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. Maret du Toit	Dr Benoit Divol
Present position	Associate-Professor	Researcher
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PROJECT INFORMATION

Project number	IWBT-B 08/08	
Project title	Assessing malolactic fermentation under winemaking conditions	
Project Keywords	Malolactic fermentation, screening for *mle* gene, pH, ethanol, expression of *mleA*	

Industry programme	**CFPA**	
	Deciduous	
	DFTS	
	Winetech	Microbiology committee
	Other	

Fruit kind(s)	Wine
Start date (dd/mm/yyyy)	01/01/2008
End date (dd/mm/yyyy)	31/12/2010

(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

This project deals with the evaluation of commercial malolactic starter cultures and the impact that specific oenological conditions will have on the regulation of the *mleA* gene. We aim to demonstrate a potential link between quick adaptation – MLF completion and high expression of the *mleA* gene. Results showed that 53% of all lactic acid bacteria tested possessed the malolactic (*mleA)* gene.

Four different MLF starter cultures were evaluated in industrial experimental fermentation in three different cultivars. Results obtained showed that there is difference in the lag phase of the cultures in the beginning of MLF, however all the cultures finished MLF. In certain instances there were significant differences in the rate of malic acid degradation between the different starter cultures, but it seems to be dependent on the wine matrix. Expression of *mle* for both *O. oeni* and *L. plantarum* was shown to be inducible by the presence of malic acid, with increased expression in the middle of MLF. Expression of *mle* was also shown to be increased at low pH values and decreased in the presence of ethanol. This indicates the role of malic acid in acid tolerance and the negative impact of ethanol on the completion of MLF. The results therefore provide further evidence that co-inoculation for MLF in high alcohol and low malic acid wines can be used as a tool to overcome problems experienced with sequential inoculations.

Wines were made with four selected *O. oeni* strains from the characterized collection. Results showed that they all had the ability to conduct MLF and that there was differences in the aroma profile of the strains.

2. Problem identification and objectives

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

Malolactic fermentation (MLF) is a key-step of winemaking. It is conducted since late 19th century in a few European regions and interpretation of the phenomenon was established in the 1920s in Burgundy and 1930s in Bordeaux, France. MLF is mainly conducted by the lactic acid bacterium *Oenococcus oeni* in order to decrease wine acidity through the biotransformation of L-malic acid in L-acetic acid. This biochemical reaction involves the malolactic enzyme (mleA), NAD^+ and Mn^{2+} being used as cofactors. In spite of the availability of the nucleotide sequence of the enzyme-encoding gene in the databases, the regulation of *mleA* remains poorly understood. In particular, the adaptation of *Oenococcus oeni* to the wines originated from warm climates (such as South Africa's) has not been studied yet.

This project deals with the evaluation of commercial malolactic starter cultures (all originally isolated in the Northern hemisphere) for warm-climate wine conditions (*i.e.* mostly with low malic acid concentration, but high ethanol content and high pH). The impact of these specific oenological conditions will then specifically be tested in regard to the regulation of the *mleA* gene. We aim to demonstrate a potential link between quick adaptation – MLF completion and high expression of the *mleA* gene.

3. Workplan (materials & methods)

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

Milestone 1: Screening commercial starter cultures for population dynamics under South African wine conditions

Task 1: A broad range of commercial strains of *Oenococcus oeni* will be evaluated for their ability to complete MLF in a short time. Different conditions will be tested by adjusting various parameters in a same standard wine: ethanol (12, 14 and 16%vol.), temperature (14, 20 and 30°C), pH (3.2, 3.5 and 3.8). Samples will be taken every 2 days and counting on plates will be carried out.

Task 2: The time of the different commercial strains to embed in the tank will also be tested by pulsed-field gel electrophoresis at various times after inoculation.

Milestone 2: Regulation of the *mleA* gene of *Oenococcus oeni* and *Lactobacillus pla*ntarum in synthetic medium

Task 1: The experiment will be conducted in a synthetic medium, supplemented with various concentrations of ethanol, sulphur dioxide, various temperatures and pH. 2 strains of *Oenococcus oeni* will be compared. Both strains will be separately inoculated into the different wines.

Task 2: Twice a week, samples will be taken in order to measure the decrease in malic acid concentration (enzymatic reaction), the number of living cells (plate assays) and cells will be pelleted and frozen at -80°C for further RNA extraction.

Task 3: After MLF completion, RNAs will be extracted from the frozen samples and reverse-transcription will be carried out.

Task 4: Quantitative real-time PCR will be used to show the evolution in *mleA* transcript quantity throughout the time. Primers already published in the literature can potentially be used, but this will be confirmed while setting the PCR conditions. It will also be verified that the *ldhD* gene can indeed be used as a housekeeping gene, as described in the literature. For this latter purpose, it will be checked that the expression of this gene does not vary during MLF.

Task 5: quantitative real-time PCR will be performed on the cDNA samples obtained after reverse transcription of the RNAs. The evolution of the *mleA* transcript concentration will be followed.

Task 6: The different data (kinetics of malic acid consumption, population dynamics and expression of the *mleA* gene) will be compiled. This should allow establishing a model, demonstrating the link between cell adaptation and growth, MLF kinetics and malolactic gene expression.

Milestone 3: Screening for the best strains adapted for the completion of MLF in South African wines

Task 1: The strains expressing the highest *mleA* expression rate will be tested for their ability to complete fast MLFs in various South African wines whose physicochemical parameters correspond to the previously tested.

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.

Milestone	**Achievement**
1. Screening commercial starter cultures for population dynamics under South African wine conditions	▪ Four commercial starter cultures were evaluated under industrial scale experimental fermentation conditions in three different grape cultivars ▪ Strains evaluated differed in their rate of MLF, but all completed ▪ Strains differed between cultivars and batches
2. Screening of strains for the *mleA* gene	▪ *mleA* primers were designed and reaction optimized ▪ 53% of LAB strains possess enzyme
3. Regulation of the *mleA* gene of *Oenococcus oeni* in synthetic wine	▪ The behaviour of two oenological *O. oeni* strains IWBT B026 and Lalvin VP41 with regard to the expression patterns of their malolactic enzyme-encoding (*mleA*) genes under the stressful conditions of pH and ethanol were tested. Using quantitative real-time PCR, we demonstrated that the *mleA* gene expression appears to be negatively affected by high ethanol content in the medium, while low pH seemed to have an enhancing effect towards the expression of the *mleA* gene particularly in the middle of MLF. Besides the effect of ethanol on *mleA* gene expression, we also observed the loss of cell viability in the presence of 15% (v/v) ethanol, with the effect less pronounced at lower ethanol content (12% v/v).
4. Regulation of the mleA gene of *Lb. plantarum* in synthetic and white wine	▪ The influence of pH and ethanol on expression of the structural malolactic enzyme gene (*mle*) from *Lactobacillus plantarum* was investigated in a synthetic wine media, as well as in wine using quantitative PCR. Expression of *mle* was shown to be inducible by the presence of malic acid, with increased expression in the middle of MLF. Expression of *mle* was also shown to be increased at low pH values and decreased in the presence of ethanol. This indicates the role of MLF in acid tolerance and the negative impact of ethanol on the completion of MLF.
5. Screening for the best strains adapted for the completion of MLF in South African wines	▪ Wines were made with a selection of 4 *O. oeni* strains and the strains performed MLF at a different rate and therefore yielded wines with different aromatic characteristics.

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)).

- Different PCR primers for malolactic enzymes of different LAB species
- qRT-PCR primers for *mle*
- Sequences of different LAB species are available
- Optimization of qRT-PCR to determine expression of *mle* gene
- Evidence for co-inoculation success for high alcohol wines

- *O. oeni* selected strains completed MLF and generated different wine styles

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.	Senzo Mtshali (PhD)	37,500
2.	Sulette Malherbe (PhD)	1,500
3.	Bronwen Miller (MSc)	5,000
4.	Elizabeth Fritz Intern from France	0
5.		

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

1. Miller, B.J., C.M.A.P. Franz, G.-S. Cho & M. du Toit. **2011**. Expression of the malolactic enzyme gene (*mle*) from *Lactobacillus plantarum* under winemaking conditions. *Curr. Microbiol.* 62: 1682-1688.
2. Malherbe, S., A.G.J. Tredoux, H.H. Nieuwoudt & M. du Toit. **2012**. Comparative metabolic profiling to investigate the contribution of *O. oeni* MLF starter cultures to red wine composition. *J. Ind. Microbiol. Biotechnol.* 39: 477-494.
3. Mtshali, P.S., M. Du Toit & B. Divol. A Survey of wine-related enzyme-encoding genes and *mleA* gene expression analysis in *Oenococcus oeni* strains. *Adv. Microbiol.* (In preparation)

Presentations/papers delivered

1. **Du Toit, M.**, S. Mtshali, C. Knoll & B. Divol. **2008**. Genetic screening of wine lactic acid bacteria for enzyme encoding genes important in winemaking. Ninth Symposium on Lactic acid and bacteria, Egmond aan Zee, The Netherlands.
2. **Mtshali, P.S.**, B.T. Divol & M. du Toit. **2009**. Genetic characterisation of *Lactobacillus lindneri* strains isolated from South African grapes and wine. 4th International SASEV Conference on Enology & Viticulture - BEYOND 2010, Cape Town, South Africa.
3. **Mtshali, P.S.**, B.T. Divol & M. du Toit. **2009**. Comparative analysis of genes coding for enzymes of oenological relevance in wine lactic acid bacteria. 4th International SASEV Conference on Enology & Viticulture - BEYOND 2010, Cape Town, South Africa.

4. Total cost summary of project

	Year	CFPA	Deciduous	DFTS	Winetech	THRIP	Other	TOTAL
Total cost in real terms for year 1	2008				R 150 000	R 71 250		**R 221 250**
Total cost in real terms for year 2	2009				R 180 000	R 90 000		**R 270 000**
Total cost in real terms for year 3	2010				R 160 000	R 61 920		**R 221 920**
Total cost in real terms for year 4	2011				0	0		**0**
Total cost in real terms for year 5								
TOTAL					**R 490 000**	**R 223 170**		**R 713 170**