Industry allocated project number	PHI allocated project number

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FINAL REPORT
(2015)

1. PROGRAMME AND PROJECT LEADER INFORMATION

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2. PROJECT INFORMATION

Research Organisation Project number	IWBT Y11-01
Project title	Investigating the impact of non-*Saccharomyces* yeasts on wine composition – focus on extracellular enzymes of oenological interest
Short title	

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

Key words	Non-*Saccharomyces* yeasts, enzymes, pectinases, glycosidases, proteases

Approved by Research Organisation Programme leader (tick box) ☐

3. EXECUTIVE SUMMARY

Over the past two decades, research has shown that certain non-*Saccharomyces* yeasts can be co-inoculated with *S. cerevisiae* in order to enhance the aromatic complexity of wines. Part of the contribution these non-*Saccharomyces* yeasts has been attributed to the secretion of extracellular enzymes (e.g. pectinases, glycosydases and proteases) that could break down certain grape compounds such as pectin, glycosylated aroma compounds and haze-forming proteins, respectively. In this study, a large collection of non-*Saccharomyces* yeasts was screened for these enzyme activities. The results show that pectinase activity is only found in *Kluyveromyces marxianus*. β-glucosidase activity was much more common and protease activity was the strongest in *Metschnikowia pulcherrima* and *Zygoascus meyerae*. Large strain variation was identified. The identification of extracellular proteins at the end of fermentation revealed that the main oenologically relevant enzymes present were glucanases and β-glucosidases. Novel protease genes from *M. pulcherrima* and *Z. meyerase* were retrieved. Their expression and the impact of their corresponding enzymes are currently under investigation in a follow-up project (IWBT W14/01). In conclusion, this study confirmed that non-*Saccharomyces* yeasts secrete a range of extracellular enzymes that could be of oenological interest. We also retrieved novel genetic genes encoding these enzymes. However, the actual secretion of these enzymes could only be partially confirmed under fermentative conditions and the actual activity and impact of these enzymes was not investigated. In future projects, we therefore propose to perform fermentations with pure and mixed yeast cultures and monitor the secretion and extracellular enzyme activity on wine substrates in order to confirm the relevance of these enzymes from an oenological perspective. In particular, the oenological potential of *K. marxianus*, *M. pulcherrima* and *Z. meyerae* should be investigated.

4. PROBLEM IDENTIFICATION AND OBJECTIVES

The inoculation of non-*Saccharomyces* yeasts together with *Saccharomyces cerevisiae* provides an opportunity to winemakers to create distinct wine styles. Non-*Saccharomyces* yeasts display divergent intracellular metabolisms that significantly contribute to these alterations, however, such activities alone cannot explain all the differences observed from the fermentations carried out by pure cultures of *S. cerevisiae*. The secretion of hydrolytic enzymes (e.g. proteases, glycosidases and pectinases) is known to be much stronger in non-*Saccharomyces* yeasts than in *S. cerevisiae*. This trait has been reported repeatedly in literature and identified as one of the main contributions of non-*Saccharomyces* yeasts. The aim of the current study was to screen a large collection of non-*Saccharomyces* yeasts for pectinase, β-glucosidase and protease activity all at pH 3.5 and to test the actual secretion of hydrolytic enzymes during fermentation of synthetic grape juice. Isolating the genetic sequences of these enzymes was also attempted in order to characterise them further and to evaluate their global impact on wine in the future.

Objectives of the study:

MILESTONE 1: Selection of non-*Saccharomyces* yeasts to be used in this study based on their phenotypes

MILESTONE 2: Optimisation of protein extraction from grape juice/wine and visualisation of proteins and enzyme activities

MILESTONE 3: Retrieving the genes encoding the enzyme of interest

MILESTONE: Gene expression study

5. WORKPLAN (MATERIALS AND METHODS)

MILESTONE 1: Selection of non-*Saccharomyces* yeasts to be used in this study based on their phenotypes

Task 1: Detection of extracellular enzyme activity on plates (semi-quantitative assays).

Task 2: Characterisation of the enzymes identified in Task 1: determination of optimal pH and temperature, influence of the medium composition (e.g. carbon and nitrogen sources), etc. Liquid medium will be used (quantitative assays). Substrate specifity will also be tested. The same assays will be conducted on both collections (IWBT and ARC-Nietvoorbij) using standardised protocols.

MILESTONE 2: Optimisation of protein extraction from wine and visualisation of proteins and enzyme activities

Task 1: optimisation of protein extraction from synthetic grape juice/wine and from real grape juice/wine

Task 2: Alcoholic fermentation will be conducted after inoculation of sterilised grape juice with either a pure *S. cerevisiae* culture, or a pure non-*Saccharomyces* culture (as selected in Milestone 1) or a mixed culture (different ratios will be considered). Populations will be monitored by plate cell counts throughout alcoholic fermentation.
In parallel, total proteins will be extracted from the fermenting juice and 1D- and 2D protein gel electrophoreses will be used to visualise extracellular proteins.
The workload in this task will be shared between IWBT and ARC-Nietvoorbij (except for protein gel electrophoreses which will be carried out at the IWBT).

Task 3: Zymography will be used on the 2D gel strips in order to evaluate the pI of the proteins of interest and on the 1D and 2D gels in order to identify the bands/spots corresponding to proteins of interests.

Task 4: the protein bands/spots will be excised from the gels and sent for identification (trypsin digestion or N-terminal sequencing) to external facilities in an attempt to confirm the identification and possibly retrieve partial sequences of these gels.

MILESTONE 3: Retrieving the genes encoding the enzyme of interest

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 the non-*Saccharomyces* wine yeast selected in Milestone 1.
- 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 extracted 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.

Note: the genome of *L. thermotolerans* has been recently sequenced and made available online. This genome will be directly mined for enzymes of interest.

MILESTONE 4: Gene expression study

If milestone 3 is successful, genetic studies will be within reach and would allow furthering the characterization of these genes and their respective enzyme activities.

Task 1: The genes will be heterologously expressed in *Saccharomyces cerevisiae* in order to verify their identity.

Task 2: The mutants generated in task 1 will also be used to characterise the activity of the enzymes. These results will be correlated to those generated in Milestone 1.

Task 3: Primers will be designed to use quantitative real-time PCR in order to investigate the expression of the genes in their native host in order to assess whether the genes are expressed under winemaking conditions and when. These results will be correlated to those generated in Milestone 2.

6. RESULTS AND DISCUSSION

MILESTONE 1: Selection of non-*Saccharomyces* yeasts to be used in this study based on their phenotypes

A large collection of non-*Saccharomyces* yeasts were screened for hydrolytic activities of oenological interest: β-glucosidase, pectinase and protease (Table 1). This was carried out by spotting the different yeasts on a solid medium containing substrates related to the activity investigated. The results show that the secretion of these enzymes is strongly strain-dependent. At times, the results were also substrate-dependent (data not shown). This was not particularly surprising since the nature of the substrate may impact the activity of enzymes in general. Moreover, the substrates tested in these assays are not found in grape juice/wine and substrate specificity might limit the activity of these enzymes against wine compounds. It must also be stressed that these enzymes are primarily involved in the construction, maintenance and remodelling of the yeast cell walls and not specifically designed to be active against wine relevant substrates. Their potential activity against the latter compounds is somewhat fortuitous. Nevertheless, the assays show whether or not the yeasts secrete the sought enzymes. Overall, the results showed that pectinase activity is rarely encountered amongst wine yeasts and was only found in *Klyveromyces marxianus* in this study. β-Glucosidase and protease activities are more frequent, but strong protease activity was only found in *Metschnikowia pulcherrima* and *Zygoascus meyerae* strains.

Table 1: Screening of non-*Saccharomyces* yeasts isolated from grape juice or wine for pectinase, glycosidase and protease activity (+: activity, -: no activity, v: strain dependent)

Yeast species	β-Glucosidase	Pectinase	Protease
*Brettanomyces anomalus**	-	-	-
Brettanomyces bruxellensis	-	-	-

*Candida asparagi**	+	-	+
*Candida azyma**	+	-	+
*Candida azymoides**	-	-	+
*Candida cantarelli**	-	-	-
*Candida intermedia**	-	-	-
Candida lusitaniae	-	-	+
Cryptococcus flavescens	+	-	-
*Filobasidium capsuligenum**	-	-	-
Hanseniaspora guilliermondii	-	-	+
Hanseniaspora opuntiae	v	-	v
Hanseniaspora uvarum	v	-	-
Hanseniaspora vinae	v	-	-
Issatchenkia terricola	-	-	+
Kazachstania aerobia	-	-	+
*Kluyveromyces marxianus**	-	+	-
Lachancea thermotolerans	v	-	-
*Metschnikowia chrysoperlae**	-	-	+
Metschnikowia fructicola	-	-	+
Metschnikowia pulcherrima	v	-	v
*Pichia caribbica**	+	-	+
*Pichia manshurica**	-	-	-
Phaemoniella prunicola	-	-	-
Rhodotorula mucilaginosa	+	-	+
Starmerella bacillaris	-	-	-
*Wickerhamomyces anomalus**	+	-	+
Zygoascus meyerae	-	-	+

* Only one strain tested

MILESTONE 2: Optimisation of protein extraction from wine and visualisation of proteins and enzyme activities

In order to investigate whether the enzymes identified above are actually secreted under winemaking conditions, fermentations were carried out in synthetic grape juice using pure cultures of *S. cerevisiae*, *M. pulcherrima* and *L. thermotolerans* as well as mixed cultures of *S. cerevisiae* with the individual non-*Saccharomyces* yeasts. The results show that, overall, the yeasts secreted a wide range of proteins, mostly including extracellular (cell wall related) enzymes, but also certain intracellular enzymes. The latter included enzymes involved in sugar metabolism (glycolysis) and it is not known whether they were released upon autolysis or if the yeasts secreted them actively. Amongst the extracellular enzymes identified, *M. pulcherrima* was found to secrete a variety of mannoproteins including several β-glucosidases and β-glucanases which could be of oenological interest. Unfortunately, no protease could be found amongst the secreted proteins probably because the *M. pulcherrima* strain used in the fermentation experiment differed from the one shown to have strong protease activity and whose protease-encoding gene had been retrieved as described above. In addition, this *M. pulcherrima* strain did not persist long in fermentation and the medium used was not real grape

must and therefore lacked the appropriate substrates that would typically induce protease activity in the yeast. Further investigations are currently on-going to identify the conditions under which *M. pulcherrima* secretes this protease. The data also revealed possible existence of different types of interactions between yeasts when two species are co-inoculated at high cell concentrations. These should be further investigated in order to maximise the positive activities of non-*Saccharomyces* yeasts while retaining fermentation reliability.

MILESTONE 3: Retrieving the genes encoding the enzyme of interest

From the screening above, the main enzyme activities secreted by non-Saccharomyces yeasts were glucosidase and protease. Pectinase was only identified in Kluyveromyces marxianus. Although it could be interesting to test the impact of a co-inoculation of K. marxianus and S. cerevisiae on wine composition, the genes encoding pectinases in K. marxianus are already known. For Milestones 3 and 4, it was therefore decided to focus on the non-*Saccharomyces* yeast strains secreting the strongest glucosidase and protease activities.
Very strong activity was also identified in *Schwanniomyces polymorphus* var. *africanus* and *Schwanniomyces pseudopolymorphus*, although these yeasts were not isolated from wine. Using molecular biology techniques, we attempted to retrieve the gene(s) encoding the extracellular β-glucosidase(s) of *S. polymorphus* var. *africanus*, but unfortunately, we were not successful. With genome sequencing becoming more affordable, we suggest that this route be taken to retrieve these genes in order to evaluate their relevance for winemaking.
Extracellular protease activity is not very common in yeasts, but we observed it in a few species. Those displaying the strongest activity were *Metschnikowia pulcherrima* and *Zygoascus meyerae*. Hanseniaspora opuntiae also exhibited some protease activity but much weaker than the former two species. We successfully isolated genes encoding extracellular acid proteases in these three species and they seem to be active against haze-forming grape proteins. Moreover, their activity against grape must proteins in general could liberate assimilable nitrogen in the form of amino acids and alter the overall aroma profiles of the wine. Full characterisation and assessment of their activity in grape juice is currently underway at the Institute for Wine Biotechnology in collaboration with the University of Bordeaux, France (Winetech project IWBT W14/01).

MILESTONE 4: Gene expression study

As Milestone 3 was only partially successful, Milestone 4 could not be fully achived. In a follow-up project, it would be of interest to perform fermentations with co-cultures of *K. marxianus* and *S. cerevisiae* and monitor the expression of the pectinase-encoding genes.
As for the protease genes of *M. pulcherrima* and *Z. meyerae*, the 2 species exhibiting the strongest protease activity, their will be monitored as part of the Winetech project IWBT W14/01.

7. COMPLETE THE FOLLOWING TABLE

Milestone	Target Date	Extension Date	Date completed	Achievement
1. Selection of non-*Saccharomyces* yeasts to be used in this study based on their phenotypes	2013	n/a	2013	A large collection of non-Saccharomyces was screened for pectinase, β-glucosidase and protease activities. The isolates exhibiting the strongest activities were selected.
2. Optimisation of protein extraction	2013	n/a	2013	Fermentations were performed with *L.*

from grape juice/wine and visualisation of proteins and enzyme activities				*thermotolerans* and *M. pulcherrima* in pure and mixed culture (with *S. cerevisiae*) situations. Extracellular proteins were identified at the end of fermentation. The main oenologically relevant enzymes secreted were glucanases and β-glucosidases. The results also show the existence of interactions between the yeast species.
3. Retrieving the genes encoding the enzyme of interest	2014	n/a	2014	Although we could not retrieve glycosydase genes, protease genes were retrieved from 3 different non-*Saccharomyces* species.
4. Gene expression study	2014	n/a	Still on-going.	This milestone was not fully achieved, but it will be performed shortly.
5. Journal publication(s) – final milestone	continuous	continuous	continuous	2 scientific articles and 1 popular article were written.

8. CONCLUSIONS

This study confirmed that non-*Saccharomyces* yeasts secrete hydrolytic enzymes of oenological interest. Pectinase activity is rare but *K. marxianus* displays a strong activity that could potentially be exploited. β-Glucosidase activity is fairly common and the corresponding enzymes are indeed secreted during fermentation when selected non-*Saccharomyces* yeasts are co-inoculated with *S. cerevisiae*. Fermentations using real grape must will now be performed to assess the activity on aroma precursors. Strong protease activity was identified in *M. pulcherrima* and *Z. meyerae* and the genes encoding the relevant enzymes have been isolated. Further investigations are currently on-going in our environment to identify the best way to exploit this interesting enzyme activity. Overall, these exciting results are a stepping stone towards a better understanding of the contribution of non-*Saccharomyces* yeasts in wine. The use of carefully selected yeasts could provide an easy-to-use tool to address various technological issues as well as to modulate the aromatic profile of wines.

9. ACCUMULATED OUTPUTS
a) TECHNOLOGY DEVELOPED, PRODUCTS AND PATENTS
b) SUGGESTIONS FOR TECHNOLOGY TRANSFER

c) HUMAN RESOURCES DEVELOPMENT/TRAINING

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					
Jana Meyer	South African		Honours	Dec 2014	
Caryn Hobbs	South African		Honours	Dec 2013	
Maryke Korsten	South African		Honours	Dec 2012	
Masters Students					
Louwrens Theron	South African		MSc	Dec 2013	
Talitha Mostert	South African		MSc	March 2013	
PhD students					
Postdocs					
Support Personnel					
Anscha Zietsman	South African			n/a	

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

Reid VJ, Theron LW, du Toit M, Divol B. (2012) Identification and partial characterization of extracellular aspartic protease genes from *Metschnikowia pulcherrima* IWBT Y1123 and Candida apicola IWBT Y1384. *Applied and Environmental Microbiology* 78, 6838–6849.

Mostert TT, Divol B (2014) Investigating the yeast secretome in fermentation. *International Journal of Food Microbiology* 171, 108-118.

Divol B, Setati ME (2015) Secretion of hydrolytic enzymes by non-*Saccharomyces* yeasts: a relevant trait for winemaking? Submitted for publication in *Wineland*

e) PRESENTATIONS/PAPERS DELIVERED

Mostert TT, Divol B (2014) Yeast coinoculation and release of proteins: a proteomic approach. Poster presented at the Third edition of the international conference series on wine active compounds at Beaune, March 2014.

Theron LW, Zietsman JJ, Divol B (2013) Optimizing recombinant expression and purification of an aspartic protease from *Metschnikowia pulcherrima*. Oral presentation at the 18th Biennial Conference of the South African Society of Microbiology (SASM 2013) at Bela-Bela, November 2013.

Theron LW, Zietsman JJ, Divol B. (2013) Utilisation of acid protease produced by non-*Saccharomyces* yeast to prevent haze and release assimilable nitrogen. Oral presentation at the 35th conference of the South African Society of Enology and Viticulture at Somerset West, November 2013.