun.ac.za

PROJECT INFORMATION

Project number	IWBT Y 08-05
Project title	Enzyme-secreting yeast
Project Keywords	Extracellular enzymes – pectinase – β-glucosidase - yeast

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

Fruit kind(s)	
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

In this project, various enzymes of oenological interest were studied. The endo-polygalacturonase-encoding gene of *Saccharomyces cerevisiae* was successfully reintroduced in strains naturally lacking this gene and therefore lacking pectinase activity. As a result, at least one recombinant strain recovered strong pectinase activity. Only slight differences were seen with regards to wine composition after fermentation using this strain. The endo-polygalacturonase encoding gene from *S. paradoxus* was also studied at genetic and enzymatic level. The gene displayed a few minor differences in its sequence when compared to that of *S. cerevisiae*. Moreover, although *S. paradoxus* strains exhibit stronger activities than those of *S. cerevisiae*, we showed that *S. cerevisiae*'s enzyme was marginally better adapted to winemaking conditions. Overexpression of either enzyme showed a significant increase in the volume of free-run wine recovered at the end of alcoholic fermentation, but no clear difference in wine composition.
One β-glucosidase gene was recovered from *Candida oleophila*, but it is not clear whether this gene is secreted and further characterisation is still needed. This part of the project was very challenging as β-glucosidase genes seem not to be highly conserved between species. As a result, we propose to use more global approaches in the future.
Two protease-encoding genes from *Metschnikowia pulcherrima* and *Candida apicola* were retrieved and partially characterised. We showed in particular that these enzymes seem to be active at wine pH and temperature and that the presence of grape proteins induced expression of at least the gene of *M. pulcherrima*. Further characterisation is now needed as these enzymes look very promising.
Finally, techniques (e.g. genomic DNA libraries and metagenomic libraries) are currently being optimised for the holistic study of enzymes of interest and to further our global understanding of non-*Saccharomyces* and their potential impact on wine.

2. Problem identification and objectives

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

Although some wine yeasts have been identified as having the genetic potential to secrete enzymes of oenological interest, the actual secretion rate and/or impact of these enzymes are limited and poorly understood. For this reason, the addition of enzymes by winemakers during alcoholic fermentation has become a common practice. These enzymes are of different nature, but mostly combine enzymes breaking the network of grape cell walls in order to release more aromas and eventually to facilitate filtration and other clarification practices.
This project deals with the improvement of enzymes secretion by wine yeasts during alcoholic fermentation in order to decrease or suppress the addition of commercial enzymes. Initially, two sub-projects were included in this project. Over the last year, two more sub-projects were added. These latter two sub-projects were regarded as preliminary investigations to broaden the "enzyme-related studies" by adding a new family of enzyme of interest (i.e. proteases) and by using more holistic approaches.

Project 1: Recovery of endo-polygalacturonase activity in wine strains of *Saccharomyces cerevisiae* devoid in this enzymatic activity

Many strains of *Saccharomyces cerevisiae*, especially wine strains, are devoid in pectinase activity. For few strains, this absence is due to the natural loss of a gene. This project aims to reintroduce this gene (and therefore pectinolytic activity) in these particular strains while keeping their strong oenological properties.

Project 2: Secretion of β-glucosidase by wine strains of *Saccharomyces cerevisiae*
It has been noticed that the secretion of β-glucosidase enzymes by *Saccharomyces cerevisiae* during alcoholic fermentation is not sufficient and/or not effective enough under winemaking conditions. We propose to clone and express the β-glucosidase-encoding gene of *Candida oleophila, Debaryomyces pseudopolymorphus* and *Debaryomyces pseudopolymorphus.* The latter has recently been described as more effective in a wine strain of *Saccharomyces cerevisiae*.

Project 3: Investigating the secretion of proteolytic enzymes by non-*Saccharomyces* yeasts isolated from wine
There are very few yeast species that secrete proteases potentially active under winemaking conditions. Proteases could nevertheless make a large impact on wine composition and on microbial interactions as they could protect wine from haze formation and release Yeast Assimilable Nitrogen. This study aims at screening non-*Saccharomyces* yeast isolate for acid protease activity and retrieving the corresponding genes. Partial characterisation of the genes/enzymes is envisaged.

Project 4: Investigating the actual impact of non-*Saccharomyces* yeasts on wine composition with a specific focus on enzymes of oenological interest

So far, most studies focusing on enzymes of oenological interest targeted specific enzymes of specific microorganisms and aimed at cloning the corresponding genes in *S. cerevisiae* for overexpression during alcoholic fermentation. Recently, the focus shifted towards the study of wine microorganisms, their genetic potential and the expression thereof or towards the even more holistic study of all wine microorganisms present in grape juice and throughout alcoholic fermentation. This study aims at starting the optimisation of techniques to further these studies in future projects.

3. Workplan (materials & methods)

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

Project 1: Recovery of endo-polygalacturonase activity in wine strains of *Saccharomyces cerevisiae* devoid in this enzymatic activity

Milestone 1: Clone and construct plasmids containing a *PGU1* gene

Milestone 2: Integration into strains lacking the *PGU1* gene and check the success of this integration

Milestone 3: Test the endo-polygalacturonase activity of these new strains in synthetic medium

Milestone 4: Wine fermentations with these new strains and analyses

Milestone 5: Study of *Saccharomyces paradoxus PGU1* gene

Project 2: Secretion of β-glucosidase by wine strains of *Saccharomyces cerevisiae*

Milestone 1: Isolation, PCR amplification and sequencing of the gene

Milestone 2: Cloning and integration in *S. cerevisiae*

Milestone 3: Control of the integration, expression and secretion of the enzyme

Milestone 4: small-scale fermentations of must with the new strains and analyses

Project 3: Investigating the secretion of proteolytic enzymes by non-*Saccharomyces* yeasts isolated from wine

Milestone 1: Investigating the protease activity of selected wine yeast isolates
Task 1: Total proteins will be extracted from various pure cultures and their amounts evaluated. Culturing method will probably have to be optimized as the amount of extracellular proteins might be low. The method used to extract protein will also be optimized.
Task 2: Protease activity will be assessed in liquid medium and various environmental parameters will be assessed in order to verify the activity of these enzymes in wine. The trinitrobenzene sulfonic acid assay will be used to test for protease activity.

Milestone 2: Visualisation of the enzymes and attempt to isolate them
Task 1: Zymography will be used to visualize protease activity on gel.
Task 2: The bands displaying protease activity will be excised and an attempt to retrieve a partial sequence of the proteins will be made.
Task 3: Should task 2 be unsuccessful, genomic libraries will be constructed (see project 4) in order to isolate the genes encoding these proteases in the selected microorganisms.

Project 4: Investigating the actual impact of non-*Saccharomyces* yeasts on wine composition with a specific focus on enzymes of oenological interest

Milestone 1: Global genetic investigation of selected non-*Saccharomyces* yeasts for oenologically relevant biocatalysts
Several yeast species isolated from grape must in previous years were shown to display a wide range of enzymatic activities of oenological importance. Such activities have also been reported in the literature. *Metschnikowia* and *Debaryomyces* are for instance known for their β-glucosidase and protease activities and *Rhodotorula* for its esterase activity. However, no genetic information is currently available on these enzymes.
Our approach will focus on genomic and transcriptomic analyses of the yeast isolates that exhibit a wide range of enzymatic activities
Task 1: Generation of genomic libraries from selected wine yeast isolates
- Genomic DNA will be extracted from various pure cultures and be sheared to generate different fragment sizes
- A clone library will be generated through ligation of these fragments into suitable vectors
Task 2: Generation of cDNA libraries from selected wine yeast isolates
- Total RNA will be isolated and mRNA will be isolated
- cDNA will be synthesized and an expression library will be generated
Task 3: Mining of the libraries for enzymes of oenological interest
- Both libraries will be sequenced using high-throughput sequencing techniques. The data obtained will be analysed for sequences orthologous to genes encoding enzymes of interest.
- Both libraries will also be screened for enzymes activities using previously optimized plate assays. The clones exhibiting desired activities will be sequenced through Sanger sequencing.

Milestone 2: Functional screening of the wine matrix for enzymes improving its organoleptic and technological properties
Culture-dependent methods provide essential information on the contribution of known and usually dominant microorganisms, while the contribution of microorganisms that are present in lower amounts is often overlooked due to the bias introduced by cultivation methods.
The recent discovery of the existence of viable but not culturable cells within yeast and bacteria strengthen the need for culture-independent approaches.

Therefore, this study will employ metagenomics and metatranscriptomics for a holistic search of the wine environment for detection and identification of microorganisms and enzymes active at different stages of winemaking.
Task 1: genomic DNA and total RNA will be extracted from white and red wine samples at different stages of winemaking. Libraries will be generated using similar techniques to those described in milestone 1.
Task 2: The libraries generated will be sequenced and screened using similar techniques to those described in milestone 1.

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: Recovery of endo-polygalacturonase activity in wine strains of *Saccharomyces cerevisiae* devoid in this enzymatic activity

Milestone	Achievement
1. Cloning and constructing plasmids containing a *PGU1* gene	The *PGU1* gene and its native regulatory sequences were successfully PCR-amplified and cloned into plasmids suitable for integration into yeast genome.
2. Integration into strains lacking the *PGU1* gene and check the success of this integration	The construction obtained in milestone 1 was successfully integrated into the genome of 5 strains naturally lacking the *PGU1* gene at the original location of *PGU1*. Expression of the gene was observed in 3 of these strains, while the other 2 did not, although it was verified that the gene was correctly reintegrated.
3. Test the endopolygalacturonase activity of these new strains in synthetic medium	The same 3 recombinant strains that were able to express the reintroduced *PGU1* gene displayed endo-polygalacturonase activity while the other 2 did not.
4. Wine fermentations with these new strains and analyses	Fermentations of grape juice were conducted using the recombinant strains as well as their corresponding wild-types. Only the wines made by the recombinant strain displaying the strongest activity showed significant differences in terms of flavor and aroma

	compounds composition. It is most likely that the conditions chosen with regards to temperature in particular were not favourable to enzyme activity.
5. *Saccharomyces paradoxus PGU1* gene study	The *PGU1* gene of *S. paradoxus* and its promoter were sequenced and compared to those of *S. cerevisiae*. A very limited number of polymorphisms were noted. Different strains of *S. paradoxus* were used and all strains possessed the same allele. Both genes were heterologously expressed in VIN13, a wine strain displaying no natural endo-polygalacturonase activity. The enzyme were then characterised with regards to kinetics properties as well as optimal pH and temperature. Surprisingly, this experiment showed that, although *S. paradoxus* strains display a stronger activity than *S. cerevisiae* strains, the enzyme secreted by *S. paradoxus* is better adapted to the wine environment. Wine was made using the different recombinant strains. No significant difference was noticed between the wines obtained from the strains overexpressing the gene of either species with regards to wine chemical composition. However, the wines made by these strains allowed the recovery of a higher volume of free-run wine than the wine made by the control strain.

Project 2: Secretion of β-glucosidase by wine strains of *Saccharomyces cerevisiae*

Milestone	**Achievement**
1. Isolation, PCR amplification and sequencing of the gene(s)	Completed for *Candida oleophila*. However,

	for the other species, the genes isolated do not encode for beta-glucosidase. New strategies are being implemented, but more time is needed to optimize such techniques (i.e. construction of genomic DNA libraries).
2. Cloning and integration in *S. cerevisiae*	Completed, but the genes isolated do not encode for beta-glucosidase. New strategies are being implemented.
3. Control of the integration, expression and secretion of the enzyme	Not completed
4. Small-scale fermentations of must with the new strains and analyses	Not completed

Beta-glucosidases seem to belong to a diverse family and it proved difficult to retrieve genes coding for such enzymes in the yeast species displaying the strongest activity under winemaking conditions. We therefore suggest that a new strategy be implemented. We have now started optimising the construction of genomic DNA library which will allow screening for other enzymes of oenological interest as well (see Project 4).

Project 3: Investigating the secretion of proteolytic enzymes by non-*Saccharomyces* yeasts isolated from wine

Milestone	**Achievement**
1. Investigating the protease activity of wine isolates	308 non-*Saccharomyces* wine isolates were screened for extracellular protease activity at pH 3.5. Three strains displayed a strong activity. They were identified as *Metschnikowia pulcherrima*, *Candida apicola* and *Aureobasidium pullulans*. Liquid assays confirmed the presence of protease activity in the extracellular environment.
2. Visualising protease activity and isolating the corresponding genes	The genes coding for extracellular aspartic (acid) proteases were retrieved using the technique of inverse-PCR from *Metschnikowia pulcherrima* IWBT Y1123 and *Candida apicola* IWBT Y1384. The sequences were analysed *in silico*. Further experiments were carried out for MpApr1 (i.e. the protease gene retrieved

	from *M. pulcherrima* IWBT Y1123). Gene function was confirmed by heterologous expression in *S. cerevisiae*. Induction of gene expression and substrate specificity was tested by using different sources of nitrogen. This experiment showed that MpApr1 was not expressed in the absence of proteins in the medium but was induced in the presence of BSA, casein and grape proteins. Further characterization is now needed to assess the expression of MpApr1 under winemaking conditions and the impact of this enzyme on wine composition and haze.

Project 4: Investigating the actual impact of non-*Saccharomyces* yeasts on wine composition with a specific focus on enzymes of oenological interest

Milestone	**Achievement**
1. Global genetic investigation of selected non-*Saccharomyces* yeasts for oenologically relevant biocatalysts	A genomic DNA library is currently under construction *for Schwanniomyces polymorphus* var. *africanus* (previously known as *Debaryomyces polymorphus* var. *africanus*), known for its strong β-glucosidase activity (and potentially strong protease activity) as a follow-up of project 2. Furthermore, the protease-encoding genes retrieved in project 3 are now under characterization with regards to enzyme properties and impact on wine (release of YAN and haze formation are particularly assessed). The construction of a genomic DNA library for *M. pulcherrima* is also under consideration to retrieve more protease-encoding genes from this species.
2. Functional screening of the wine matrix for enzymes improving its organoleptic and	A Fosmid metagenomic library containing an

technological properties	average insert size of 30 Kb was prepared with genomic DNA extracted from cabernet sauvignon must. However, the library only represented approximately 1/10 of the required clone size, and more optimization is currently underway to prepare a larger library and commence with screening for different enzymes.

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)).

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.	Van Wyk H. (MSc)	15,000
2.	Eschstruth A. (special student)	122,104
3.	Reid V.J. (MSc)	17,000
4.	Ghosh S. (PhD)	40,000
5.		0

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

van Wyk H. and Divol B. (2010) Recovery of endo-polygalacturonase activity in wine yeast and its effect on wine aroma. *FEMS Yeast Research*, 10, 58-71.

Eschstruth A. and Divol B. (2011) Comparative characterization of endo-polygalacturonase (Pgu1) from *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* under winemaking conditions. *Applied Microbiology and Biotechnology*. 91, 623–634.

Divol B., van Rensburg P. and Zietsman AJ.J. (2012) Releasing the good, destroying the bad and preventing the ugly (Part 2): the pectinase activity of *Saccharomyces* spp. *Wineland* (May 2012).

Reid V.J., du Toit M. and Divol B. Identification and partial characterization of extracellular aspartic protease genes from *Metschnikowia pulcherrima* IWBT Y1123 and *Candida apicola* IWBT Y1384. Submitted for publication in *Applied and Environmental Microbiology*.

Presentations/papers delivered

Van Wyk H. and Divol B. (Sept 2008) Recovery of endo-polygalacturonase activity in recombinant wine strains of *Saccharomyces cerevisiae*. Food Microbiology 2008, Aberdeen, Scotland.

Van Wyk H. and Divol B. (Nov 2008) Recovery of endo-polygalacturonase activity in wine yeast strains and its effect on wine aroma. Poster at the 31st SASEV conference in Somerset West, Cape Town, South Africa

Eschstruth A. and Divol B. (2010) Altering wine bouquet by overexpressing non-*Saccharomyces* yeasts beta-glucosidases. Genetics of Industrial Microorganisms 2010, Melbourne, Australia.

Eschstruth A. and Divol B. (2011) Expression of *Saccharomyces cerevisiae* and *Saccharomyces paradoxus*' endo-polygalacturonase-encoding genes enhances juice extraction without much alteration of wine composition. ŒNO 2011, Bordeaux, France.

4. Total cost summary of project

	Year	CFPA	Deciduous	DFTS	Winetech	THRIP	Other	TOTAL
Total cost in real terms for year 1	2008				R 130,000	R 61,750		**R 191,750**
Total cost in real terms for year 2	2009				R 140,400	R 70,200		**R 210,600**
Total cost in real terms for year 3	2010				R 154,440	R 77,220		**R 231,660**
Total cost in real terms for year 4	2011				R 250,000	R 125,000		**R 375,000**
Total cost in real terms for year 5	2012				-	-		-
TOTAL					**R 674,840**	**R 334,170**		**R 1,009,010**