Industry allocated project number	PHI allocated project number

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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
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Title, initials, surname			
Present position			
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2. PROJECT INFORMATION

Research Organisation Project number	IWBT MG 11-01
Project title	**Metagenomic and metatranscriptomic analyses of microbial population dynamics on grapes and in musts derived from conventional, organic and biodynamic vineyards**
Short title	

Fruit kind(s)	Wine		
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.*

This report provides a synthesis of the research that was carried out over a 3 year period investigating the microbial community structures associated with grapes and the impact of agricultural practices commonly used in vineyard management on the community composition. In addition, the functional potential of the microbial community was explored. A combination of cultivation-dependent basic microbiological analyses, DNA-based community fingerprinting and high-throughput whole community genome sequencing approaches were employed to generate a more comprehensive overview of the grapevine microbial community and the dynamics of the yeast population during fermentation. The project investigated microbial diversity in vineyards managed through biodynamic farming practice and Integrated Production of Wine guidelines. In addition, the influence of leaf removal strategies on microbial community structures was evaluated. Key findings in the current project revealed the following:

(i) vineyards employing different farming practices harbour distinct fungal communities even though there are significant overlaps between vineyards, and within a vineyard there is significant variability in fungal community structures that might result in tank variation in fermentation profiles,

(ii) several non-Saccharomyces yeasts with known potential impact on wine quality can persist in high numbers for a significant part of the fermentation; and knowledge of the initial yeast community would be an important management tool for winemakers in order to enhance or suppress existing dominant species

(iii) South African vineyards may harbour unique uncultured plant pathogens that remain uncharacterized, for instance, metagenomic data revealed a high incidence of *Kabatiella microsticta* known to cause leaf spot in several plants but never reported in grapevine.

(iv) leaf removal strategies may result in changes in microbial community composition resulting in a high incidence of *Rhodotorula* spp and *Cryptococcus* species on exposed bunches than shaded bunches

The data obtained in the current project has been synthesized into five manuscripts (1 published, 2 accepted, 2 in preparation).

4. PROBLEM IDENTIFICATION AND OBJECTIVES

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

Vineyards harbour a wide variety of microorganisms that play a pivotal role in pre- and post-harvest grape quality and will contribute significantly to the final aromatic properties of wine. The diversity of microorganisms associated with grape berries may be affected by viticultural practices such as pruning, as well as farming systems e.g. organic, biodynamic, conventional, or integrated pest management practices. Viticulturalists are constantly trying to improve the quality of grapes by applying different pruning strategies, while others switch from conventional to more environmentally friendly farming methods. However, there is no clear understanding of the impact of these practices on grape microbiota, and therefore their subsequent impact on wine fermentation and wine quality.

The objectives of this study are:

1. To evaluate the impact of conventional, organic and biodynamic farming practices on grape berry surface microbial diversity
2. To determine the functional potential of spontaneously fermenting grape must derived from biodynamically produced grapes
3. To evaluate the impact of viticultural practices such as pruning and canopy management on the diversity of indigenous yeasts on grape berries

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.

Milestone 1: Comparative analysis of the fungal diversity on grapes derived from biodynamic and conventional vineyards

i. Microbiological analysis of fungi (yeast and moulds) associated with grapes from three neighbouring vineyards

Grapes were collected from a certified biodynamic vineyard and two conventional vineyards bordering the biodynamic vineyard. Thirty bunches were collected per vineyard at selected rows and panels. The grapes were rinsed with saline solution containing 0.1% Tween 80, followed by serial dilution of the wash solution and spread-plating on Wallerstein nutrient agar supplemented with Biphenyl 150 mg/L and 34 mg/L chloramphenicol for isolation of yeast. The isolates were identified by standard phenotypic characterization as well as PCR-RFLP of the ITS1-5.8S-ITS2 gene using *Hae*III, *Hin*fI and *Cfo*I followed and sequencing of the gene for confirmation of identities.

ii. Comparison of inter and intra-vineyard variability

Automated Ribosomal Intergenic Spacer analysis (ARISA) was used for fungal community fingerprinting (Slabbert et al., 2010). Genomic DNA was extracted from the wash solutions obtained above. The ITS1-5.8S rRNA-ITS2 gene sequence was amplified using a caboxy-fluorescein labelled forward primer (ITS1-6FAM) and ITS4 reverse primer. PCR amplicons were resolved through capillary electrophoresis and analysed with GeneMapper 4.1 software. Correlation networks were employed to compare the fungal community structures within and between vineyards.

iii. Evaluating the diversity and dynamics of the yeasts involved in wine fermentation

Samples were collected in two consecutive years (2012 and 2013) and used for micro-vinification. For each vineyard, 5 kg of Cabernet sauvignon grapes were collected and transported to the laboratory in sterile "Ziploc®" bags. The grapes were asceptically hand-destemmed and crushed within 1 h of collection. The chemical composition of the must was analysed by Fourier Transform Infrared (FT-IR) spectroscopy using the GrapeScan 2000 instrument (FOSS Electric, Denmark). Fifty millilitre samples were collected from the fresh must and used for yeast isolation and enumeration. The remaining must was transferred into duplicate 2 L fermentation bottles and set-up for natural fermentation at 25°C to allow sufficient growth of the non-*Saccharomyces* population without negatively affecting the development of the indigenous *S. cerevisiae* strains. Alcoholic fermentation was followed by measuring the loss of weight resulting from CO_2 release. In addition samples were withdrawn regularly for sugar analysis. The glucose and fructose concentrations were measured using the Enytec™ *Fluid* D-Glucose Id-No: 5140 and Enytec™ *Fluid* D-Fructose Id-No: 5120 (R-Biopharm AG,Germany) enzymatic kits on the Arena™ 20XT Photometric analyser (Thermo Electron, Oy, Finland). Yeast dynamics were evaluated after 12.5%, 30%, 50% and 70% sugar consumption. Yeast isolation and identification was performed as described in (a) above.

Milestone 2: In-depth analysis of the functional diversity of grape must obtained from conventionally and biodynamically produced grapes

i. Construction of a metagenomic fosmid library from conventional and biodynamic grape must

Genomic DNA was extracted from the grape must of the vineyard which demonstrated higher cultivable yeast diversity (Biodynamic vineyard) and used for metagenomic analysis. Two approaches were employed, firstly a fosmid library containing insert fragment ranging from 25 – 40 kb was constructed and cloned in *E. coli* using the CopyControl™Fosmid library production kit (EPICENTER, Madison, WI). The

fosmid clones were selected on Luria Bertani (LB)-Chloramphenicol (12.5 µg/mL) plates. The clones were then screened on LB agar supplemented with chloramphenicol and either 1% (w/v) carboxy-methyl cellulose (CMC), 0.1% (w/v) laminarin, 0.5% (w/v) arbutin, 1.25 (w/v) polygalacturonic acid (PGA), 1.14% (w/v) skimmed milk and 0.45% (w/v) chitin to screen for glucanases, β-glucosidases, pectinases, acid proteases and chitinases respectively. Fosmids of interest were further sequenced using the Ion Torrent Proton Semiconductor Sequencer (Applied Biosystems) at the Central Analytical Facility, Stellenbosch University, Stellenbosch, South Africa. The contigs were assembled using the DNA Dragon–DNA sequence Contig Assembler Software (http://www.dna-dragon.com/) (www.sequentixde) and compared with sequences available on the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/genome) using the Basic Local Alignment Search Tool X (BLASTX) algorithm. In order to construct the physical map of the fosmid insert, the contigs were matched manually and arranged accordingly.

ii. Whole metagenome shotgun sequencing and analysis

In addition to the Fosmid libraries, genomic DNA was subjected to direct sequencing on the Roche GS-FLX 454 platform. The data was processed through the freely available server-based MetaGenome Rapid Annotation Subsystems Technology (Meyer et al., 2008). The data underwent quality control (QC) analyses including quality filtered, length filtered, and dereplication. Taxonomic analysis was done by comparison of the metagenome data with the M5RNA database available on MG-RAST using a minimum identity cut-off of 97% and a minimum alignment length of 100 bp. To characterize the gene content of the grape must, all reads were functionally annotated by means of the Clusters of Orthologous Groups of proteins database (COG) (Tatusov et al., 2000, 2001). Organism and functional identifications were performed using a BLAT [Basic Local Alignment Search Tool (BLAST)-like alignment tool] search of the integrative MG-RAST M5NR database, which is a non-redundant protein database that combines sequences from multiple common sources. Identifications were made using a maximum e-value of $1e^{-8}$, a minimum identity cut-off of 60% and a minimum alignment length of 50 bp. The relative abundance of each gene or species was determined by dividing the number of hits of that particular gene or species by the total number of hits.

Milestone 3: Analysis of microbial population diversity on grapes produced under full canopy and exposed bunches

Grape must samples were collected after crushing of grapes obtained from vines with full canopy and partial canopy treatments. These treatments were performed in the research of Prof. Deloire and Prof.

Vivier at the IWBT. The I also attempted to compare the results with the treatments performed as part of the ARC Winetech funded project (ww12/12 – Alternative pruning – D van Schalkwyk). DNA was extracted from fresh must as described in 1.2. PCR amplifications with labelled and unlabelled primers will be performed. Automated ribosomal intergenic spacer analysis (ARISA) analysis was employed to compare fungal community composition. Culture-dependent microbiological analysis was also performed on the must to identify culturable yeasts that were then be added to the culture collections at ARC-Nietvoorbj and IWBT.

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

Comparative analysis of the fungal diversity on grapes derived from biodynamic and conventional vineyards

(i) Investigating the spatial distribution of microbial communities within and between individual vineyards that employed different farming practices (biodynamic, conventional, integrated production of wine).

(ii) Evaluating whether the differences in the three vineyards were also evident in the yeast populations constituting the wine microbial consortium and how differences in the initial yeast population composition and concentration would influence the fermentation kinetics

Our data demonstrated that farming systems have a significant impact on fungal diversity but more importantly that there is significant species heterogeneity between samples in the same vineyard. This suggested that the frequently reported heterogeneity of tank samples of grapes harvested from single vineyards at the same stage of ripeness might therefore, at least in part, be due to the differing microbiota in different sections of the vineyard. In addition, the fermentation data highlighted two parameters, initial cell concentration and yeast community composition as important fermentation drivers, and open the possibility to predict fermentation behaviour based on the initial composition of the yeast community.

This study highlighted the need for a rapid and reliable culture-independent method that can be used as a tool to determine the initial yeast population in must as this would help inform winemakers regarding the community composition, its potential influence on the fermentation, especially in cases where the winemakers might want to rely on natural fermentation.

This part of the project generated two manuscripts, trained two interns from Cape Peninsula University of Technology, graduated one BScHons student and 1 MSc student.

In-depth analysis of the functional diversity of grape must obtained from conventionally and biodynamically produced grapes

This milestone was divided into two tasks:

(i) Evaluating microbial diversity in wine using automated ribosomal intergenic spacer analysis and culture-based methods

(ii) Assessing the functional potential of the wine microbiome using culture-based methods and metagenomics

The study investigated the microbial diversity present in grape juice and in the early stage of alcoholic fermentation of Cabernet sauvignon from the biodynamic vineyard in three consecutive harvests (2012, 2013 and 2014). Although our data demonstrated the suitability of ARISA for studying microbial diversity and dynamics in grape must and during wine fermentation, it also revealed that a reliable database would be required in order to accurately identify the microorganisms present in must. Such a database has now been constructed and will be used during the 2015 harvest to determine yeast diversity in different grape must samples.

In order to tap into the broader microbial diversity in grape must, previously uncharacterized yeast isolates were screened for various activities including β-glucosidases, β-1,4-glucanases, β-1,3-1,6-glucanases, chitinases, proteases, and inhibitory activity against *Brettanomyces bruxellensis*. One yeast species was found to inhibit *B. bruxellensis* probably through the production of biosurfactants. However, this needs further investigation. In addition, several yeast species including *Pichia burtonii*, *Hyphopichia pseudoburtonii* and *Candida oleophila* were shown to secrete glucanases.

The distribution of various enzyme encoding genes in the wine metagenome was evaluated through screening Fosmid libraries and also direct sequencing. A few clones displaying β-glucosidase activity were identified. Most importantly, the direct sequencing data revealed the high frequency of glucosidases and glucanases of fungal origin. The data demonstrated that a metagenomic approach will be the best approach to retrieve various biocatalysts from the must. In addition, metagenomic data revealed the presence of several fungal grapevine endophytes including yeasts known to play an important role in plant protection clearly highlighting that if better sequence depth is achieved, pivotal information regarding microbial community structure in grapevine and the functional role of plant pathogens and protectants could be unearthed.

This part of the project graduated 1 BScHons and 1 PhD student. In addition, 1 manuscript has been published and two are in preparation.

Analysis of microbial population diversity on grapes produced under full canopy and exposed bunches

This part of the project evaluated the impact of canopy treatment on yeast diversity associated with grapes. Our data based on culture-dependent method showed that exposed and shaded grapes exhibited similar yeast populations. However, the exposed bunches showed a higher incidence of *Rhodotorula* spp. and *Cryptococcus* spp. This preliminary data suggested that canopy treatment has an impact on microbial diversity. Consequently, culture-independent metagenomic approaches are employed to get a broad overview of the fungal and bacterial communities. This study is still on-going.

7. COMPLETE THE FOLLOWING TABLE

Milestone	Target Date	Extension Date	Date completed	Achievement
1. Comparative analysis of the fungal diversity on grapes derived from biodynamic and conventional vineyards	2012		2012	One manuscript published, two and one final year intern trained
2. In-depth analysis of the functional diversity of grape must obtained from conventionally and biodynamically produced grapes	2014		2014	One MSc and BScHons student graduated I record time
3. Analysis of microbial population diversity on grapes produced under full canopy and exposed bunches	2014		Incomplete	
5. Journal publication(s) – final milestone	2014		2015	The project generated 4 manuscripts within the first two milestones.

8. CONCLUSIONS

This project reached most of the targets set out in the initial proposal although one milestone remains incomplete. It is our intention to complete this milestone under a different funding stream. We believe that

all the remaining tasks e.g. submitting a popular article will be finalised before the end of the year 2015. This project has generated valuable information that can be useful to the South African wine industry and will continue to contribute positively towards knowledge generation. Some aspects of the project, e.g. Metatranscriptomic analysis of the wine microbiome had to be removed from the project due to challenges regarding method optimization. This approach will be revisited in future once protocols are well established.

9. ACCUMULATED OUTPUTS

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

The project generated 4 manuscripts and graduated 1 PhD student, 1 MSc student, 2 BScHons students as well as 1 final year intern from Cape Peninsula University of Technology. In addition, the findings of this research were presented at 1 international conference and two national conferences.

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 not yet delivered any products.

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 data generated from metagenomic assessment of the microbial diversity associated with grapes under various farming practices and pruning strategies is currently being analysed to compare the diversity with that observed on grapes in other countries. The aim is to generate an inventory of microbiota that perhaps represent the microbial terroir of South African grapes and also to highlight any potential plant pathogens that may otherwise not be detected in common culture dependent methods. Once this analysis is complete, the key findings of the current project will be put together in one report for publication in a popular wine industry magazine.

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					
Horatio Herbert Morgan	South African	BScHons	Completed	Dec/2014	
Caryn Hobbs	South African	BScHons	Completed	Dec/2013	

MSc students					
Bahar Bagheri	Iranian	MSc	Completed	March 2014	
PhD Students					
Soumya Ghosh	Indian	PhD	Completed	March 2015	
Postdocs					
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***

- Setati M.E., Jacobson D, Bauer F.F. (2015) Sequence-based analysis of the Vitis vinifera L. cy Cabernet sauvignon in three neighbouring South African vineyards. Frontiers in microbiology. *Submitted*
- Ghosh S., Bagheri B., Morgan H.H., Divol B., Setati M.E. (2015) Assessment of microbial diversity in wine using ARISA. Ann. Microbiol. *Accepted.*
- Bagheri B., Bauer B., Setati M.E. (2015) The diversity and dynamics of indigenous yeast communities in grape must from vineyards employing different agronomic practices and their influence on wine fermentation. S. Afr. J Enol. Vitic. *Accepted.*
- Setati M.E., Jacobson D., Andong U-C., Bauer F.F. (2012) The vineyard yeast microbiome, a mixed-model map. **PLoS ONE,** 7(12):e52609. Doi:10.1371/journal.pone.0052609.

e) PRESENTATIONS/PAPERS DELIVERED
Please list using the format illustrated in the example below.

- Setati, ME, Bagheri B, Bauer FF (2013) The wine microbial consortium and its evolution during spontaneous fermentation. SASM 2013 From Africa to the World: Trending Microbiology, Bela-Bela, South Africa. (24 – 27 November 2013). ***Key note***

- Ghosh, S., Divol, B. & Setati, M.E. 2013. Using metagenomics to explore the functional potential of the wine microbiome. SASM 2013 From Africa to the World: Trending Microbiology, Bela-Bela, South Africa. (24 – 27 November 2013). ***Poster***
- Setati, M.E. 2013. Viticulture and microbial biodiversity – the vineyard as a complex and patchy ecosystem. 35th SASEV/WINETECH International Conference, Lord Charles Hotel, Somerset-West, South Africa. (13-15 November 2013). ***Oral***
- Setati ME, Bagheri B, Ghosh S, Divol B, Bauer FF (2013) Taxonomic and functional diversity of the grape must ecosystem. Funtional Metagenomics. Pretoria, South Africa 2 – 5 June. ***Oral***
- Ursula Andong, Soumya Ghosh, Evodia Setati, Dan Jacobson, Florian Bauer (2011) Structural diversity of yeast associated with grapes in biodynamically and conventionally managed vineyards. SASM Conference, Cape Town. ***Oral***

10. BUDGET