THE UNIVERSITY OF ADELAIDE
AUSTRALIA

A dissertation submitted for the degree of
Doctor of Philosophy

Identification Of Yeast Genes Enabling Efficient Oenological Fermentation Under Nitrogen-Limited Conditions

Josephine Jasmine Peter
M. Sc.

Department of Wine and Food Science
Faculty of Sciences
The University of Adelaide

August 2017

Table of Contents

Declaration 4

Acknowledgement 5

Abstract 6

List of abbreviations 8

CHAPTER 1 Literature review and summary of research aims 10

1. Introduction 11

1.1 Yeast and winemaking 11

1.2 Importance of nitrogen during alcoholic fermentation 13

1.3 Adverse effects of limited nitrogen 14

1.4 Nitrogen supplementation and side effects 14

1.5 Yeast nitrogen transport and metabolism 15

1.5.1 Nitrogen Catabolite Repression (NCR) 17

1.5.2 Plasma membrane Ssy1-Ptr3-Ssy5 (SPS) sensor 18

1.5.3 Nitrogen metabolism 19

1.6 Improvement of industrial wine yeast strains 20

1.7 Using a gene deletion library as a tool to study gene function 21

1.7.1 Previous studies on deletion libraries under fermentation conditions 21

1.8 Limitations of current laboratory yeast deletion libraries 22

1.8.1 Requirement for prototrophy and the available prototrophic libraries 22

1.9 Nitrogen efficient genes and strains of *Saccharomyces cerevisiae* 23

1.10 Bioinformatics as tools in determining gene function 24

1.11 Aims and objectives of the project 25

CHAPTER 2 Identification of yeast genes related to fermentation efficiency in limited and sufficient nitrogen 27

2.1 Introduction 28

2.2 Materials and methods 30

2.3 Results 33

2.4 Discussion 45

2.5 Conclusion 52

CHAPTER 3 Use of wine yeast deletion collection reveals genes that influence fermentation performance under low nitrogen conditions 53

3.1 Abstract 57

3.2 Introduction 58

3.3 Material and methods 59

3.4 Results 64

3.5 Discussion 73

3.6 Conclusion 78

CHAPTER 4 Investigstion of the effect of an *MFA2* deletant on fermentation duration 80

4.1 Abstract 84

4.2 Introduction 85

4.3 Materials and methods 86

4.4 Results 89

4.5 Discussion 93

CHAPTER 5 General discussion, conclusions and future directions 99

5.1 General discussion and conclusions 100

5.1.1 Functional genomic tools identify key genes influencing fermentation in sufficient and limiting nitrogen conditions 100

5.1.2. Screening of the wine yeast deletion library for fermentation efficient mutants in limiting nitrogen 101

5.1.3 Investigation of an *MFA2* deletion in AWRI1631 102

5.1.4 Fermentation performance of *mfa2*Δ is dependent on strain background, mating type and gene dosage 102

5.2 Future directions 103

Appendices 107

Appendix 1 ..... **108**

Appendix 2 ..... **149**

Appendix 3 ..... **163**

Appendix 4 ..... **177**

Appendix 5 ..... **184**

Appendix 6 ..... **187**

Appendix 7 ..... **189**

Appendix 8 ..... **190**

Appendix 9 ..... **191**

Appendix 10 ..... **192**

Appendix 11 ..... **194**

Appendix 12 ..... **195**

References **196**

Declaration

I certify that this work contains no materials which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without prior approval of The University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

I acknowledge that copyright of published works contained within this thesis resides with the copyright holders of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Josephine Jasmine Peter

August 2017.

Acknowledgement

First of all, I am grateful to the almighty God for enabling me to complete this study. I would like to express my sincere gratitude to my principal supervisor Prof. Vladimir Jiranek for his continuous support throughout my Ph.D. study. The guidance helped me during my research and writing this thesis. I would like to thank Vlad for believing in my potential and helping me to identify them, and to succeed. I could not have imagined having a better advisor and mentor for my Ph.D. study. Besides my principal supervisor, I would like to thank the rest of my supervisory team. Dr. Tommaso L Watson for his guidance mainly during the initial days of my Ph.D. and screening exercises. Dr. Michelle Walker for her insightful comments, support, coffee breaks and constant encouragement, thank you for being so patient with me and Dr. Jennie Gardner, for all her timely advice and inputs to make this project a successful one.

My sincere thanks go to Associate Prof. Paul Grbin, who wished me well and continuously supported me at various times during my study. I would like to thank my present and past fellow lab mates for all their help, especially Jo, Krista, Renata, Fede, Nick, Tom, Pat, Louise, Ana, Ee Lin, Jiao, Jin, Max, Cris, Mandy, Dan, Greg, Jin-Chen and Simon. Also, I thank my friends from level 4 (especially, Joanna Gambetta and Hanru) and AWRI for all their support. A special thanks to Sandra from school for being a kind friend and helping me with children during the final stages of my Ph.D.

I would like to thank my better half - my husband Vinodh Nair, without whom this Ph.D. study would have been impossible. Thanks for encouraging, supporting and believing in me and above all, wishing me to achieve a Ph.D. more than what I did. I am grateful to my mum Mrs. Felsi Peter and my dad Mr. Peter, for this life, love and wishing me to successfully complete this study. I extend my gratitude to my mum-in-law Mrs. Sarojini Sukumar and dad-in-law Mr.Sukumar Nair, for their love and support. I would like to thank my brother Benjamin Peter and my sister Ferdine Peter, for being there when needed. Finally, I would like to thank my beautiful girls, Laya Vinodh and Toshi Vinodh for all the sacrifices they have made in the last four years for me to achieve this thesis. Thanks for all the love, care, laughter and fun which has made this journey as easy one. Love you both.

Abstract

Nitrogen deficiency can often lead to slow or sluggish fermentation, resulting in wine out of specification and at risk of oxidation and microbial contamination. Problems due to nitrogen deficiency can be rectified by optimising grape chemistry (through vineyard fertilization), or more commonly supplementing the fermentation with ammonium salts. An alternative is to use wine yeast that can utilize nitrogen efficiently and complete fermentation more reliably. However, to develop 'nitrogen efficient' yeast, it is important to understand how such yeast can utilize nitrogen effectively by identifying genes that influence fermentation performance over a range of nitrogen concentrations. Past research related to the identification of genes influencing nitrogen efficiency under fermentative conditions is largely confined to laboratory yeast.

Investigation of the ~5,000 non-essential genes in yeast is possible through research tools such as deletion libraries (collections of strains, each with a single gene deletion). Several genome-wide studies have successfully used deletion libraries in the auxotrophic background of laboratory yeast to investigate phenotypes in response to exposure to single stress factors associated with fermentation. However, the need to supplement with amino acids to overcome auxotrophies makes quantitative physiological studies in nitrogen limiting conditions impractical. Therefore, in this study, we have used a prototrophic deletion collection in both laboratory and wine yeast backgrounds to identify genes influencing fermentation performance.

Screening (micro-fermentation; 600 µL) of the prototrophic laboratory yeast deletion library (BY4741; 5,372 deletants) and the partial wine yeast library (AWRI1631; 1,844 deletants) for growth and consumption of sugar and nitrogen under limiting (75 mg FAN L^{-1}) and non-limiting nitrogen (450 mg FAN L^{-1}) conditions identified deletants with improved fermentation. To better understand the role of individual genes in fermentation, candidate gene sets from each screen were compared to each other and to other published data sets from genome wide transcriptomic analyses related to fermentation.

Wine yeast deletants that enabled shortened micro-fermentation duration in low nitrogen conditions were further investigated, since the experiment best represented nitrogen deficient grape must associated with problematic fermentation. Fifteen deletants completed fermentation quicker than the wildtype (*c.a.* a 15-59% time reduction) when tested in larger (100 mL) fermentations. This group of genes were annotated to biological processes including protein modification, transport, metabolism and ubiquitination (*UBC13, MMS2, UBP7, UBI4, BRO1, TPK2, EAR1, MRP17, MFA2 and MVB12*), signalling *(MFA2)* and amino acid metabolism (*AAT2*). Among

the genes identified, *MFA2* (mating a-factor), which conferred a 34% decrease in fermentation duration, was further investigated. We were interested to understand how deletion of this mating type gene affected fermentation since a link between these metabolic pathways would be novel.

The 15 strains identified in this study, which were fermentation proficient in a 'wine-like', limited nitrogen condition, provide a basis to better understand how yeast adapt to nitrogen limitation during fermentation. Furthermore, the corresponding genes can be targeted in second generation strain improvement programs, using tools such as CRISPR (yet to be approved by relevant regulatory bodies) to generate nitrogen efficient yeast to reduce the need to supplement low nitrogen fermentations.

List of abbreviations

Abbreviation	Full term
Δ	Gene deletion
°C	Degree celsius
3'	Three prime of the nucleic acid sequence
5'	Five prime of the nucleic acid sequence
AAPs	Amino Acid Permeases
APO	Ascomycete Phenotype Ontology
ATP	Adenosine Triposphate
AUC	Area Under the Curve
Bp	Base pair
CDGJM	Chemically Defined Grape Juice Medium
cDNA	Complementary deoxyribonuclease acid
CO_2	Carbon dioxide
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DAP	Diammonium Phosphate
DNA	Deoxyribonucleic Acid
E	Environment
FAN	Free Amino Nitrogen
FD	Fermentation Duration
G	Genotype
GE	Genotype and environment
GM	Genetically Modified
GO	Gene Ontology
GS	Glutamate Synthetase
HMR	Hidden MAT right
HN	High Nitrogen
HOG	High Osmolarity Glycerol
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LN	Low Nitrogen
LYDL	Laboratory Yeast Deletion Library
MAPK	Mitogen Activated Protein Kinase
N	Nitrogen
NCR	Nitrogen Catabolite Repression
NOPA	o-phthaldialdehyde/N-acetyl-L-cysteine
NREL	Normalized Relative Expression Level
OD	Optical Density
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PM	Plasma Memebrane

qPCR	Quantitative Polymerase Chain Reaction
QTL	Quantitative Trait Loci
RNA	Ribonucleic Acid
RNA-Seq	RNA sequencing
SD	Standard Deviation
SGD	*Saccharomyces* Genome Database
SNPs	Single Nucleotide Polymorphisms
SPS	Ssy1-Ptr3-Ssy5
TCA	Tricarboxylic Acid Cycle
WYDL	Wine Yeast Deletion Library
YAN	Yeast Assimilable Nitrogen
YPD	Yeast Extract Peptone Dextrose