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 2012

PROGRAMME & PROJECT LEADER INFORMATION

	Programme leader	Project leader
Title, initials, surname	Prof FF Bauer	Dr D Rossouw / Dr E Setati
Present position	Professor	Post-Doctoral Fellow / Senior Researcher
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PROJECT INFORMATION

Project number	IWBT 08/01
Project title	Low ethanol yielding yeast
Project Keywords	Yeast, reduced ethanol, mutant screens, control of metabolic flux

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

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

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

(Give a summary of the project to date in no more than 300 words. Please start the summary with 1 or 2 sentences that summarise the overall objective of this project so that this summary can be read in context of the whole project).

The initial project was targeted specifically at identifying new strategies to develop yeast strains with high fermentative ability, but low ethanol yields. The project pursues an integrated approach, using the tools of systems biology to identify targets and using new methodologies to achieve the desired aims.
The project was initially subdivided into four sub-projects, and a fifth subproject was added as part of the extended work-plan in 2011.
In previous progress reports, the finalisation of two of the intial sub-projects was reported:
In subproject1, a mutant.library screen of all central carbon metabolism genes (approx 150) led to the identification of pathways offering the best possible carbon sinks to direct metabolism away from ethanol. The most promising targets were identified within the trehalose biosynthetic pathway.
In subproject 2, the identification of genes involved with increased fermentative efficiency during competitive growth under wine making conditions resulted in the isolation f several strains with significantly improved fermentative capacity..
Three subprojects were continued on the basis of the amended work-plan as presented in the 2011 progress reports, and are also part of the new approved Winetech program since the beginning of 2012.
Subproject 3: Redirecting carbon flux towards the intracellular synthesis of levans. New genes required for levan biosynthesis have been cloned into *S. cerevisiae* expression systems (MSc student Bianca Brandt since 01/01/2011). The new strains were able to synthesise levans at high levels under respiratory conditions. Ms Brandt graduated in 2012, and a paper has been submitted to PLOS One (Franken et al. 2013)
Subproject 4: This subproject is based on the genes identified in Subproject 1. Several constructs combining specific target genes were combined with fermentation phase-specific promoters. In 2011, these constructs were successfully transformed into industrial wine yeast strains and these strains are currently undergoing full assessment of ethanol yields and aroma production (MSc student Hutton Heyns since 01/01/2010, Dr D. Rossouw). This project yielded excellent results, with wine yeast strains that produce lower levels of ethanol when trehalose metabolism is adjusted slightly. Ms Heyns has graduated in 2012. A paper has been pubnlished in AEM (Rossouw et al. 2013.)
Subproject5 was added as part of the amendment to the workplan in 2011. In this subproject, a metagenomic approach will be implemented to identify and isolate enzymatic activities. Metagenomic libraries from cellar and vineyard environments have been constructed. (Collaboration with Dr E. Setati, B.Divol), This work is on-goinfg as part of the new Wunetech funded projects

2. Problem identification and objectives

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

The average ethanol content of South African and international wines have increased significantly over the past decade. This appears to be mainly due to a shift in viticultural practices, with grapes being left to ripen for longer periods of time resulting in higher levels of

fermentable sugars in the must. Higher initial sugar concentrations in the must logically lead to a higher ethanol level in the wine. In many cases, such high ethanol levels have a negative impact on wine quality and consumer perceptions. An additional problem associated with high initial sugar concentrations in must is the increased risk of experiencing problem fermentations, either sluggish or stuck, and ending up with wines with high residual sugar concentrations (residual sugars > 5 g/l). Discussions with wine makers indicate that these problems are some of the most serious issues affecting the future competitiveness of the SA wine industry.

Several mechanical-physical methods for the reduction of ethanol have been developed, including methods based on reverse osmosis and on centrifugal forces in a spinning cone. However, these methods of ethanol removal are labour-intensive, expensive and may in some cases have a significant impact on the final quality of wine, resulting in altered taste, aroma or texture.

Wine yeast strains have been evaluated for their intrinsic ethanol yields (the amount of ethanol that is produced per unit of sugar consumed). These studies show that the ethanol yields of individual strains are dependent on environmental conditions (including availability of oxygen, temperature and others) and that different strains have different ethanol yields. However, all variations fall within a narrow band of values, and the differences are too small to have a significant impact on final ethanol concentrations. For this reason, approaching the problem from a biotechnological perspective appears the most promising option to provide an easy to implement and cost-effective solution.

The aims of the project are to identify biotechnological targets for strain improvement (ethanol yield and fermentation efficiency) and to apply these insights to develop strains that have high fermentation efficiency combined with significantly lower ethanol yields.

This report includes some newly added milestones that were approved as part of the one year extension of the running of this program. These milestones all have been carried over into the newly approved Winetech program that started in 2012.

3. Workplan (materials & methods)

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

The project used all standard microbiological and *in vitro* nucleic acid manipulation and analysis tools, including PCR, restriction digestions, ligations and cloning. Transformation of yeast and bacteria followed standard procedures, including chemical transformation methods (calcium chloride for bacteria, lithium acetate for yeast) and electroporation (for industrial yeast strains). Metabolite concentrations were determined either enzymatically or through HPLC, GF-FID or GC-MS, dependent on the type of metabolite and the matrix to be analysed. Data were analysed using standard statistics and multivariate data analysis tools for the more complex data sets.

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.

The sub project 1, which was concerned with the **Systematic screening of genes involved in the central carbon metabolism and associated pathways,** the previous reports described the identification of several genes whose deletion led to significant changes in ethanol concentration after small scale fermentation in synthetic must. In 2009, the phenotypes were further investigated in different wine fermentation conditions. All selected

strains fermented more sugars than the wild-type. These genes were representative of trehalose biosynthesis (*TPS1*, trehalose-6-phosphate synthase), glycolysis (*TDH3*, glyceraldehyde-3-phosphate dehydrogenase), the oxidative pentose phosphate pathway (*ZWF1*, glucose-6-phosphate dehydrogenase) and the TCA cycle (*ACO1* and *ACO2*, aconitase isoforms 1 and 2). Two strains exhibited lower ethanol yields (*tps1-Δ* and *tdh3-Δ*), with the remaining three having higher ethanol yields than the wild-type. Phenotypes of *tps1-Δ* and *tdh3-Δ* were confirmed by construction of new knockout strains. A draft article describing this work was attached to the report. To assess the impact of these gens on ethanol yields in industrial wine yeast strains, a new original approach was designed. The approach is based on changing expression intensity and timing of specific genes. Indeed, in the past,all approaches used a relatively blunt strategy of disruption (absence of a gene) or general overexpression (always more of a gene product). Transcriptome data by DNA microarray generate by Dr Rossouw and using diverse wine yeast strains during fermentation has provided us with significant insights into subtle regulatory processes. Such more subtle alterations may be more effective in re-orientating metabolic flux away from ethanol than previous approaches used by our and other research teams. The new milestones for 2011 therefore target several of the genes identified in our screens for promoter replacement strategies. The new promoters will impart fermentation stage –specific gene expression patterns.

Dr Sue Bosch had been on maternity leave for six month and decided not to pursue a career in science. Dr Debra Rossouw, was appointed in 2010 to supervise the work. .

Mr Madlanga has graduated (MSc) in March 2009. A new MSc student, Ms Hutton Heyns, has been appointed to follow up on the new milestones in 2010. Ms Heyns work is described as part of the report on Subproject 4 below.

In sub-project 2 the **Identification of genes involved with increased fermentative efficiency during competitive growth under wine conditions** several mutant libraries, including a transposon deletion library and the full deletion library, have been screened in competitive conditions (sequential wine fermentations). After 25 generations (corresponding to five sequential batch fermentations), a significant enrichment was observed for two strains of the deletion library, which made up 3 or 2 out of 20 randomly picked colonies in two independent selection processes.

In 2009, The screening resulted in 7 potential deletion mutants namely *Δfcy22*, *Δyhl005c*, *Δfaa3*, *Δdjp1*, *Δyjl022w*, *Δecm25* and *Δpda1*. The first 6 mutants showed improved fermentative efficiencies in particular towards a higher V_{max} for CO_2 production and shorter lag phase and fermentation time when compared to its reference strain. The *Δpda1* strain showed a longer lag phase but then increased fermentative ability during mid exponential phase. However, upon reconfirmation of these mutants in the same genetic background, the phenotypes observed were no longer prevalent. This led us to conclude that secondary genetic mutations may have occurred within the Euroscarf deletion mutants. Among abnormalities observed were mixed molecular barcode sequences and the ability of these supposedly haploid strains to sporulate.

We have decided not to pursue this line of work any further since the delete strain library does not appear a suitable tool to differentiate strains for small phenotypic differences. Confidential communications by other research groups using the deletion strain library suggest that this problem has been encountered by a large number of researchers. A paper to describe these pitfalls was attached to the report.

In sub-project 3: **Redirecting carbon flux towards the intracellular synthesis of levans,** all genes required for levan biosynthesis have been cloned into *S. cerevisiae* expression systems. The sucrose synthetase, SUSY, and a sucrose transporter have been successfully expressed and we have been able to show that transformants contain a high intracellular

sucrose concentration. These strains have been transformed with levan biosynthesis genes and we are assessing the outcomes of these combinations.

In 2009, we successfully integrated four different levan sucrases isolated from different organisms for the production of levans from the synthesized sucrose. However, we were unsuccessful in producing any detectable fructose polymers in *S. cerevisiae*. The reasons for this absence of production are unclear, since the strains produce significant amounts of sucrose, and the data show correct sequences and expression levels of the transgenic constructs. After further attempts during the end of 2009 (change of promoters, change to N-terminal sequences for cytoplasmic localisation, no improvements were observed, and the project was discontinued. However, new levan biosynthesis genes wit different kinetic properties were described by various groups and made available to us. Since we dispose of the sucrose producing strains, it was decided to assess these novel genes in this molecular background (MSc project of Bianca Brandt). The new genes have been cloned into S. cerevsiae expression systems, and will be transformed into the sucrose-producing strains.
These strains have been shown to synthesise significant amount of levans with excellent sugar conversion yields (>30% of sucrose). (Figure 1). This is the first report of the successful biosynthesis of such potentially valuable polymers in *S. cerevisiae*.

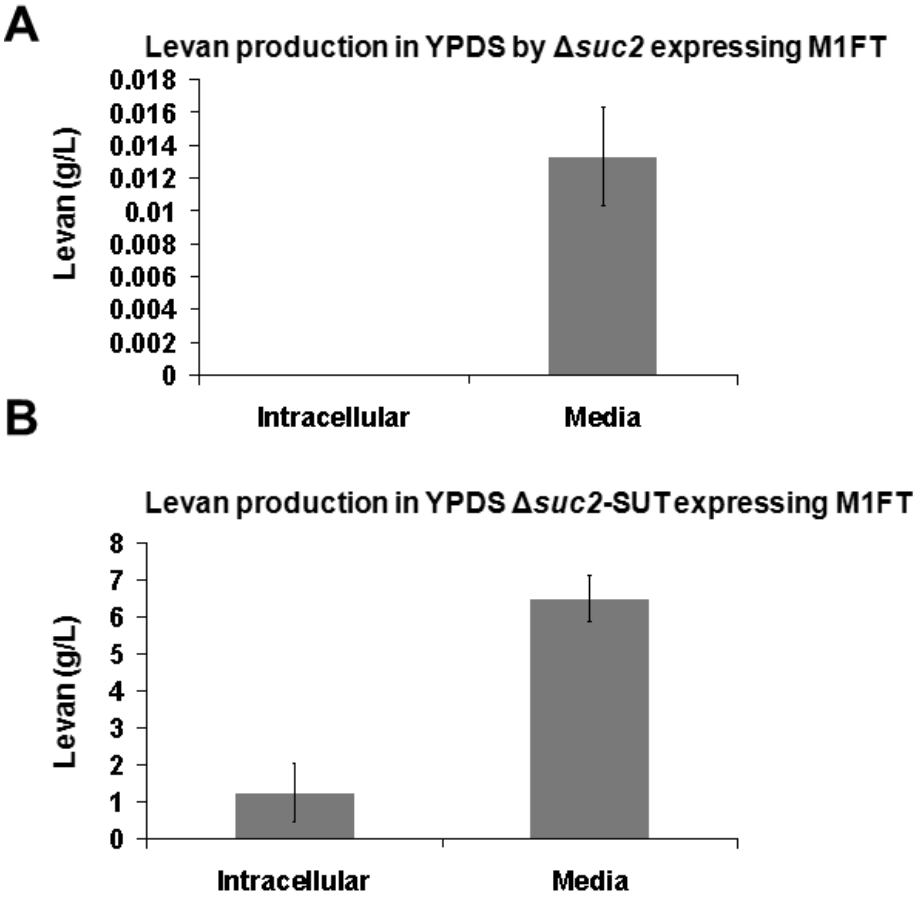

Figure 1. Quantification of levan production by M1FT transformed strains in rich media. Levan was quantified by densitometric analysis of the polymers as visiualized on TLC plates (A) Levan produced by M1FT expressed in the BY4742Δ*suc2* genetic background. (B) Levan production by M1FT expressed in the BY4742Δ*suc2*-SUT genetic background.

A manuscript has been submitted for publication. Further work is being implemented to

Subproject 4: Several constructs combining specific target genes identified in our screenings are combined with fermentation phase-specific promoters. These constructs are designed for transformation in industrial strains, and construction of these constructs and the transformation of these strains was successfully completed in 2011. All the constructs and transformed strains are currently being assessed in several industrial strains (Vin13 and one or two additional strains). An MSc student, Hutton Heyns, is finalising the evaluation of strains that have been constructed using a new approach based on minor adjustments of transcriptional activity. The data show that the generated strains show a significantly reduced ethanol yield, while fermenting with similar kinetics than the wild type (Figure 2). These finding offer exciting new opportunities for generating yeast strains with reduced ethanol yields without using genetic modification.. A manuscript has been published in 2013.

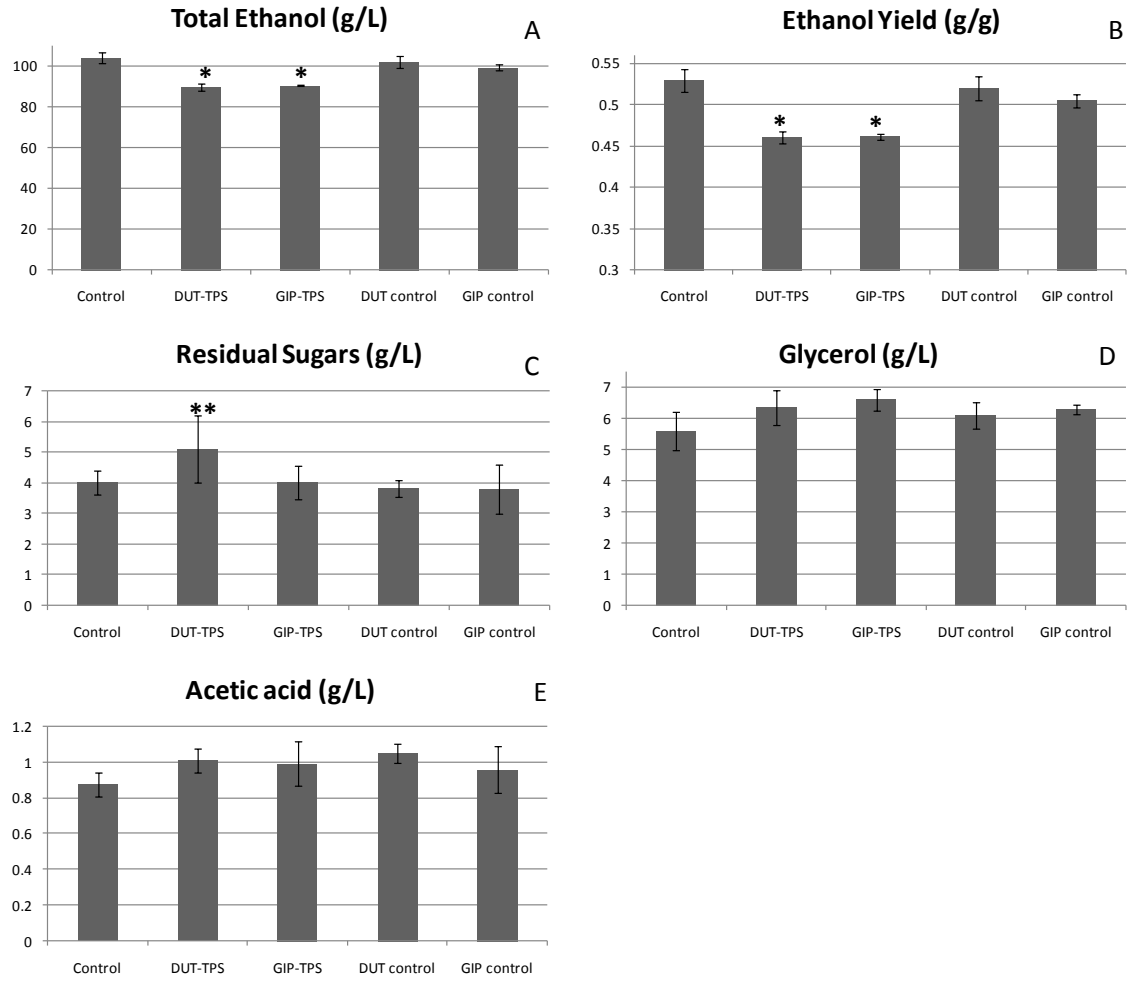

Figure 2. Ethanol (frame A), acetic acid (frame E) and glycerol levels (frame D) at the end of fermentation (T22). Sugar utilization and ethanol yield as determined at the end of fermentation are depicted in frames B and C respectively. Values are the average of three biological repeats ± standard deviation. The student t-test was used to establish significant differences between fermentations conducted with the TPS1 overexpressing strains and their respective empty plasmid controls. * denotes $p<0.05$ (95% confidence) and ** denotes $p<0.1$ (90% confidence)

A new subproject, Project 5, was part of the modified workplan for 2011. In this subproject, a metagenomic approach has been implemented to identify and isolate enzymatic activities. This approach is useful for several currently funded wine tech projects of Profs FF Bauer, Maret du Toit and Dr Benoit Divol, and the costs are shared between the different project leaders as indicated in the individual applications approved in 2011. Metagenomics is a new, extremely powerful tool to find novel enzymes, and is defined as the study of an entire ecosystem on the level of DNA. For the purpose of the " Low ethanol programme", the metagenomic approach will target the isolation of genes that encode enzymes related to the synthesis of intracellular polymeric sugars, in particular polymers that are used as storage carbohydrates. Our screen has shown that targeting carbohydrate synthesis is probably the most promising target for reducing ethanol yields. While the levan approach has failed, it is clear that the synthesis of other polymers could be more effective targets for reducing ethanol yields. With the metagenomics approach, such enzymes will be part of the wine holo-genome, meaning that the genes are present naturally during fermentation. The details are described in the following sections.

Project 1: Systematic screening of genes involved in the central carbon metabolism and associated pathways

Milestone	**Achievement**
1. Preliminary screen for differences in metabolism of selected mutant yeast (all mutant strains deleted for genes in central carbon metabolism and associated pathways, including pentose phosphate pathway, the glycerol pathway, all pathways diverting from pyruvate, and storage carbohydrate accumulation pathways) and the parental wild-type strain	(already achieved)
2. Thorough screen of all selected mutant strains in synthetic wine must and full metabolite analysis (2008)	Sixty six mutants were screened. (Finalized (see previous report)
3. Engineering of double knock-outs in a lab strain and establishing functional genetic linkages (2008-2009)	Five of these strains were selected for further investigation because of interesting properties. All five fermented more sugars than the wild-type. These genes were representative of trehalose biosynthesis (*TPS1*, trehalose-6-phosphate synthase), glycolysis (*TDH3*, glyceraldehyde-3-phosphate dehydrogenase), the oxidative pentose phosphate pathway (*ZWF1*, glucose-6-phosphate dehydrogenase) and the TCA cycle (*ACO1* and *ACO2*, aconitase isoforms 1 and 2). Two strains exhibited lower ethanol yields (*tps1-Δ* and *tdh3-Δ*), with the remaining three having higher ethanol yields than the wild-type.

Project 2: Identification of genes involved with increased fermentative efficiency during competitive growth under wine conditions

Milestone	**Achievement**
1. Obtaining yeast pools (i) yeast deletion library (ii) yeast transposon library (iii) multicopy yeast genomic library (2007)	Achieved
2. Competitive fermentation of yeast pools in sequential batches for enrichment of high performance fermentation yeast in synthetic wine must and isolation of best performing strains (2008-2009)	Competitive fermentations have been conducted with all libraries. In all cases, significant enrichments for certain mutants was observed.
3. Identification of genes that contribute to increased fermentative efficiency (2009)	In 2009, The screening resulted in the identification of 7 potential deletion mutants namely *Δfcy22*, *Δyhl005c*, *Δfaa3*, *Δdjp1*, *Δyjl022w*, *Δecm25* and *Δpda1*. The first 6 mutants showed improved fermentative efficiencies in particular towards a higher V_{max} for CO_2 production and shorter lag phase and fermentation time when compared to its reference strain. The *Δpda1* strain showed a longer lag phase but then increased fermentative ability during mid exponential phase. However, upon reconfirmation of these mutants in the same genetic background, the phenotypes observed were no longer prevalent. This led us to conclude that secondary genetic mutations may have occurred within the Euroscarf deletion mutants. Among abnormalities observed were mixed molecular barcode sequences and the ability of these supposedly haploid strains to sporulate.

Project 3: Redirecting carbon flux towards the intracellular synthesis of levans

Milestone	**Achievement**
1. Obtaining genes required for (i) sucrose transport into yeast (ii) sucrose synthase gene which synthesises sucrose from glucose and fructose (iii) production of levans – levan synthase (currently on-going)	All selected genes have been cloned. In particular, three different levan synthases of bacterial origin were selected for further analysis.
2. Construction of lab strains with relevant genes required for levans production in yeast (2008)	All genes have been integrated in a S. cerevisiae expression cassette and transformed in combination (sucrose ttransporter or sucrose synthase combined with one of three levan synthase have been co-expressed in S. cerevisiae. The expression of these genes has been assessed by Northern blot.
3. Validate the production of polymers and assess quantitatively the distribution of carbon fluxes in synthetic wine must (2008)	We integrated four different levansucrases isolated from different organisms for the production of levans from the synthesized sucrose. However, we were unsuccessful in producing any detectable fructose polymers in *S. cerevisiae*. The reasons for this absence of production are unclear, since the strains produce significant amounts of sucrose, and the data show correct sequences and expression levels of the transgenic constructs. All attempts to change genes to make levan production more likely failed.
4. Controlled ethanol yield via inducible promoters for desired ethanol degree in wine (2008-2009)	Could not be pursued since no levan synthesis was observed
5. New levan biosynthesis genes with different properties were described in literature (2010). Cloning of these genes.was started in 2011 (New MSc student, Bianca Brandt).	Three new levan synthase genes have been cloned and are now being asse for their ability to convert sucrose to longer-chain levans in sucrose producing *S. cerevsiae* strains. The new levan synthesing gene was successfully expressed in yeast, and significant amounts of levan were produced in respiratory conditions. However, no levan was produced under fermentative conditions. The strains may however generate significant commercial interest, since levans are potentially valuable biopolymers.

Project 4: Engineering of a yeast strain with improved fermentative efficiency and reduced ethanol yield (This project is now part of the new Winetech program on "Metabolic flux control in wine yeast strains" of Prof FF Bauer)

Milestone	Achievement
1. Modifications of lab strains with knowledge obtained from Projects 1, 2 and 3 (2009)	Progress 2009: Phenotypes of *tps1-Δ* and *tdh3-Δ* were confirmed by construction of new knockout strains. Besides the ethanol and residual sugar phenotypes, these two strains fermented faster and had higher glycerol levels. The combined fermentation parameters of the deletion phenotypes of *TPS1* and *TDH3* make them appropriate targets for application to an industrial wine yeast strain.
2. Assessing viability of strain for improved fermentative efficiency and reduced ethanol yield (2009)	Continued in 2010: All strains have been tested and the genes whose mutations produced the most desirable phenotypes have been selected.
3. Construction of expression cassettes combining the identified genes with different promoters (2010)	A new MSc student, Hutton Heyns, has taken charge of the project. The two genes have been cloned with two different promoters, ensuring expression at medium levels during either exponential growth or during stationary phase of the *TPS1* and *TDH3* genes.
4. Transformation of industrial yeast strains with constructs (2011) and assessment of impacts of modified gene expression.	Vin13 has been transformed with the eight different constructs, and is currently undergoing assessment regarding ethanol yields, trehalose production and fermentation kinetics.
5. Assessment of industrial strains	

Project 5: Identification of novel enzymes through wine metagenomics IThis subproject is now part of the Winetech program on exploitation of biodiversity of Drs Setati, Divol, and Profs du Toit and Bauer)

Milestone	Achievement
1. Sampling of vineyards and cellars for assessment of biodiversity	Samples were taken from various vineyards and cellar following a statistically designed
2. Assessment of biodiversity through traditional microbiological techniques (culture-based)	Biodiversity has been assessed in vineyards that have been managed according to different guidelines, from biodynamic to conventional.

3. Establishment of metagenomic library	The first metagenomic libraries representing the species present at the beginning of alcoholic fermentation have been constructed. Their quality and representativity is currently undergoing assessment.

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. Numerous metabolically engineered yeast strains have been generated. Some of these strains show higher fermentation efficiency.
2. Importantly, we generated strains that produce valuable biopolymers (levans) in high amounts. We are currently investigating industrial applications of such strains.
3. We also showed that limited modifications of trehalose biosynthesis had a significant impact on ethanol yields without impacting on fermentative behaviour. This opens new opportunities to generate non-GM low ethanol strains.
4. New resources, in particular metagenomic libraries with significant value to the SA wine industry were generated.

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.	MSc by H. Madlanga (2010)	No direct costs but for bursary supplement
2.	MSc by E. H. Heyns (2012)	Bursary supplement
3.	MSc by Bianca Brandt (2012)	Bursary supplement

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

(1) Rossouw, D., R. Olivares-Hernandes, J. Nielsen **& F.F. Bauer. 2009.** A comparative 'omics' approach to investigate differences in wine yeast physiology and metabolism during fermentation*. Applied and Environmental Microbiology* **75:**6600-6012.

(2) Rossouw, D., A.H. van den Dool, D. Jacobson & **F.F. Bauer. 2010.** Comparative Transcriptomic and Proteomic Profiling of Industrial Wine Yeast Strains. *Applied and Environmental Microbiology* **76:**3911-3923.

(3) Jain, V.K., B. Divol, B.A. Prior & **F.F. Bauer. 2012.** Effect of alternative NAD+ regenerating pathways on the formation of primary and secondary aroma compounds

in a *Saccharomyces cerevisiae* glycerol defective mutant. *Applied Microbiology and Biotechnology* **93**:131-141.

(4) Rossouw, D., D. Jacobsen & **F.F. Bauer. 2012**. Transcriptional regulation and the diversification of metabolism in wine yeast strains. *Genetics* **190:**251-261.

(5) Rossouw, D, E.H. Heyns, E. Setati, S. Bosch & **F.F. Bauer. 2013.** Adjustment of trehalose metabolism in Wine *Saccharomyces cerevisiae* strains to modify ethanol yields. *Applied and Environmental Microbiology*. 17:

(6) Franken, J., B.A. Brandt, S.L. Tai & **F.F. Bauer. 2013.** Biosynthesis of Levan, a bacterial extracellular polysaccharide, in the yeast *Saccharomyces cerevisiae. Submitted PLoS ONE.*

Presentations/papers delivered

Bosch, S, and FF Bauer. (2008) Understanding carbon flux in *Saccharomyces cerevisiae*: The case of fermentations with high initial sugar content. Yeast Genetics and Molecular Biology Meeting, Toronto, Canada.

Rossouw, D. & F.F. Bauer. 2010. Comparative omics of wine yeast strains. Internationaler IVIF Kongress Intervitis Interfructa Stuttgart. In: Micro-organisms – alcoholic fermentation. pp 1-10 (on invitation).

Vivier, M.A., M du Toit & F.F. Bauer. 2011. Wine Metabolomics: How yeast and bacteria transform the grape metabolome. Proceedings of 2nd edition of the international conference series on Wine Active Compounds. pp 11-13 (on invitation).

Bauer, F.F. 2011. Yeast – the wine builder. Proceedings of the 34th World Congress of Vine and Wine of the OIV – The construction of wine. Porto, Portugal. pp 1-10 (on invitation).

4. Total cost summary of project

	Year	CFPA	DFPT	DFTS	Winetech	THRIP	Other	TOTAL
Total cost in real terms for year 1	2008				R 390 000	R 185 250		**R 575 250**
Total cost in real terms for year 2	2009				R 421 000	R 210 500		**R 631 500**
Total cost in real terms for year 3	2010				R 463 320	R 231 660		**R 694 980**
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								
TOTAL					**R 1 274 320**	**R 627 410**		**R 1 901 730**

	Year
Total cost in real terms for year 1	
Total cost in real terms for year 2	
Total cost in real terms for year 3	
Total cost in real terms for year 4	
Total cost in real terms for year 5	
TOTAL	