Biochemical and Molecular Characterisation of Oenologically Important Enzymes Identified in Lactic Acid Bacteria

Angela H. Matthews

A thesis submitted for the degree of Doctor of Philosophy in the School of Agriculture, Food and Wine Faculty of Science The University of Adelaide

November 2007
# TABLE OF CONTENTS

| ABSTRACT | .................................................................................................................. 1 |
| DECLARATION OF AUTHORSHIP | .............................................................................................................. 3 |
| ACKNOWLEDGEMENTS | ........................................................................................................ 4 |

## CHAPTER 1  LITERATURE REVIEW

1.1 Introduction ........................................................................................................... 5
1.2 The Malolactic Enzyme ......................................................................................... 7
1.3 Proteolytic and Peptidolytic Enzymes ................................................................. 8
1.4 Glycosidases .......................................................................................................... 23
1.5 Polysaccharide-Degradning Enzymes ..................................................................... 26
1.6 Esterases .................................................................................................................. 28
1.7 Ureases .................................................................................................................... 35
1.8 Phenoloxidases ....................................................................................................... 35
1.9 Lipases ...................................................................................................................... 37
1.10 Conclusion .............................................................................................................. 42

## CHAPTER 2  A SURVEY OF LACTIC ACID BACTERIA FOR ENZYMES OF INTEREST TO OENOLOGY

2.1 Introduction ............................................................................................................. 43
2.2 Materials and Methods ............................................................................................. 45
  2.2.1 LAB strains .................................................................................................... 45
  2.2.2 Preculturing conditions ................................................................................... 45
  2.2.3 Biomass preparation ......................................................................................... 45
  2.2.4 Replication ....................................................................................................... 45
  2.2.5 Esterase activity ............................................................................................... 47
  2.2.6 Lipase activity ................................................................................................... 47
  2.2.7 Tannase activity ............................................................................................... 47
  2.2.8 Activity of polysaccharide-degrading enzymes ............................................... 48
  2.2.9 Cellulases ......................................................................................................... 48
  2.2.10 β-Glucanases ................................................................................................. 48
  2.2.11 Xylanases ....................................................................................................... 49
2.3 Results ..................................................................................................................... 50
  2.3.1 Esterases ......................................................................................................... 50
  2.3.2 Lipases ............................................................................................................. 50
  2.3.3 Tannases ......................................................................................................... 50
  2.3.4 Polysaccharide-degrading activities ................................................................. 53
2.4 Discussion ................................................................................................................ 55
2.5 Conclusions ............................................................................................................ 60
CHAPTER 3  
BIOCHEMICAL CHARACTERISATION OF THE ESTERASE ACTIVITIES OF LACTIC ACID BACTERIA

3.1 Introduction .......................................................... 61
3.2 Materials and Methods .............................................. 64
   3.2.1 Bacterial strains used in this study .......................... 64
   3.2.2 Preculturing conditions and preparation of biomass ........ 64
   3.2.3 Determination of esterase activity ............................ 65
   3.2.4 Influence of pH on esterase activity ......................... 65
   3.2.5 Influence of temperature on esterase activity ............... 66
   3.2.6 Influence of ethanol on esterase activity .................... 66
   3.2.7 Determination of ester substrate specificity ................. 66
   3.2.8 Statistical analysis ........................................... 66
3.3 Results ................................................................. 67
   3.3.1 Influence of pH on esterase activity ......................... 67
   3.3.2 Influence of temperature on esterase activity ............... 67
   3.3.3 The influence of ethanol on esterase activity ............... 70
   3.3.4 Substrate specificity of esterases ............................ 70
3.4 Discussion .............................................................. 73
3.5 Conclusions ............................................................ 76

CHAPTER 4  
INFLUENCE OF O. OENI ON WINE ESTERS DURING MALOLACTIC FERMENTATION AND THE ESTER-SYNTHESIS ACTIVITY OF O. OENI

4.1 Introduction .......................................................... 77
4.2 Materials and Methods .............................................. 86
   4.2.1 Bacterial strains used in this study .......................... 86
   4.2.2 Preparation of wine for MLF trials ........................... 86
   4.2.3 Preparation of bacteria and inoculation of wine .......... 87
   4.2.4 Conduct of malolactic fermentation ............................ 87
   4.2.5 Determination of ester concentrations in wine ............ 87
   4.2.6 Preculturing conditions of bacteria for ester- synthesis trials ............................................... 88
   4.2.7 Preparation of substrates for ester-synthesis trials ....... 88
   4.2.8 Extraction of esters for analysis ............................... 89
   4.2.9 Gas chromatography-flame ionisation detection of esters ........................................... 89
4.3 Results ................................................................. 90
   4.3.1 Changes in concentrations of esters during MLF .......... 90
   4.3.2 Ester-synthesis activity of O. oeni ........................... 91
4.4 Discussion ........................................................................... 93
4.5 Conclusions ......................................................................... 96

CHAPTER 5

MOLECULAR CHARACTERISATION OF PUTATIVE ESTERASE ENZYMES OF O. OENI

5.1 Introduction ......................................................................... 97
5.2 Preliminary Work - Identification of Putative Esterase Sequences in O. oeni .................................................. 104
5.3 Materials and Methods .......................................................... 106
  5.3.1 Bacterial strains .............................................................. 106
  5.3.2 Isolation of genomic DNA .............................................. 106
  5.3.3 Primer design and PCR conditions ............................... 106
  5.3.4 DNA electrophoresis ..................................................... 108
  5.3.5 Nucleotide sequencing .................................................. 108
5.4 Results and Discussion .......................................................... 110
5.5 Conclusions ........................................................................ 122

CHAPTER 6

OVEREXPRESSION OF PUTATIVE ESTERASE ENZYMES OF O. OENI IN E. COLI

6.1 Introduction ........................................................................ 123
6.2 Materials and Methods .......................................................... 125
  6.2.1 Preliminary cloning work ................................................ 125
  6.2.2 Bacterial strains ............................................................ 126
  6.2.3 Cloning vector .............................................................. 127
  6.2.4 Restriction enzyme digestion and dephosphorylation .... 128
  6.2.5 Ligation ......................................................................... 128
  6.2.6 Preparation of E. coli DH5α competent cells ................... 129
  6.2.7 Transformation of E. coli DH5α ..................................... 129
  6.2.8 Screening of transformants ............................................ 129
  6.2.9 Transformation of E. coli Rosetta2 (DE3) for overexpression .......................................................... 130
  6.2.10 Overexpression protocol .............................................. 130
  6.2.11 Preparation of total cell extracts ................................... 130
  6.2.12 SDS-PAGE analysis of the cell extracts ...................... 131
  6.2.13 Western blot ............................................................... 131
  6.2.14 Esterase assay of total cell extracts and whole cells following induction ........................................... 132
  6.2.15 Purification of the target proteins ................................... 132
  6.2.16 Thrombin treatment of the cell extracts ....................... 132
6.3 Results ........................................................................................................... 134

6.3.1 Preliminary cloning work ............................................................................ 134
6.3.2 Screening of pET-43.1b transformants ..................................................... 136
6.3.3 SDS-PAGE analysis of the cell extracts ...................................................... 139
6.3.4 Western blot ................................................................................................. 140
6.3.5 Esterase activity assay ............................................................................... 141
6.3.6 Purification of the target proteins ............................................................... 141
6.3.7 Thrombin treatment of the total cell extracts ............................................ 143

6.4 Discussion ...................................................................................................... 145

6.5 Conclusions .................................................................................................. 149

CHAPTER 7 GENERAL DISCUSSION AND FUTURE DIRECTIONS .................. 150
APPENDIX MEDIA, BUFFERS AND SOLUTIONS .............................................. 158
REFERENCES ..................................................................................................... 160
ABSTRACT

Enzyme concentrates are available for use in commercial wineries to aid in wine processing, or to enhance wine quality. However, pectolytic enzyme remains the sole product routinely used in most wineries. One disadvantage of some of the products currently available commercially is they contain enzymes sourced from microorganisms not usually associated with grape juice or wine, typically fungi such as Aspergillus species. As a result, enzymes are inefficient catalysts under the harsh oenological conditions. In addition, some products contain secondary, and potentially undesirable, contaminant enzyme activities. Clearly there is the potential to develop enzyme preparations specifically for use in grape juice and wine. A potential source of such enzymes are the lactic acid bacteria (LAB), the organisms more commonly associated with the conduct of the malolactic fermentation (MLF) during vinification. In this study, the production of cell-associated enzymes with potential oenological applications by LAB was investigated.

A screening of 50 LAB isolates for the production of lipases, esterases, tannases, and polysaccharide-degrading enzymes revealed wine LAB can produce enzymes of oenological importance. In general, activity towards polysaccharide substrates was more frequent among the lactobacilli and pediococci strains. Lipase activity was observed in three lactobacilli, and all strains were found to have tannase activity. Similarly, all strains displayed some esterase activity, although the activity was markedly stronger among the Oenococcus oeni.

On the basis of the initial screen, a more detailed characterisation of the esterase activity of selected LAB isolates was conducted. Esterase activity was examined across a range of pH, temperature, and ethanol concentrations - all important oenological parameters. In addition, substrate specificity was determined using six ester substrates. In general, activity was maximal at pH values close to 6.0, and temperatures close to 40°C, although exceptions were observed with some strains. Increases in ethanol concentration resulted in lower activity for most lactobacilli and pediococci, but stimulated the esterase activity of all O. oeni.

Work conducted with dairy LAB isolates has suggested esterases may be capable of both hydrolysing and synthesising esters. In the wine industry, the results of some volatile-profiling studies tend to support this theory, with concentrations of esters being reported to both increase and decrease during MLF. Malolactic fermentation trials were conducted in
wine with six strains of O. oeni and GCMS was used to quantify particular esters before and after MLF. Some esters were found to increase in concentration during MLF, while others were found to decrease. These findings suggest LAB esterases are in fact capable of both synthesising and hydrolysing ester substrates in wine.

To further dissect the esterase make-up of selected LAB strains, attempts to clone and heterologously express three structural genes for these enzymes were made. Three putative esterase genes were identified in O. oeni and cloned. Sequencing was completed and alignment with published esterase sequences used to reveal theoretical proteins of the O. oeni genes with high homology with those from other organisms. Of note, key features, such as active site motifs, were conserved in each O. oeni sequence. Expression of the recombinant proteins in E. coli resulted in higher esterase activity in one of the clones compared to the host. These results indicate that the open reading frame of one esterase gene in O. oeni has been identified.