Industry allocated project number	PHI allocated project number

SATI	CFPA	SAAPPA/SASPA	DFTS	Winetech
tarryn@satgi.co.za Tel: 021 863-0366	inmaak@mweb.co.za Tel: 021 872-1501	theresa@hortgro.co.za Tel: 021 882-8470	dappies@dtd.co.za Tel: 021 870 2900	andraga@winetech.co.za Tel: 021 276 0499

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

NB: The instructions in red, throughout the template, should be omitted from the final document.

FINAL REPORT
(2015)

1. PROGRAMME AND PROJECT LEADER INFORMATION

	Research Organisation Programme leader	Research Team Manager	Project leader
Title, initials, surname	Prof FF Bauer		Dr D Rossouw
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Title, initials, surname			
Present position			
Address			
Tel. / Cell no.			
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2. PROJECT INFORMATION

Research Organisation Project number	**IWBT Y 11-04**
Project title	**Fermentation, metabolic flux and biomass formation:** Balancing the major products of fermentative metabolism to reduce ethanol and volatile acidity formation
Short title	Lower ethanol fermentation

Fruit kind(s)			
Start date (mm/yyyy)	01/2012	**End date** (mm/yyyy)	12/2014

Key words	

Approved by Research Organisation Programme leader (tick box) ☐

THIS REPORT MUST INCLUDE INFORMATION FROM THE **ENTIRE** PROJECT

3. EXECUTIVE SUMMARY

*This must report on the **ENTIRE** project. Address the objectives and milestones of the project as well as the impact of the study on the industry.*

In the research presented here, the problem of high ethanol levels in wine was approached from three very different angles. In first instance, a relatively simple 'cellar practice' approach was employed, by investigating the effect of different yeast strains and fermentation parameters on ethanol yields in a multifactorial framework. In principal, the outcomes from such as study would have been the easiest to implement in practice, as most of the parameters investigated could be controlled by winemakers. However, the results of this research showed that the complexity of the wine production system does not allow for the controlled modification of ethanol production by fine-tuning of oenological settings (given our present knowledge and available data).

A second, biotechnological approach was also considered, which investigated the metabolic and genetic regulation of central carbon metabolism and ethanol production by yeast. The role of different genes in ethanol production was studied and genes selected as candidates for modulation of ethanol production by genetic engineering. In particular, our strategy focussed on targeting genes which could direct carbon from wine sugars to alternative metabolic end-products other than ethanol. In this regard we were successful in directing carbon to trehalose in yeast as an alternative end product, leading to reduced ethanol production without affecting the fermentation rates and total sugar utilisation of the engineered wine yeast strains. Though successful, the challenge remains to translate this technology in a non-GM fashion in future. A strategy to achieve such an outcome through directed evolution has been designed, and will be implemented if funding can be secured.

Lastly, a microbiological approach to the problem of high ethanol levels was pursued: Wine isolates of non-*Saccharomyces* yeasts from various genera and species groups were evaluated for their fermentative ability and the ability to produce low levels of ethanol. Several strains were found which showed substantial reductions in the final ethanol content of sequential fermentations, as well as unique aroma compound production profiles. Some of these strains were selected for comprehensive wine trials in both red and white grape musts, complete with microbial, chemical and sensory analyses of the wines produced. Work in this regard thus presents a full bench-to-bottle characterisation of non-*Saccharomyces* strains showing the most potential for commercial application. The findings of this study enlarge the potential range of oenological applications for non-*Saccharomyces* yeast, while also suggesting the potential usefulness of several yeast species that have previously not been considered for wine making applications.

4. PROBLEM IDENTIFICATION AND OBJECTIVES

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

Currently there is a growing consumer demand for high quality low ethanol wines, while ethanol contents of wines worldwide continue to increase in recent years. This can be attributed to viticultural practices, whereby grapes are kept on vines for an extended period of time to ensure phenolic ripeness and fuller wines with improved flavour attributes. However, this results in an increase in the sugar content of the grape must, and ultimately a higher ethanol yielding wine.

Canopy management practices such as leaf removal also lead to increased sugar levels in the must. Other reasons for the increased ethanol in wines may be due to global warming, as increased temperatures lead to increased sugar accumulation. A high ethanol content does not only have health consequences (higher calorie intake and risk of alcohol related diseases), but also negatively affects the overall bouquet and flavour balance of the wine. The risk for stuck fermentations also increases with increasing ethanol concentrations.

Many physical systems do already exist to remove or decrease ethanol levels in wine. These methods are either expensive or not permitted for use in the South African wine industry. Another drawback especially with post-fermentation methods is that the wine quality may be compromised. Several genetic modification strategies have also been endeavoured to produce lower ethanol –yielding yeast strains, with moderate success. Most often the reduction in ethanol levels is accompanied by the production of unwanted off flavours such as acetaldehyde and butandiol. These methods are also labelled as GM and therefore the yeast strain generated in these studies cannot be used commercially.

Therefore the need exists for an inexpensive and accessible approach for ethanol reduction in wine, without negatively impacting wine quality. At the start of this project no information was available on how must composition and environmental factors interact with individual yeast strains to influence metabolic and regulatory circuits, and how precisely such interactions impact on the final production levels of carbon compounds (such as ethanol) in a specific must. The first objective of our project was thus to investigate the causative factors (other than initial sugar content) of high ethanol yields in grape musts, and provide specific guidelines on how to reduce ethanol yields in high sugar grape juices.

This milestone involved screening 15 widely used wine yeast strains, with regard to their ethanol yields during fermentation. The next step was to investigate the effects of different wine fermentation conditions on ethanol yields. Eight synthetic grape juices were selected to represent a limited number of conditions which represent the most controllable and most relevant parameters (low (3) or high (4) pH, low or high (15 and 25℃) temperature, low or high YAN (120 and 400 mg/l), and low and high sugar (150 and 250 g /l). Different yeast strains were inoculated into the different musts and the fermentations characterised with regards to metabolite production. The factors above were also used in various combinations to generate 27 different chemically defined musts and fermentation conditions to create a multifactorial matrix to evaluate metabolite (most importantly ethanol) production by different yeast strains under these conditions. Subsequently, selected yeast strains were assessed in four different real grape musts derived from controlled experimental vineyards (Sauvignon blanc, Chardonnay, Cabernet and Shiraz) and covering a wide range of grape juice compositions.

The second objective was to investigate the genetic regulation of ethanol production and possibilities to alter carbon balance in yeast to reduce wine ethanol levels. To this end, the objective was to identify metabolic genes which impact ethanol production during fermentation in laboratory yeast strains. Selected genes served as targets for metabolic engineering of the wine yeast strain VIN13 for altered ethanol levels.

The third objective was to pursue an alternative microbiological solution to high ethanol levels, by identifying other species of yeast which are able to survive and utilise sugars under fermentative conditions and produce lower ethanol yields, without imparting negative sensory impacts or producing undesirable compounds.

5. WORKPLAN (MATERIALS AND METHODS)

List trial sites, treatments, experimental layout and statistical detail, sampling detail, cold storage conditions and examination stages and parameters. Add additional rows if required.

- PART 1: Oenological control of ethanol production
    - Fermentations were conducted in synthetic grape must using 15 different commercial wine yeast strains. Selected physio-chemical parameters of the must were varied (pH, temperature, nitrogen levels, sugar levels) in both univariate and multivariate designs. The fermentations were monitored for weight loss and samples analysed by HPLC for residual sugars, glycerol and ethanol at the end of alcoholic fermentation.
    - Ethanol yields of different yeast strains under varying physio-chemical conditions (based on the settings of the synthetic must experiments) were investigated in real grape must. The aim was to evaluate the effects of wine-relevant changes in must composition (pH, temperature and nitrogen) of real white and red grape musts (using different yeast strains) on the ethanol yields of these fermentations. The parameters selected were those that can in principal be controlled by the wine maker to some extent. Four yeast strains were used (EC1118, VIN7, VIN13 and 228) in Sauvignon Blanc, Chardonnay, Shiraz and Cabernet Sauvignon wine musts from the 2013 harvest season. Apart from the control (unmodified) musts, two different temperature settings, two different pH settings, and two different nitrogen settings were selected as experimental factors.

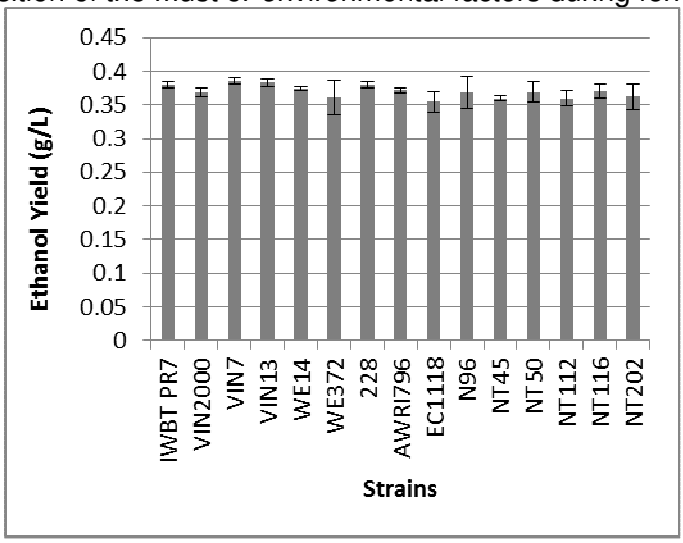
- PART 2: Carbon balance adjustment as means to decrease ethanol production
    - Fermentations were conducted using 66 deletion mutant strains of S. cerevisiae to identify genes with a strong impact on ethanol production during fermentation.
    - Based on these outcomes, the TPS1 gene, encoding an enzyme responsible for trehalose production, was selected as a target gene for modification to direct carbon flux to trehalose (a non-reducing disaccharide) as opposed to ethanol by yeast.
    - TPS1 was overexpressed in the wine yeast VIN13 using newly constructed cassettes for controlled and fermentation stage – specific overexpression of the TPS1 gene. Fermentations were conducted with the genetically modified strains (as well as controls) and sugars, ethanol and trehalose quantified at various time points throughout fermentation in synthetic must.

- PART3: Microbiological tools to reduce wine ethanol

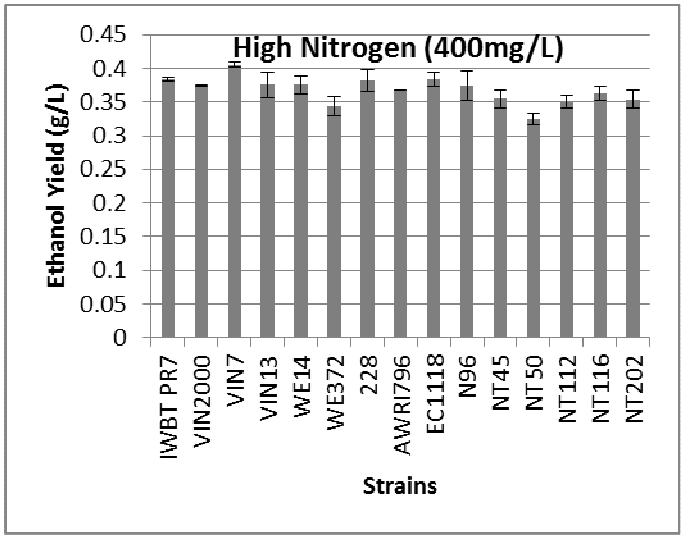
    - 91 non-*Saccharomyces* isolates were evaluated in triplicate in small scale fermentations, with end point sugars, acetic acid, glycerol and ethanol analysed by HPLC.
    - End point samples of these fermentations were also analysed by GCMS to evaluate the potential of these isolates to produce interesting or novel aroma compounds not commonly associated with wine yeast strains of *S. cerevisiae*.
    - A selected subset of 23 isolates (showing lower ethanol yields based on the small scale fermentations) were used in co-fermentation with *S. cerevisiae* (sequentially inoculated after 7 days) in larger volume fermentations. Fermentation kinetics and primary metabolite profiles were analysed at different time points throughout fermentation. End point aroma analysis was performed by GCMS.
    - A final subset of 4 strains were selected for real wine trials (4 repeats each of 20L) in 2013/2014 Pinotage and Sauvignon Blanc grape must. Full microbial, chemical and sensory analyses were performed on these fermentations.

6. RESULTS AND DISCUSSION

State results obtained and list any industry benefits. If applicable, include a short discussion covering ALL accumulated results from the start of the project. Limit to essential information only

- The aim of the first phase of the proposed study was to investigate differences in ethanol yield by a variety of different yeast strains, in response to changes in the chemical composition of the must or environmental factors during fermentation.
  - 

  - The results indicate that only minor variations exist between different commercial yeasts in terms of their intrinsic ethanol yields from sugars consumed. However certain chemical parameters (particularly temperature and nitrogen) seem to play a role in determining the final ethanol yields of fermentations when applied in the single factorial context. However, when different factors are combined with one another the differences in ethanol yields between treatments often disappear, and the predictability of the system diminishes.



  - Ethanol yields of different yeast strains under varying physio-chemical conditions were also investigated in real grape must. The aim was to evaluate the effects of wine-relevant changes in must composition (pH, temperature and nitrogen) of real white and red grape musts (using different yeast strains) on the ethanol yields of these fermentations.
  - The results showed that strains behaved differently with regard to ethanol yield and total ethanol produced in musts from different cultivars. Strains showed contradictory trends in response to chemical parameter changes in the different wine musts, highlighting the important impact of the wine matrix on ethanol production by specific yeast strains. Impacts appear strain –specific, with no

apparent conserved trends between treatments and/or strain, highlighting the complexity of the metabolic regulation of ethanol production.

- The second approach to the problem of wine ethanol levels involved a metabolic flux engineering approach to steer yeast carbon balance in such a manner to reduce ethanol production.
    - Several genes were identified from the genetic analyses which may potentially impact on ethanol production by yeast during fermentation.
    - One strong target, TPS1 (encodes trehalose phosphate synthase, a key enzyme in trehalose biosynthesis) was identified with the potential to direct carbon flux away from ethanol as an end product by producing higher levels of the sugar trehalose.
    - Novel constructs were made using growth stage specific promoter constructs (DUT1 and GIP2 promotor regions) for regulated overexpression of the TPS1 gene. Successful transformants of VIN13 were generated using these constructs.
    - Gene overexpression (stage –specific) was confirmed by real-time PCR analysis. Fermentation analyses confirmed an increase in trehalose production by the modified VIN13, and a concomitant decrease in ethanol production.

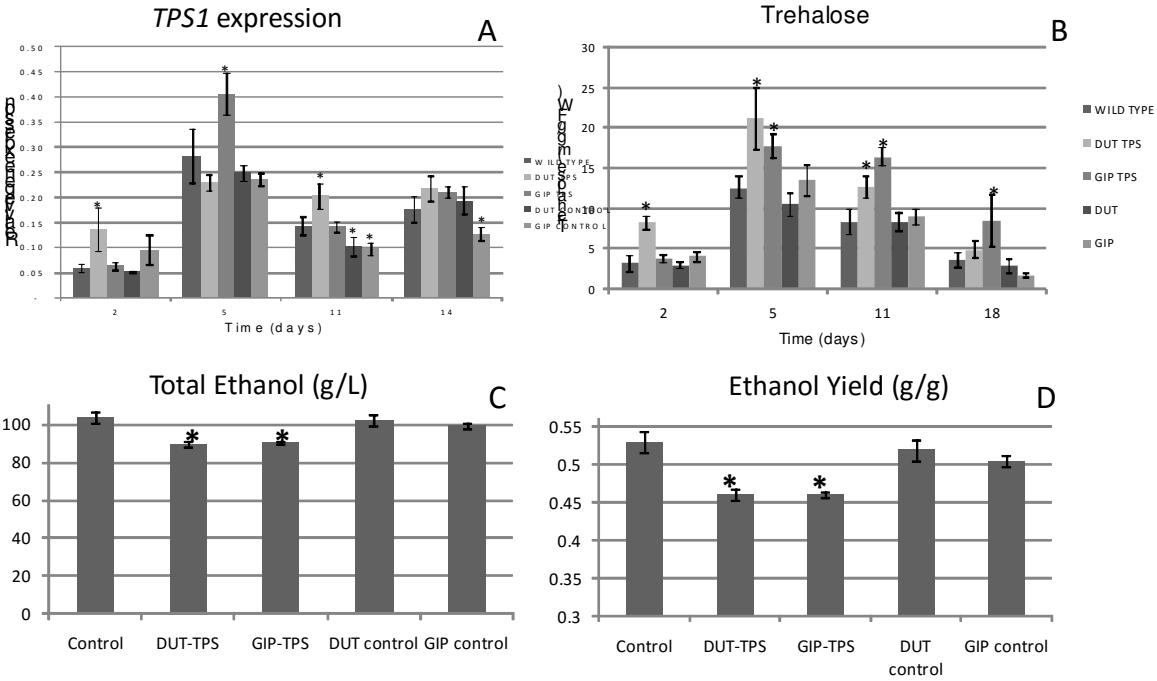
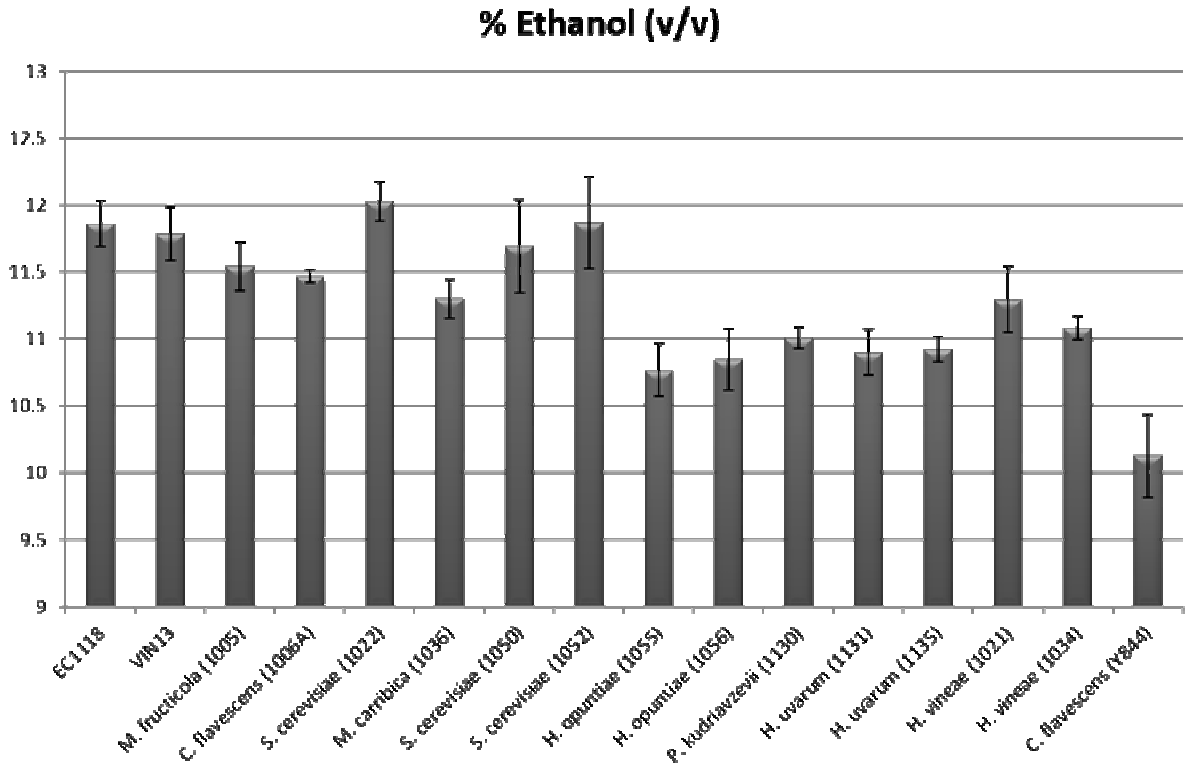


Figure: Relative gene expression levels at different stages of wine fermentation (Frame A); trehalose levels (Frame B), total ethanol (Frame C) and ethanol yield (Frame D) at the end of fermentation. GIP-TPS and DUT-TPS represent data for the modified VIN13 strain overexpressing the trehalose synthesizing enzyme TPS1.

- Lastly, the third approach to wine ethanol level reduction looked at the potential use of non-*Saccharomyces* yeasts in co-fermentation with *S. cerevisiae*.
    - A large-scale screen of 91 non-*Saccharomyces* yeast strains was carried out (vineyard and winery isolates) in order to identify strains with a satisfactory fermentative capacity and lower yields of ethanol per unit of sugar utilised.
    - Several of the original 91 isolates screened in our study produce monoterpenes by *de novo* biosynthesis of these compounds

- Of the 91 isolates evaluated initially, 21 showed lower ethanol yields when compared to commercial wine yeast strain controls. The low ethanol phenotypes were confirmed in sequentially inoculated fermentations (in combination with the wine yeast VIN13).



- Real wine trials in red and white wines were conducted with four selected non-*Saccharomyces* strains. In the Pinotage fermentations, the greatest reduction in final ethanol concentrations (%) was observed for *H. uvarum*, followed by *H. opuntiae* (0.8 and 0.6 percent v/v respectively). In the Sauvignon Blanc, these strains again led to the lowest final ethanol concentrations, with a reduction of up to 1.3 percent (v/v) compared to the wild type. Significantly reduced ethanol levels were also observed for the other two strains used (*P. kudriavzevii* and *C. flavescens*).
- Besides the impact on ethanol yields, inoculation of the non-*Saccharomyces* yeasts resulted (in many cases) in arguably positive aroma impacts and sensory outcomes.

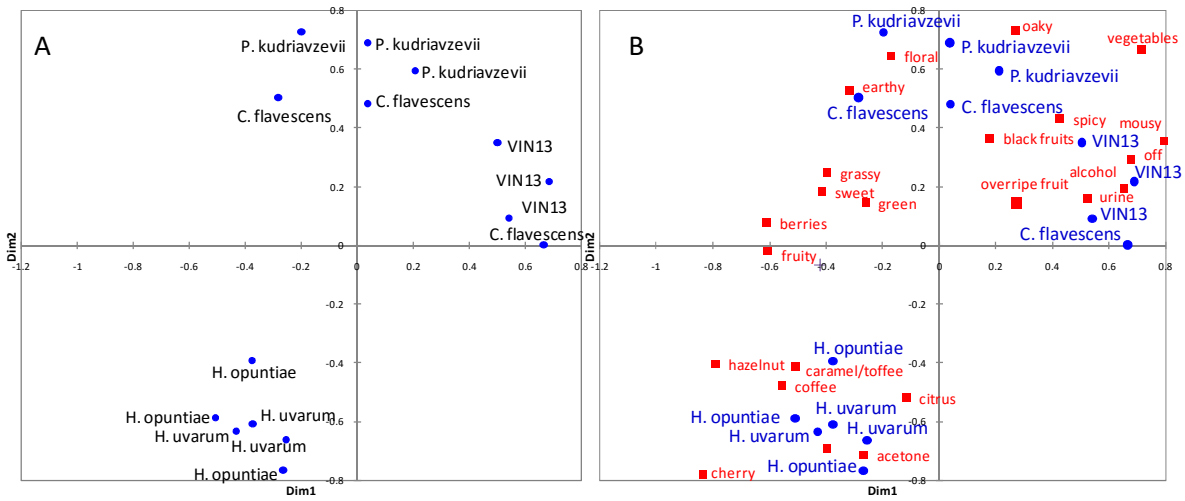


Figure: Results of sensory analysis based on the aroma of Pinotage wines produced by the different non *Saccharomyces* treatments and control. The correspondence analysis is based on aroma descriptors assigned to these fermentations.

7. COMPLETE THE FOLLOWING TABLE

Milestone	Target Date	Extension Date	Date completed	Achievement
1.1 Evaluate EtOH yields of different strains			May 2012	
1.2 Evaluate EtOH yields of different strains in different musts			August 2012	
1.3 Multifactorial analysis of fermentation conditions and yeast			February 2013	
1.4 Chemical analysis of aroma compounds from fermentations			Dec 2013	
1.5 Extend experimental design to real wine must fermentations			July 2013	The outcomes of milestones 1.1-1.5 were compiled into a thesis and resulted in the graduation of one MSc student.
2.1 Screen gene deletion mutants of S. cerevisiae			Before reporting period	
2.2 Construct TPS1 gene			Jun 2012	

overexpression vectors and transform VIN13				
2.3 Fermentation trials and analyses of modified VIN13 and control strains			Dec 2012	The outcomes of milestones 2.1-2.3 were compiled into a thesis and resulted in the graduation of one MSc student, as well as two scientific publications.
3.1 Screen non-Saccharomyces yeasts under fermentative conditions for decreased ethanol yields			Dec 2013	
3.2 Evaluate target strains in larger volume sequential fermentations (plus detailed chemical analysis)			March 2014	
3.3 Real wine trials of selected non-Saccharomyces isolates			Dec 2014	The outcomes of milestones 3.1-3.3 have been submitted for publication.

8. CONCLUSIONS

9. ACCUMULATED OUTPUTS

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

a) TECHNOLOGY DEVELOPED, PRODUCTS AND PATENTS

Indicate the commercial potential of this project, e.g. Intellectual property rights or commercial product(s).

The project has developed several S. cerevisiae yeast prototypes that yield significantly lower ethanol levels.

While these yeast are GM yeast at this stage, the insight into the impact of redirecting carbon flux towards trehalose suggests strategies on how such yeast could be obtained through classical approaches based on directed evolution.

We furthermore isolated several species and strains of non-Saccharomyces yeast that showed significant impact on ethanol, yields and tested such strains in wine fermentation. The data reveal several promising new species and strains, including several species which have not previously been proposed for wine fermentation.

b) SUGGESTIONS FOR TECHNOLOGY TRANSFER

Provide steps taken to ensure the transfer of the gained/new information/knowledge to ultimately benefit the South African fresh fruit industry.

The non-Saccharomyces species and strains should be further investigated:

- Such strains represent an opportunity to develop new tools for oenology.
- Some of these strains appear specific to SA vineyards, and may contribute to the typicality of SA wines (the concept of microbial terroir).
- On-going experiments evaluating the impact of various nutrients and of environmental parameters suggest ways to favour the contribution of wanted species within the natural microflora, suggesting strategies to benefit from such yeast strains without having to inoculate.

c) HUMAN RESOURCES DEVELOPMENT/TRAINING

Complete the following table, adding more lines if necessary.

Student Name and Surname	Student Nationality	Degree (e.g. MSc Agric, MComm)	Level of studies in final year of project	Graduation date	Total cost to industry throughout the project
Honours students					
Masters Students					
Olaf Morgenroth (MSc)	*South African*	MSc Agric		March 2014	
Bianca Brandt (MSc)	*South African*	MSc Agric		Dec 2013	
Hutton Heyns (MSc)	*South African*	MSc Agric		Dec 2012	
Natasha Luyt	*South African*	MSc		Dec 2014	
PhD students					
Postdocs					
Debra Rossouw	South African				
Jaco Franken	South African				
Support Personnel					

d) PUBLICATIONS (POPULAR, PRESS RELEASES, SEMI-SCIENTIFIC, SCIENTIFIC)

Please list using the format illustrated in the example below. ***ATTACH PDF COPIES OF ANY PAPERS***

ALREADY PUBLISHED

2013 Adjusting trehalose metabolism to modify ethanol yields in wine yeast strains. **Debra Rossouw, Hutton Heyns, Evodia Setati, Sue Bosch and Florian F Bauer**. *Applied and Environmental* Microbiology 17:5197-5207

2013 Finding novel carbon sinks in *S. cerevisiae*. **Florian F Bauer, Debra Rossouw and Jaco Franken.** *First International Symposium on Alcohol level reduction in wine,* Bordeaux, France. pp 38-48. [ISBN: 2-915883-11-4]

ACCEPTED FOR PUBLICATION:

2015 Exploring the phenotypic space of non-Saccharomyces wine yeast biodiversity: Finding strains for both low ethanol wines and improved aroma. **Debra Rossouw and Florian F Bauer**. *Food Microbiology*. Accepted for publication.