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Impact and identification of inhibitory peptides released by *Saccharomyces cerevisiae* on the malolactic fermentation

Ziad Rizk

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de Toulouse

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Abstract

The production of most red wines and certain white and sparkling wine styles requires two consecutive fermentation steps. The first one is the alcoholic fermentation (AF) and is carried out mainly by *Saccharomyces cerevisiae*. At the end of the AF, the wines undergo malolactic fermentation (MLF) carried out mainly by *Oenococcus oeni*. However, the MLF is often difficult to trigger and accomplish because of the individual or synergistic antibacterial activity of several physical chemical wine parameters and yeast inhibitory metabolites.

In this context, the study of the interactions that may occur between specific strains of yeasts and bacteria is important for choosing the adequate strain combination and inoculation strategy.

In the present work, *S. cerevisiae* strain D strongly inhibited *O. oeni* strain X during sequential fermentations performed in synthetic grape juice (SGJ) media whereas *S. cerevisiae* strain A stimulated it. Protease and heat treatments of the SGJ media fermented by strain D showed the protein nature of the yeast inhibitory metabolites. Fractionation by ultrafiltration of the same media revealed that an extracellular peptidic fraction of 5-10 kDa was responsible for the inhibition. It was gradually released during AF and reached its highest concentration at late stages of the stationary phase. The MLF inhibition was maintained in natural grape juices and grape musts (Cabernet-Sauvignon and Syrah) presenting low and high phenolic contents. Therefore, the activity of the inhibitory peptides was not affected by grape phenolic compounds. The 5-10 kDa fraction was tested *in vitro* on cell-free bacterial cytosolic extracts containing the malolactic enzyme in a pH range between 3.5 and 6.7. Results showed that it was able to directly inhibit the malolactic enzyme activity with an increasing inhibitory kinetic correlated to the AF time at which it was collected.

The 5-10 kDa peptidic fraction of the 60-80 % ammonium sulfate precipitate was submitted to analyses by both anionic and cationic exchange chromatography (AEXC and CEXC). Eluates recuperated with 0.5 M NaCl from both AEXC and CEXC contained inhibitory peptides and were further migrated by SDS-PAGE. The bands of interest were excised and sequenced by LC1D-nanoESI-LTQ-Orbitrap. Results gave 12 different peptidic fractions that may have worked synergistically. 2 GAPDH fragments of 0.9 and 1.373 kDa having a *pI* of 9.074 and a Wtm2p fragment of 2.42 kDa having a *pI* of 3.35 were involved in the MLF inhibition.

Keywords: *Saccharomyces cerevisiae*, *Oenococcus oeni*, malolactic fermentation, malolactic enzyme, antibacterial yeast peptides, microbial interactions

Résumé

La production de la majorité des vins rouges et de certains types de vins blancs et de vins pétillants requiert deux étapes fermentaires successives. La première est la fermentation alcoolique (FA) réalisée principalement par *Saccharomyces cerevisiae*. A la fin de la FA, les vins sont soumis à la fermentation malolactique (FML) réalisée principalement par *Oenococcus oeni*. Cependant, la FML est souvent difficile à déclencher et accomplir à cause de l'activité antibactérienne individuelle ou synergique de différents paramètres physico-chimiques du vin et de métabolites levuriens inhibiteurs.

Dans ce contexte, l'étude des interactions qui peuvent avoir lieu entre les souches de levures et de bactéries est importante pour le choix adéquat des souches et de la stratégie d'inoculation.

Dans le présent travail, *S. cerevisiae* souche D a fortement inhibé *O. oeni* souche X pendant les fermentations séquentielles réalisées dans des milieux synthétiques jus de raisin (MSJ) alors que la souche levurienne A s'est révélée stimulatrice. Des traitements protéasiques et thermiques des MSJ fermentés par la souche D ont démontré la nature protéique des métabolites levuriens inhibiteurs. Le fractionnement par ultrafiltration de ces mêmes milieux a montré qu'une fraction peptidique extracellulaire de 5-10 kDa était responsable de l'inhibition de la FML. Elle a été graduellement relarguée au cours de la FA et a atteint sa concentration maximale à un stade avancé de la phase stationnaire. L'inhibition de la FML a été maintenue dans les jus de raisin commerciaux et les moûts naturels (Cabernet-Sauvignon et Syrah) présentant des teneurs faibles et élevées en composés phénoliques. Ces derniers n'ont pas pu donc modifier l'activité biologique des peptides inhibiteurs. La fraction 5-10 kDa a été testée *in vitro* sur un extrait cellulaire contenant l'enzyme malolactique dans une gamme de pH comprise entre 3,5 et 6,7. Les résultats ont montré qu'elle a pu directement inhiber l'activité de l'enzyme malolactique avec une cinétique d'inhibition croissante corrélée à l'avancement de la fermentation alcoolique.

La fraction 5-10 kDa du précipité au sulfate d'ammonium (60-80 %) a ensuite été analysée par chromatographie échangeuse d'ions. Les éluats récupérés avec 0,5 M NaCl par chromatographie anionique et cationique ont renfermé des peptides inhibiteurs et ont été migrés sur SDS-PAGE. Les bandes d'intérêt ont ensuite été coupées et séquencées par LC1D-nanoESI-LTQ-Orbitrap. Les résultats ont montré 12 peptides qui ont probablement travaillé en synergie. 2 fragments de GAPDH de 0,9 et 1,373 kDa ayant un *pI* de 9,074 et un fragment de Wtm2p de 2,42 kDa ayant un *pI* de 3,35 sont impliqués dans l'inhibition de la FML.

Mots-clés : *Saccharomyces cerevisiae*, *Oenococcus oeni*, fermentation malolactique, enzyme malolactique, peptides levuriens antibactériens, interactions microbiennes

Table of Contents

Acknowledgments

Abstract

Résumé

Abbreviations

INTRODUCTION..... 2

CHAPTER I. STATE OF THE ART

| | |
|---|----|
| I.1 ECOLOGY OF WINEMAKING YEASTS | 8 |
| I.2 THE ALCOHOLIC FERMENTATION | 10 |
| a- Glycolysis | 18 |
| b- Alcoholic fermentation | 12 |
| c- Glyceropyruvic fermentation | 12 |
| d- Acetic acid formation | 13 |
| I.3 ECOLOGY OF WINEMAKING LACTIC ACID BACTERIA | 14 |
| I.4 CHARACTERISTICS OF <i>OENOCOCCUS OENI</i> | 16 |
| I.4.1 Sugar metabolism in <i>O. oeni</i> | 18 |
| I.4.2 Nutritional requirements of <i>Oenococcus oeni</i> | 21 |
| I.4.3 The malolactic fermentation | 23 |
| I.4.4 Role of the membrane ATPase of <i>O. oeni</i> | 24 |
| I.5 IMPACT OF MLF ON THE OVERALL WINE COMPOSITION AND QUALITY | 27 |
| I.5.1 Loss of acidity and increase in pH..... | 27 |
| I.5.2 Microbial stability..... | 27 |
| I.5.3 Sensorial modifications | 27 |
| I.5.4 Impact of MLF on red wine color | 28 |
| I.6 BIOGENIC AMINES..... | 29 |
| I.7 INHIBITORS OF MALOLACTIC FERMENTATION | 30 |
| I.7.1 Temperature..... | 31 |
| I.7.2 pH | 32 |
| I.7.3 Oxygen..... | 33 |

| | |
|---|----|
| I.7.4 Sulfur dioxide | 33 |
| I.7.5 Ethanol..... | 35 |
| I.7.6 Medium chain fatty acids (MCFA) | 37 |
| I.7.7 Antimicrobial peptides (AMP)..... | 38 |
| I.7.8 Nutrient depletion..... | 38 |
| I.7.9 Lees compaction and residual lysozyme activity | 39 |
| I.7.10 Pesticides | 40 |
| I.7.11 Phenolic compounds..... | 40 |
| I.7.12 Bacteriophages..... | 43 |
| I.8 STRESS RESISTANCE MECHANISMS OF LAB DURING MLF | 44 |
| I.9 WINE MICROBIOME, A DYNAMIC ENVIRONMENT OF MICROBIAL INTERACTIONS | 47 |
| 1. Yeast-yeast interactions | 49 |
| a. Negative effect: killer toxins produced by <i>Saccharomyces</i> and non- <i>Saccharomyces</i> with activity against sensitive yeasts | 49 |
| b. Positive effect: <i>Saccharomyces</i> and non- <i>Saccharomyces</i> | 50 |
| 2. Yeast-bacteria interactions..... | 51 |
| a. Negative effect: AMP produced by <i>S. cerevisiae</i> with activity against <i>O. oeni</i> | 51 |
| b. Positive effect: stimulation of <i>O. oeni</i> by <i>S. cerevisiae</i> | 53 |
| 3. Bacteria-bacteria interactions..... | 54 |
| I.10 AIMS OF THE STUDY | 54 |

CHAPTER II. MATERIALS AND METHODS

| | |
|---|----|
| II.1. MATERIALS | 59 |
| II.1.1 Microorganisms..... | 59 |
| II.1.2 Chemicals | 59 |
| II.1.3 Culture media | 59 |
| II.1.3.1 Conservation and reactivation media for yeasts..... | 59 |
| II.1.3.2 Culture media and reactivation media for bacteria | 60 |
| II.1.3.3 Synthetic medium for the preparation of yeast and bacterial starter cultures | 60 |
| II.1.3.4 Synthetic medium used for yeast and bacterial cultures | 61 |
| a. Synthetic grape juice medium (SGJ)..... | 61 |
| b. Synthetic wine medium (SWM) | 62 |
| c. Natural grape juice medium (NGJ)..... | 62 |
| d. Cabernet Sauvignon and Syrah musts..... | 62 |
| e. Modified MRS broth..... | 63 |
| f. SGJ medium with yeast nitrogen base ins stead of yeast extract | 63 |

| | |
|--|----|
| II.2 METHODS..... | 65 |
| II.2.1 Fermentations..... | 65 |
| II.2.1.1 Alcoholic fermentation (AF)..... | 65 |
| II.2.1.2 Malolactic fermentation (MLF)..... | 65 |
| II.2.1.3 Sequential fermentation..... | 66 |
| II.2.2 ANALYTICAL METHODS..... | 66 |
| II.2.2.1 Cell enumeration..... | 66 |
| II.2.2.2 Yeast viability..... | 67 |
| II.2.2.3 Optical density (OD) or absorbance measurement..... | 67 |
| II.2.2.4 Biomass or dry weight measurement..... | 67 |
| II.2.2.5 DNS method for the determination of the sugar concentration..... | 68 |
| II.2.2.6 Glycerol and ethanol measurement by HPLC..... | 68 |
| II.2.2.7 Fatty acids measurement by GC..... | 69 |
| II.2.2.8 Determination of the SO ₂ concentration..... | 69 |
| II.2.2.9 Measurement of polysaccharides..... | 70 |
| II.2.2.10 Determination of the protein concentration by the Lowry method..... | 71 |
| II.2.2.11 Determination of the protein concentration at 280 nm..... | 72 |
| II.2.2.12 Determination of total polyphenols..... | 72 |
| II.2.2.13 Enzymatic assays for the determination of the L-malic acid, L-lactic acid, D-lactic acid, acetic acid, citric acid and ethanol concentrations..... | 73 |
| II.2.3 CHARACTERIZATION OF THE YEAST ANTI-MLF PROTEINACEOUS COUPOUNDS..... | 73 |
| II.2.3.1 Heat and protease treatments of the SGJ media fermented by <i>S.cerevisiae</i> strain D..... | 73 |
| II.2.3.2 Fractionation of the yeast fermented media by ultrafiltration..... | 74 |
| II.2.3.3 Timing of the release of the 5-10 kDa inhibitory fraction by <i>S. cerevisiae</i> strain D..... | 75 |
| II.2.3.4 Fractionation of the yeast fermented media by ammonium sulfate precipitation..... | 76 |
| II.2.3.5 Protein purification by Ion Exchange Chromatography..... | 77 |
| II.2.3.6 Protein purification by Aurum TM Ion Exchange Mini Kits and columns..... | 79 |
| II.2.3.7 Protein purification by Tricine SDS-PAGE..... | 82 |
| II.2.4 PEPTIDES/PROTEINS SEQUENCING BY LC1D-NANO ESI-LTQ-VELOS ORBITRAP..... | 84 |
| II.2.5 IN VITRO EVALUATION OF THE INHIBITORY EFFECT OF THE YEAST PEPTIDIC FRACTIONS ON THE MALOLACTIC ENZYME ACTIVITY..... | 85 |
| II.2.5.1 Preparation of the cell-free bacterial enzymatic extract..... | 84 |
| II.2.5.2 Monitoring of the L-malic acid consumption <i>in vitro</i> | 84 |
| II.2.6 WELL PLATE TEST..... | 87 |

| | |
|--|------------|
| II.2.7 STATISTICAL ANALYSES..... | 88 |
| CHAPTER III. RESULTS AND DISCUSSION..... | 90 |
| PART A- DETERMINATION OF THE KINETIC PARAMETERS AND THE BIOCHEMICAL PROFILES OF THE OENOLOGICAL STRAINES USED DURING THE ALCOHOLIC AND THE MALOLACTIC FERMENTATIONS..... | 92 |
| I. INTRODUCTION..... | 93 |
| II. ALCOHOLIC FERMENTATIONS (AF)..... | 94 |
| II.1. Kinetic parameters of the AF carried out by <i>S. cerevisiae</i> strains D and A in SGJ media ... | 94 |
| II.2. Biochemical parameters of the AF carried out by <i>S. cerevisiae</i> strains D and A in SGJ media | 97 |
| III. MALOLACTIC FERMENTATIONS | 101 |
| III.1. Pure cultures of <i>O. oeni</i> strain X in synthetic grape juice and synthetic wine media..... | 101 |
| III.2. Sequential fermentations with the pairs <i>S. cerevisiae</i> strain A/ <i>O. oeni</i> strain X and <i>S. cerevisiae</i> strain D/ <i>O. oeni</i> strain X in synthetic and natural grape juices | 101 |
| PART B- PRODUCTION OF ANTI-MLF PEPTIDES BY S. CEREVISIAE STRAIN D | 108 |
| I. INTRODUCTION..... | 109 |
| II. PROTEASE AND HEAT TREATMENTS OF THE SGJ MEDIA FERMENTED BY <i>S. CEREVISIAE</i> STRAIN D | 109 |
| III. FRACTIONATION OF THE SGJ MEDIA FERMENTED BY <i>S. CEREVISIAE</i> STRAIN D BY ULTRAFILTRATION..... | 113 |
| IV. TIMING OF THE RELEASE OF THE 5-10 KDA ANTI-MLF PEPTIDIC FRACTION PRODUCED BY <i>S. CEREVISIAE</i> STRAIN D DURING AF | 115 |
| V. MAINTENANCE OF THE ANTI-MLF EFFECT OF THE 5-10 KDA PEPTIDIC FRACTION PRODUCED BY <i>S. CEREVISIAE</i> STRAIN D IN NATURAL WINEMAKING CONDITIONS | 118 |
| VI. FRACTIONATION OF THE SGJ MEDIA FERMENTED BY <i>S. CEREVISIAE</i> STRAIN D BY AMMONIUM SULFATE PRECIPITATION | 122 |
| PART C-IN VITRO EVALUATION OF THE MALOLACTIC ENZYME INHIBITION BY THE 5-10 KDA PEPTIDIC FRACTIONS AND THE 60-80% PRECIPITATE..... | 126 |
| I. INTRODUCTION..... | 127 |
| II. IN VITRO EVALUATION OF THE MALOLACTIC ENZYME INHIBITION BY THE 5-10 KDA PEPTIDIC FRACTIONS | 128 |

| | |
|---|------------|
| III. IN VITRO EVALUATION OF THE MALOLACTIC ENZYME INHIBITION BY THE PROTEINACEOUS FRACTIONS OBTAINED BY AMMONIUM SULFATE PRECIPITATION..... | 131 |
| PART D- PURIFICATION OF THE ANTI-MLF PEPTIDES OF THE 5-10 KDA FRACTION FROM THE 60-80% PRECIPITATE BY ION EXCHANGE CHROMATOGRAPHY (IEXC) FOLLOWED BY SDS PAGE..... | 134 |
| I. INTRODUCTION..... | 135 |
| II. COMPARISON OF THE PROTEIN PROFILES OF <i>S. CEREVISIAE</i> STRAINS A AND D BY TRICINE SDS-PAGE | 135 |
| III. PURIFICATION OF THE ANTI-MLF PEPTIDES OF THE 5-10 KDA FRACTION FROM THE 60-80 % PRECIPITATE BY ION EXCHANGE CHROMATOGRAPHY (IEXC) FOLLOWED BY SDS-PAGE..... | 138 |
| | |
| CHAPTER IV. CONCLUSIONS AND PERSPECTIVES | 145 |
| CHAPTER V. REFERENCES | 153 |
| | |
| APPENDICES..... | 175 |
| APPENDIX OF PART A: ALCOHOLIC AND MALOLACTIC FERMENTATION APPENDIX..... | 188 |
| APPENDIX A-I. CORRELATION CURVES BETWEEN THE BIOMASS AND THE OD, THE CELL CONCENTRATION AND THE OD AND THE BIOMASS AND THE CELL CONCENTRATION OF <i>S. CEREVISIAE</i> STRAIN D..... | 177 |
| APPENDIX A-II. PURE CULTURE OF <i>S. CEREVISIAE</i> STRAIN D IN SGJ MEDIA CONTAINING YEAST NITROGEN BASE WITHOUT AMINO ACIDS AND PEPTIDES. | 191 |
| APPENDIX A-III. PURE CULTURE OF <i>S. CEREVISIAE</i> STRAIN D IN SGJ MEDIA CONTAINING YEAST NITROGEN BASE WITH AMINO ACIDS BUT WITHOUT PEPTIDES | 192 |
| APPENDIX A-IV. CORRELATION CURVES BETWEEN THE BIOMASS AND THE OD, THE CELL CONCENTRATION AND THE OD AND THE BIOMASS AND THE CELL CONCENTRATION OF <i>S. CEREVISIAE</i> STRAIN A | 193 |
| APPENDIX A-V. STANDARD CURVE FOR THE SUGAR ANALYSIS BY DNS | 194 |

| | |
|--|------------|
| APPENDIX A-VI. CORRELATION CURVES BETWEEN THE BIOMASS AND THE OD, THE CELL CONCENTRATION AND THE OD AND THE BIOMASS AND THE CELL CONCENTRATION OF <i>O. OENI</i> STRAIN X..... | 195 |
| APPENDIX A-VII. SEQUENTIAL FERMENTATION WITH THE PAIR <i>S. CEREVISIAE</i> STRAIN A/ <i>O. OENI</i> STRAIN X..... | 196 |
| APPENDIX A-VIII. SEQUENTIAL FERMENTATION WITH THE PAIR <i>S. CEREVISIAE</i> STRAIN D/ <i>O. OENI</i> STRAIN X..... | 197 |
| APPENDIX A-IX. SEQUENTIAL FERMENTATIONS IN THE NATURAL RED GRAPE JUICES | 185 |
| APPENDIX OF PART B: PRODUCTION OF ANTI-MLF PEPTIDES BY <i>S. CEREVISIAE</i> STRAIN D | 187 |
| APPENDIX B-I. PROTEASE AND HEAT TREATMENTS OF THE SGJ MEDIA FERMENTED BY <i>S. CEREVISIAE</i> STRAIN D..... | 187 |
| APPENDIX B-II. FRACTIONATION OF THE SGJ MEDIA FERMENTED BY <i>S. CEREVISIAE</i> STRAIN D BY ULTRAFILTRATION..... | 189 |
| APPENDIX B-III. FRACTIONATION OF THE SGJ MEDIA FERMENTED BY <i>S. CEREVISIAE</i> STRAIN A BY ULTRAFILTRATION..... | 190 |
| APPENDIX B-IV. TIMING OF THE RELEASE OF THE 5-10 KDA ANTI-MLF PEPTIDIC FRACTION PRODUCED BY <i>S. CEREVISIAE</i> STRAIN D DURING AF | 190 |
| APPENDIX B-V. FRACTIONATION OF THE SGJ MEDIA FERMENTED BY <i>S. CEREVISIAE</i> STRAIN D BY AMMONIUM SULFATE PRECIPITATION..... | 191 |
| APPENDIX B-VI. SCREENING OF THE ANTIMICROBIAL ACTIVITY OF <i>S. CEREVISIAE</i> STRAIN D DURING WELL PLATE TESTS | 192 |
| APPENDIX OF PART C: IN VITRO EVALUATION OF THE MALOLACTIC ENZYME INHIBITION BY THE 5-10 KDA PEPTIDIC FRACTIONS AND THE 60-80 % PRECIPITATE | 194 |
| APPENDIX OF PART D: PURIFICATION OF THE ANTI-MLF PEPTIDES OF THE 5-10 KDA PEPTIDIC FRACTION ISOLATED FROM THE 60-80 % AMMONIUM SULFATE PRECIPITATE | 195 |
| Purification of the anti-MLF peptides of the 5-10 kDa fraction from the 60-80 % precipitate by Ion Exchange Chromatography (IEXC) | 195 |
| LIST OF PUBLICATIONS AND SCIENTIFIC CONTRIBUTIONS..... | 199 |

List of Figures

| | |
|--|-----|
| Figure I.1. Exponentially growing <i>Saccharomyces cerevisiae</i> . Cells stained with DAPI (blue) showing the nuclei and phalloidin (green) showing actin structures..... | 8 |
| Figure I.2. Glycolysis and alcoholic fermentation pathway..... | 11 |
| Figure I.3. Glyceropyruvic fermentation pathway..... | 13 |
| Figure I.4. Acetic acid formation pathways in yeasts..... | 14 |
| Figure I.5. A chain of <i>Oenococcus oeni</i> cells..... | 17 |
| Figure I.6. The three possible carbohydrate fermentation pathways in wine LAB..... | 20 |
| Figure I.7. Malolactic fermentation reaction..... | 24 |
| Figure I.8. ATP generation during MLF..... | 26 |
| Figure I.9. A schematic representation of the production and modification of flavor-active compounds by LAB..... | 28 |
| Figure I.10. Precursor amino acids of biogenic amines..... | 29 |
| Figure I.11. Different forms of SO ₂ in wines..... | 34 |
| Figure I.12. Three mechanisms that are important in conferring, in <i>Oenococcus oeni</i> , the ability to survive in wine..... | 46 |
| Figure II.1. Ultrafiltration of the yeast fermented SGJ medium for harvesting different peptidic fractions..... | 75 |
| Figure II.2. Aurum ion exchange mini kit components..... | 81 |
| Figure III.1. Growth of <i>S. cerevisiae</i> strain D (●) and sugar consumption (■) during AF in SGJ media..... | 94 |
| Figure III.2. Growth of <i>S. cerevisiae</i> strain A (●) and sugar consumption (■) during AF in SGJ media..... | 95 |
| Figure III.3. Comparison of the growth kinetics of <i>S. cerevisiae</i> strain D (◆) and strain A (■) and the sugar consumption kinetics during AF in SGJ media..... | 96 |
| Figure III.4. Growth of <i>O. oeni</i> strain X in SGJ media (■) and in SW media (▲) and corresponding malate consumption. Each value is the mean of triplicate experiments ± SD.... | 103 |

| | |
|--|-----|
| Figure III.5. Growth of <i>O. oeni</i> strain X and malate consumption in the controls and in the SGJ and NGJ media fermented by <i>S. cerevisiae</i> strains A and D. (◆) Bacterial control culture in the SGJ medium, (■) Bacterial control culture in the SW medium, (▲) NGJ fermented by <i>S. cerevisiae</i> strain A, (●) SGJ fermented by <i>S. cerevisiae</i> strain A, (○) NGJ fermented by <i>S. cerevisiae</i> strain D, (x) SGJ fermented by <i>S. cerevisiae</i> strain D | 105 |
| Figure III.6. Growth and malic acid consumption kinetics of <i>O. oeni</i> strain X in the untreated and treated yeast fermented media and in the bacterial control cultures. (■) SGJ control, (▲) SW control, (◆) Untreated fermented SGJ medium, (○) Untreated fermented NGJ medium, Treated fermented SGJ media: (□) Papain treatment, (●) Pepsin treatment, (Δ) Trypsin treatment, (x) Heat treatments (mean values of the treatments at 100°C/30 min and 121°C/20 min). | 110 |
| Figure III.7. Growth and malic acid consumption kinetics of <i>O. oeni</i> strain X in the control and in the presence of four different MW fractions from the SGJ media fermented by <i>S. cerevisiae</i> strain D. (◆) Control: modified MRS medium, (■) MW ≤ 3 kDa, (▲) MW between 3 and 5 kDa, (x) MW between 5 and 10 kDa, (○) MW ≥ 10 kDa | 113 |
| Figure III.8. Growth and malic acid consumption kinetics of <i>O. oeni</i> strain X in the control and in the presence of the 5-10 kDa inhibitory fractions collected at different intervals of the AF. (◆) Control: modified MRS medium, (■) 5-10 kDa collected after 24 h of the AF, (▲) 5-10 kDa collected after 48 h of the AF, (x) 5-10 kDa collected after 72 h of the AF, (○) 5-10 kDa collected after 96 h of the AF, (●) 5-10 kDa collected after 120 h of the AF | 115 |
| Figure III.9. Growth and malic acid consumption kinetics of <i>O. oeni</i> strain X in the control and in the presence of the proteinaceous fractions obtained by ammonium sulfate precipitation in the SGJ media fermented by <i>S. cerevisiae</i> strain D. (◆) Control: modified MRS medium; precipitated fractions at different saturation degrees: (■) 0-20 %, (▲) 20-40 %, (○) 40-60 %, (x) 60-80 %, (●) 80-100 % | 122 |
| Figure III.10. Silver stained Tricine SDS-PAGE gel. Lane A corresponds to the dual-xtra MW standard 2-250 kDa (Bio-Rad). Lane B corresponds to the 5-10 kDa fraction of the 60-80 % precipitate from <i>S. cerevisiae</i> strain D. Lane C corresponds to the 5-10 kDa fraction of the 60-80 % precipitate from <i>S. cerevisiae</i> strain A (reference strain) | 136 |
| Figure III.11. Coomassie G-250 stained Tricine SDS-PAGE gel. Lane A contains dual-xtra MW standard 2-250 kDa (Bio-Rad). Lane B corresponds to eluate 1 from CEXC. Lane C corresponds to eluate 1 from AEXC | 140 |

List of Tables

| | |
|--|-----|
| Table I.1. Contribution of non- <i>Saccharomyces</i> strains to wine aroma..... | 9 |
| Table I.2. Main species of lactic acid bacteria in grape must and wine | 16 |
| Table II.1. Composition of the YEPD medium | 59 |
| Table II.2. Composition of the MRS broth..... | 60 |
| Table II.3. Composition of the synthetic medium used for yeast and bacterial starter cultures ... | 61 |
| Table II.4. Composition of the synthetic grape juice medium..... | 61 |
| Table II.5. Composition of the synthetic wine medium..... | 62 |
| Table II.6. Composition of both types of yeast nitrogen base | 64 |
| Table II.7. Common buffers used for ion exchange chromatography | 80 |
| Table II.8. Protein precipitation solution or TCA solution composition | 82 |
| Table II.9. Laemmli sample buffer composition | 83 |
| Table II.10. Running buffer composition | 83 |
| Table II.11. Reaction mixture for the monitoring of the L-malic acid consumption <i>in vitro</i> | 86 |
| Table III.1 Growth and sugar consumption kinetics of <i>S. cerevisiae</i> strain D during AF in SGJ media..... | 94 |
| Table III.2 Growth and sugar consumption kinetics of <i>S. cerevisiae</i> strain A during AF in SGJ media..... | 95 |
| Table III.3 The biochemical profile of <i>S. cerevisiae</i> strains D and A during AF in SGJ media .. | 98 |
| Table III.4 Kinetic parameters of the pure cultures of <i>O. oeni</i> strain X in synthetic grape juice and synthetic wine media..... | 102 |
| Table III.5 Some biochemical parameters of <i>O. oeni</i> strain X during its pure cultures in the SGJ and the SW media | 103 |
| Table III.6 Determination of the average specific growth rates and the average specific rates of malate consumption during the MLF carried out by <i>O. oeni</i> strain X in the SGJ and NGJ fermented by <i>S. cerevisiae</i> strains A and D as well as in the controls..... | 105 |

| | |
|--|-----|
| Table III.7 Determination of the average specific growth rates and the average specific rates of malate consumption during the MLF carried out by <i>O. oeni</i> strain X in the treated and untreated fermented media as well as in the controls | 110 |
| Table III.8 Determination of the average specific growth rates and the average specific rates of malate consumption during the MLF carried out by <i>O. oeni</i> strain X in the modified MRS media containing the 5-10 kDa fractions collected each 24 h of the AF as well as in the control..... | 116 |
| Table III.9 Total phenolic content (mg/l equivalent gallic acid) in Cabernet Sauvignon and Syrah wines at the end of the AF carried out by <i>S. cerevisiae</i> strains A and D..... | 119 |
| Table III.10 Growth of <i>O. oeni</i> strain X and malate consumption during MLF performed in the Syrah and Cabernet Sauvignon wines obtained after AF by <i>S. cerevisiae</i> strains A or D..... | 120 |
| Table III.11 Protein concentration (mg/l) of the different precipitates before addition to the MRS media..... | 123 |
| Table III.12 Amount of L-malic acid consumed (g/l) and L-lactic acid produced (g/l) during the enzymatic reaction <i>in vitro</i> performed at different pH in the presence of the 5-10 kDa fractions (ABP) collected each 24 h of the AF | 128 |
| Table III.13 Amount of L-malic acid consumed (g/l) and L-lactic acid produced (g/l) during the enzymatic reaction <i>in vitro</i> performed at different pH in the presence of different proteinaceous fractions obtained by ammonium sulfate precipitation at the end of the AF (120 h) | 131 |
| Table III.14 Consumed L-malic acid (g/l) and produced L-lactic acid (g/l) in the control and in the presence of the eluates obtained by AEXC and CEXC | 139 |
| Table III.15 Protein concentration of the eluates obtained by AEXC and CEXC..... | 139 |
| Table III.16 Sequence analysis by LC1D-nanoESI-LTQ-Orbitrap of the peptides corresponding to the 5.1 kDa and 7.2 kDa of the cationic eluate | 141 |
| Table III.17 Sequence analysis by LC1D-nanoESI-LTQ-Orbitrap of the peptides corresponding to the 5.1 kDa of the anionic eluate | 141 |

Abbreviations

DNA: Deoxyribonucleic Acid
RNA: Ribonucleic Acid
ATP: Adenosine Triphosphate
PMF: Proton Motive Force
BA: Biogenic Amines
BSA: Bovine Serum Albumin
NAD: Nicotinamide Adenine Dinucleotide
FAD: Flavine Adenine Dinucleotide
FMN: Flavine Mononucleotide
HM⁻: mono protonated malate
HL: mono protonated lactate
°C: degree Celsius
CFU: Colony Forming Unit
kDa: Kilo Dalton
bp: base pair
Mb: Millions of base pairs
MW: Molecular Weight
OD: Optical density
ORFs: Open Reading Frames
S: Svedberg unit
RAPD: Random Amplified Polymorphism DNA
ARDRA: Amplified Ribosomal DNA Restriction Analysis
AF: Alcoholic fermentation
MLF: Malolactic fermentation
LAB: Lactic Acid Bacteria
MLB: Malolactic Bacteria
MLE: Malolactic Enzyme
x g: centrifugal gravity force
rpm: revolutions per minute
h: hour
M: Molarity

mM: millimolar

N: Normality

min: minute

g: gram

mg: milligram

ml: milliliter

l: liter

μm , μ , μl : micrometer, micron, microliter

nm, nl: nanometer, nanoliter

ppm: parts per million

MRS: *De Man Rogosa Sharpe*

SGJ: Synthetic Grape Juice

SW: Synthetic Wine

NGJ: Natural Grape Juice

YEPD: Yeast Extract Peptone Dextrose

ABP: Antibacterial Peptides

AMP: Antimicrobial Peptides

GAPDH: Glyceraldehyde -3-Phosphate Deshydrogenase

O. oeni: *Oenococcus oeni*

S. cerevisiae: *Saccharomyces cerevisiae*

Sch. pombe: *Schizosaccharomyces pombe*

NS: *Non-Saccharomyces*

Lc.: *Lactococcus*

Ln.: *Leuconostoc*

L.: *Lactobacillus*

pH: Hydrogen potential

pHi: internal pH

pHex: external pH

pKa: Dissociation constant (acid)

K_M : Michaelis constant

$\Delta\phi$: membrane potential

‰: percentage

(v/v): volume per volume

(w/v): weight per volume

(X): times concentrated

Tris: 2-Amino-2-(hydroxymethyl) propane-1, 3-diol

SDS: Sodium Dodecyl Sulfate

PAGE: Polyacrylamide Gel Electrophoresis

di H₂O: de-ionized water

TCA: Trichloroacetic acid

DTT: Dithiothreitol

pI: Isoelectric point

IEXC: Ion Exchange Chromatography

AEX: Anionic Exchange Chromatography

CEX: Cationic Exchange Chromatography

μ : average specific growth rate (h^{-1})

X_f: final bacterial or yeast biomass (g/l)

X₀: initial bacterial or yeast biomass (g/l)

dt₁: growth duration (h)

Q_s: average specific rate of malate consumption ($\text{g g}^{-1} \text{h}^{-1}$)

S₀: initial malate or sugar concentration (g/l)

S_f: final malate or sugar concentration (g/l)

dt₂: duration of MLF (h)

Y_(X/S): Biomass yield (g/g)

Y_(P/S): Ethanol yield (g/g)

P_f: final ethanol concentration (g/l)

P₀: initial ethanol concentration (g/l)

Introduction

According to OIV (International Organization of Vine and Wine), wine is the beverage resulting exclusively from the partial or complete alcoholic fermentation of fresh grapes, whether crushed or not, or of grape must. Its alcohol content shall not be less than 8.5 % (v/v). Nevertheless, taking into account the climate, soil, vine variety, special qualitative factors or traditions specific to certain vineyards, the minimum total alcohol content can be reduced to 7 % (v/v) by legislation particular to the region considered. There are five basic stages or steps to making wine: harvesting, crushing and pressing, fermentation, clarification, aging and then bottling. Undoubtedly, one can find endless deviations and variations along the way that make each wine unique and ultimately contribute to the greatness of any particular wine (white, red, rosé or sparkling wines).

Wine composition and quality are functions of many different intrinsic and extrinsic variables, many of which are microbiologically mediated. Winemaking is usually conducted under non-sterile conditions; therefore a huge variety of microorganisms is present and can influence the process. Microbial interactions during winemaking have long been studied and their weight on microbial succession during wine fermentations has been examined. Some of the studies evaluated yeast-yeast interactions mainly the impact of *Saccharomyces* on non-*Saccharomyces* (e.g., *Hanseniaspora*, *Candida*, *Kluyveromyces* and *Torulaspora*) behavior (Albergaria et al., 2010; Branco et al., 2014), while some others examined yeast-lactic acid bacteria interactions mainly the impact of *Saccharomyces cerevisiae* on the growth and metabolism of *Oenococcus oeni* (Beelman et al., 1982; King and Beelman, 1986; Lemaresquier, 1987; Lonvaud-Funel et al., 1988, Wibowo et al., 1988; Cannon and Pilone, 1993; Henick-Kling and Park, 1994; Taillandier et al., 2002; Larsen et al., 2003; Comitini et al., 2005; Osborne and Edwards, 2006; Comitini and Ciani, 2007; Nehme et al., 2008; Mendoza et al., 2010). Different strain combinations and inoculation strategies (sequential and mixed cultures) were tested by these authors and the results showed that the type of interaction (neutralism, inhibition or stimulation) and its extent were strongly dependent on the selected strains in a combination.

In this context, it is usually known that after the accomplishment of alcoholic fermentation (AF) carried out mainly by *S. cerevisiae*, most of the red wines and certain acid white wines and sparkling wines are spontaneously, or purposely, taken through a malolactic fermentation (MLF)

step, mostly by indigenous or inoculated lactic acid bacteria of the *O. oeni* species. Sometimes, some strains of *Lactobacillus* or *Pediococcus* can also participate to the MLF.

The MLF, an enzymatic decarboxylation of L-malic acid (one of the harshest three acids naturally found in the grape, the other two being citric and tartaric) into L-lactic acid (the softer acid) and CO₂, diminishes wine acidity and improves its sensorial characteristics and its microbial stability (Kunkee 1984, 1991; Lonvaud-Funel 1999, 2002; Bartowski et al., 2002). However, it is often difficult to trigger and accomplish because of the individual or synergistic antibacterial activity of several physical chemical wine parameters and yeast inhibitory metabolites. Of all the species of lactic acid bacteria, *O. oeni* is probably the best adapted to withstand the harsh environmental wine conditions and therefore represents the majority of commercial MLF starter cultures.

Some of the MLF inhibitory factors have been intensively investigated such as low pH (Britz and Tracey, 1990; Vaillant et al., 1995), inadequate temperature (Britz and Tracey, 1990), nutrient depletion (Tracey and Britz 1989; Nygaard and Prah, 1996; Patynowski et al., 2002; Saguir and Manca de Nadra 2002), endogenous and exogenous SO₂ (Henick-Kling and Park, 1994; Carreté et al., 2002; Larsen et al. 2003; Osborne and Edwards 2006), phenolic compounds (Reguant et al., 2000), high ethanol content (Britz and Tracey, 1990; Vaillant et al., 1995) and medium chain fatty acids (Edwards and Beelman 1987; Lonvaud-Funel et al., 1988; Capucho and San Romão 1994).

While the anti-MLF role of the previous compounds is already well established, there are gradually growing evidences suggesting the involvement of yeast peptides/proteins in the inhibition of *O. oeni* growth and malic acid consumption. Few authors such as Dick et al., (1992), Comitini et al., (2005), Osborne and Edwards (2007), Mendoza et al., (2010), Nehme et al., (2010) and Branco et al., (2014) demonstrated that some *S. cerevisiae* strains were able to produce extracellular proteinaceous compounds of different MW active against some *O. oeni* strains with no conclusive results on their mechanism of action.

The current project is a continuation of the work already initiated by Nehme et al., (2008; 2010) who found that *S. cerevisiae* strain D strongly inhibited the growth of *O. oeni* strain X and its ability to consume L-malic acid during sequential fermentations performed in synthetic grape juice media. An extracellular yeast proteinaceous compound was involved in the MLF inhibition and was found to work synergistically with ethanol. The present project aimed at revalidating the

MLF inhibition with the same pair and further characterizing the yeast inhibitory proteinaceous compound. After interrupting the work for almost six years, the first concern was to demonstrate that neither the yeast strain D lost its ability to release the proteinaceous compound nor did the bacteria acquire resistance. After having shown that the anti-MLF activity for the couple chosen was still present, our objectives were first to better characterizing the peptidic compounds: are there one or many molecules? What is their apparent MW? Do they derive from an already known proteins or peptides? On the other hand we wanted to better understand the mechanisms of interaction: is there a constant activity during the different phases of the alcoholic fermentation? The effect is the same on whole cells of *Oenococcus oeni* during MLF or on the malolactic enzyme?

In the first chapter, the biochemistry of alcoholic and malolactic fermentations and the ecology of the major microorganisms involved in winemaking are described. The importance of the malolactic fermentation, its impact on the overall wine composition and quality and its main inhibitors are presented. The stress resistance mechanisms in lactic acid bacteria and the different types of microbial interactions during winemaking are also detailed.

In the second chapter, the materials and methods used for the accomplishment of the experiments are described in detail.

The third chapter shows the results obtained in this work combined with the discussions. It is divided into four parts. The first part gives the kinetic and biochemical parameters of the alcoholic and malolactic fermentations during pure and sequential cultures. The second part deals with the characterization of the anti-MLF peptides produced by *S. cerevisiae* strain D. Their proteinaceous nature, apparent MW, timing of release during AF and the reproducibility of the results in synthetic and natural grape juices are evaluated and discussed. The third part focuses on their mechanism of action and describes their direct effect on the malolactic enzyme activity. The fourth part deals with their purification by SDS-PAGE and Ion Exchange Chromatography followed by sequencing in order to determine their nature and real MW.

Finally, in the fourth chapter, the general conclusions and the perspectives for future works are given.

Chapter I. State of the Art

I.1 Ecology of winemaking yeasts

Yeasts are widespread in nature and are found in soils, on the surface of vegetables and in the digestive tract of animals. Wind and insects disseminate them. They are distributed irregularly on the surface of a grape vine and are found in small quantities on leaves, stems and unripe grapes. They colonize the grape skin during maturation (Pretorius, 2000; Munõz et al., 2011).

Saccharomyces cerevisiae (Figure I.1), is without a doubt, the most important species for humanity because of its multiple usage in different sectors, such as beverages (wine, beer), bakeries, milk and dairy products, pharmaceuticals and biotechnology Rainieri and Pretorius, 2000; Ribéreau-Gayon et al., 2006).

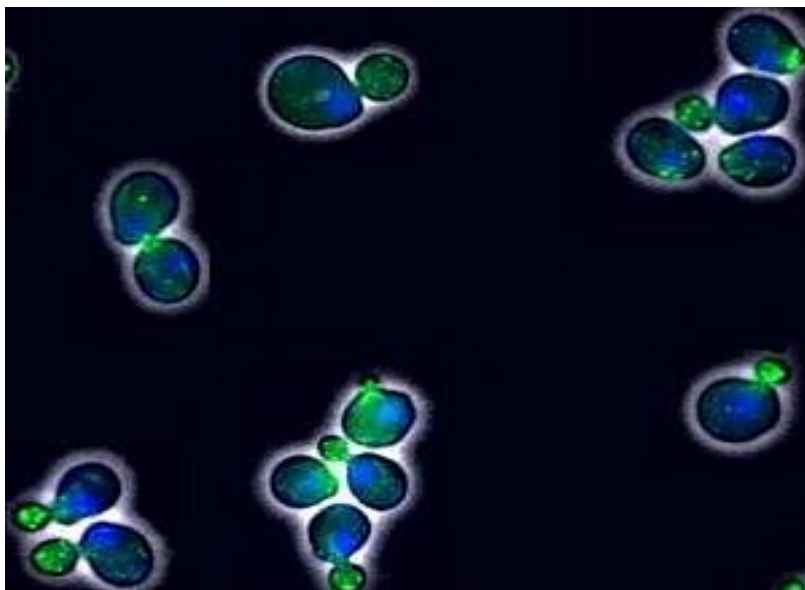


Figure I.1. Exponentially growing *Saccharomyces cerevisiae*. Cells stained with DAPI (blue) showing the nuclei and phalloidin (green) showing actin structures. Photo by Mark Bisschops, TU Delft Netherlands

It has been known for a long time that freshly crushed grape juice harbors a diversity of yeast species, principally within the genera *Hanseniaspora* (anamorph *Kloeckera apiculata*), *Pichia*, *Candida*, *Metschnikowia*, *Kluyveromyces* and *Saccharomyces*. Occasionally, species in other genera such as: *Zygosaccharomyces*, *Saccharomycodes*, *Torulaspota*, *Dekkera*, *Schizosaccharomyces* and the strictly oxidative yeasts belonging to the genus *Rhodotorula*, may be present (Romano and Suzzi, 1993; Fleet and Heard, 1993; Fleet, 2008; Li et al., 2010). These yeasts originate from the microbial communities of the grape berries and the microbial communities of the winery environment. It is also well known that many of these non-*Saccharomyces* species (especially

species of *Hanseniaspora*, *Candida*, *Pichia* and *Metschnikowia*) initiate spontaneous alcoholic fermentation of the juice, tolerate 4 to 5% ethanol (v/v), but are very soon overtaken by the growth of *S. cerevisiae* that dominates the mid to final stages of the process, most often being the only species found in the fermenting juice at these times (Fleet and Heard, 1993; Fleet, 2008). Based on these early ecological studies, *S. cerevisiae* and the related species *Saccharomyces bayanus* and *Saccharomyces uvarum* were considered to be the yeasts of main relevance to the process. *S. cerevisiae* strains ferment grape juice vigorously, leave small amounts of unfermented sugars in the medium and produce wines with high ethanol concentrations (Masneuf, 1996; Rainieri and Pretorius, 2000).

The non-*Saccharomyces* species usually achieve a maximum population of 10^7 CFU/ml or more in the early stages of fermentation before they die off. It is concluded that this amount of biomass is sufficient to impact the chemical composition of wine, such as in Chardonnay and Shiraz, and that the contribution of these yeasts to the overall wine character (aromas and flavors) is much more significant than thought previously (refer to Table I.1). Under certain circumstances, such as fermentation at lower temperatures, some non-*Saccharomyces* species do not die off and remain at high populations in conjunction with *S. cerevisiae* until the end of fermentation. Moreover, it was shown that these indigenous non-*Saccharomyces* yeasts also grow in grape juice fermentations inoculated with starter cultures of *S. cerevisiae* (Cocolin et al., 2002; Romano et al., 2003; Ribéreau-Gayon et al., 2006; Ciani et al., 2009; Zott et al., 2011; Medina et al., 2013; Holt et al., 2013; David et al., 2014).

Table I.1. Contribution of non-Saccharomyces strains to wine aroma

| | |
|---|---|
| <i>Schizosaccharomyces pombe</i> | Deacidification (Taillandier et al., 1995) |
| <i>Kluyveromyces thermotolerans</i> | Acidification (Mora et al., 1990; Ciani et al., 2006) |
| <i>Candida zemplinina</i> (<i>Starmerella bacillaris</i>) | 2 times more production of glycerol (Soden et al., 2000; Beltramo et al., 2002; Grangeteau et al., 2016) |
| <i>Pichia kluyverri</i> | Hydrolysis precursors (terpenes, thiols...) (Fleet and Heard, 1993) |
| <i>Hanseniaspora osmophila</i> | 10 times more production of phenyl-2-ethanol (Fleet and Heard, 1993; Ciani et al., 2006; Ocon et al., 2010) |
| <i>Torulaspota delbrueckii</i> | Fruity esters (Ciani et al., 2006 ; Renouf et al., 2006 ; Taillandier et al., 2014) |
| <i>Metschnikowia pulcherrima</i> | Hydrolysis precursors (terpenes, thiols...) (Fleet and Heard, 1993; Ocon et al., 2010; Sadoudi et al., 2016) |
| <i>Candida zemplinina</i> , <i>Lachancea thermotolerans</i> | Different aromatic board (terpenes, lactones) (Fleet and Heard, 1993; Ocon et al., 2010, Sadoudi et al., 2016) |

I.2 The alcoholic fermentation

Winemaking is the biotransformation of must into wine performed principally by *S. cerevisiae*. The sugar concentration in the grape must is between 160 and 220 g/l. The biochemical steps of sugar degradation are described below:

a- Glycolysis

Glycolysis is carried out entirely in the cytosol of the cell. The different steps are described in Figure I.2. During glycolysis, each mole of oxidized glucose releases 2 moles of pyruvic acid and 2 moles of NADH, H⁺ with a net gain of 2 moles of ATP. The NADH, H⁺ should be re-oxidized for the maintenance of the redox balance (Heux et al., 2006).

Aerobically, the coenzyme re-oxidation is performed by the respiratory chain localized in the internal mitochondrial membrane if the sugar concentration enables it (Pasteur Effect). If the oxidative respiration is repressed under high sugar concentrations and in the presence of oxygen (Crabtree effect), the re-oxidation occurs by reducing pyruvate during alcoholic fermentation.

Aerobically and during oxidative respiration, the pyruvic acid is oxidized to water and CO₂ and gives the precursors of anabolic reactions through the Krebs cycle. Under anaerobic conditions or Crabtree effect, the pyruvate is decarboxylated to acetaldehyde which is then reduced to ethanol, thus re-oxidizing the NADH, H⁺ during alcoholic fermentation.

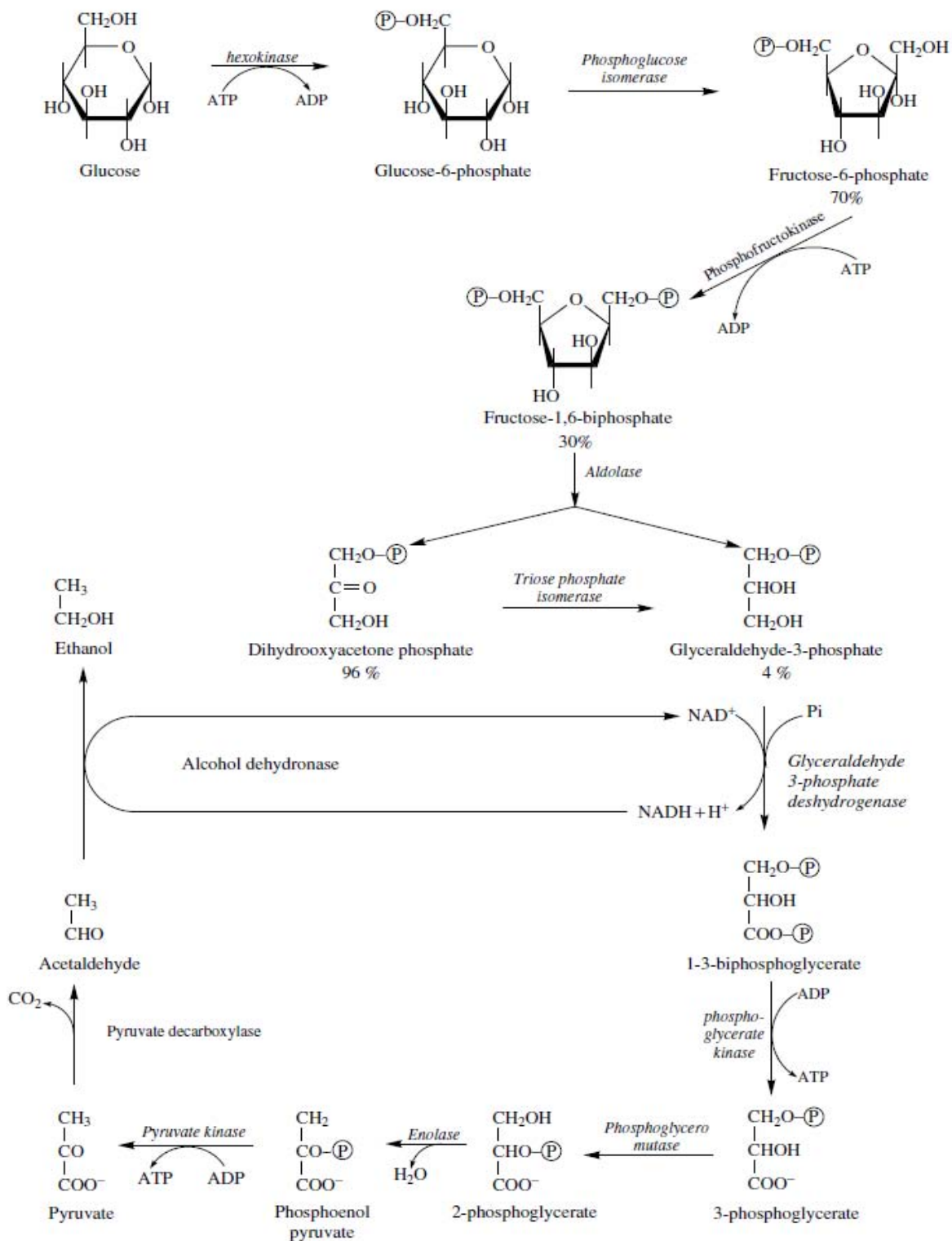
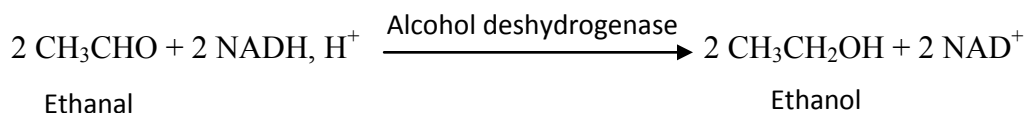
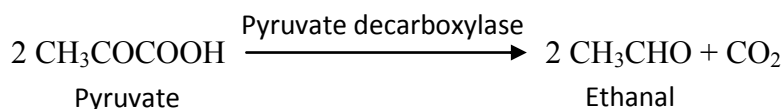


Figure I.2. Glycolysis and alcoholic fermentation pathway (Ribéreau-Gayon et al., 2006)

b- Alcoholic fermentation

At the end of glycolysis, the pyruvate is decarboxylated via the pyruvate decarboxylase into acetaldehyde (ethanal) which is then reduced to ethanol by the alcohol deshydrogenase and NADH, H⁺ (Larue et al., 1984). The pyruvate is the origin of secondary products that play a major role in wine aroma (glycerol, acetaldehyde, higher alcohols, esters, organic acids...) which will be further described.

An assimilable nitrogen (mineral and organic) concentration of 140-150mg/l is necessary to complete fermentation because it stimulates biomass production. Otherwise, it deprives glucose intake and can induce a sluggish fermentation (Wang et al., 2003; Palma et al., 2012). A small amount of pantothenic acid (10 µg/l) influences viability and fermentation rate (Wang et al., 2003). Oxygen is required for membrane lipid synthesis. However, in high quantities, it may favor the oxidation of wine phenolic compounds (Salmon, 2006).

**c- Glyceropyruvic fermentation**

Glycerol is the most important by-product of alcoholic fermentation after ethanol and carbon dioxide (Figure I.3). It is mainly produced in red wine at concentrations between 2 and 11 g/l, depending on the yeast strain, grape must composition, degree of ripeness (sugar content), mold infection and fermentation conditions such as pH, sulfite levels, aeration and temperature (Pretorius, 2000). 4 to 10% of the sugar's carbons are converted to glycerol and its level increases with fermentation temperature (Torija et al., 2003c). Wine yeast strains overproducing glycerol would improve the organoleptic quality of wine.

In the presence of sulfite, the fermentation of glucose by yeasts produces equivalent quantities of glycerol and acetaldehyde in its bisulfite form. Since the acetaldehyde combined with sulfite

cannot be reduced into ethanol, dihydroxyacetone-1-phosphate becomes the final electron acceptor. It is reduced to glycerol 3-phosphate which is then dephosphorylated into glycerol (Ribéreau-Gayon et al., 2006). Glycerol 3-phosphate deshydrogenase is the key enzyme in the production of glycerol and is encoded by *GPD1* and *GPD2* genes that play an important role in osmoregulation and intracellular redox balance, respectively. *HOG1* (*High Osmotic Glycerol*) encodes for a kinase that controls both genes and is involved in the cell response to hyperosmotic stress and glycerol production during wine fermentation (Remize et al., 2003).

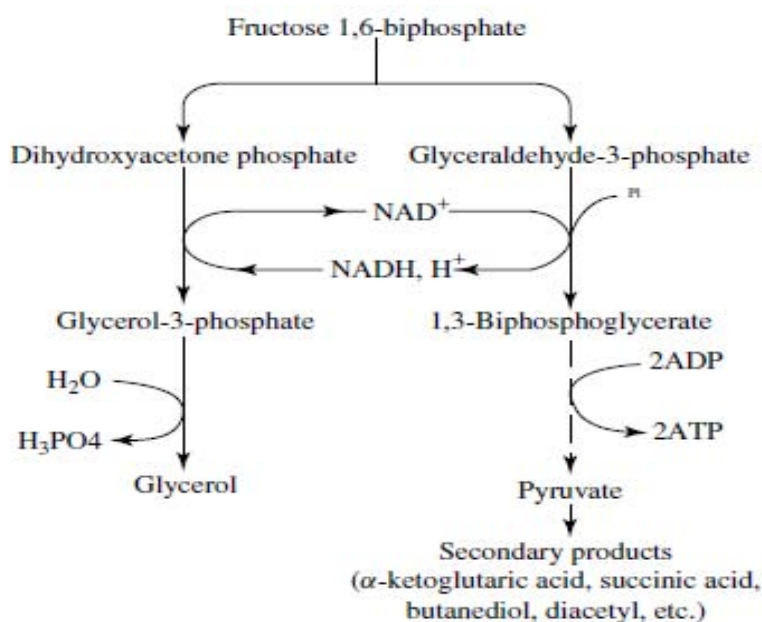


Figure I.3. Glyceropyruvic fermentation pathway (Ribéreau-Gayon et al., 2006)

Glyceropyruvic fermentation does not produce ATP and does not provide biologically assimilable energy for yeasts. The glyceropyruvic fermentation occurs also when yeast starters are grown in the presence of oxygen. Their pyruvate decarboxylase and alcohol deshydrogenase are weakly expressed (Ribéreau-Gayon et al., 2006).

d- Acetic acid formation

Acetic acid is the principal volatile acid in wine. It is produced by yeasts during alcoholic fermentation by different pathways and has a detrimental organoleptic effect on wine quality. In healthy grape must with a moderate sugar concentration, *S. cerevisiae* produces relatively small quantities (100-300 mg/l), varying according to the strain (Ribéreau-Gayon et al., 2006). The biochemical pathway for the formation of acetic acid in wine yeasts is described in Figure I.4.

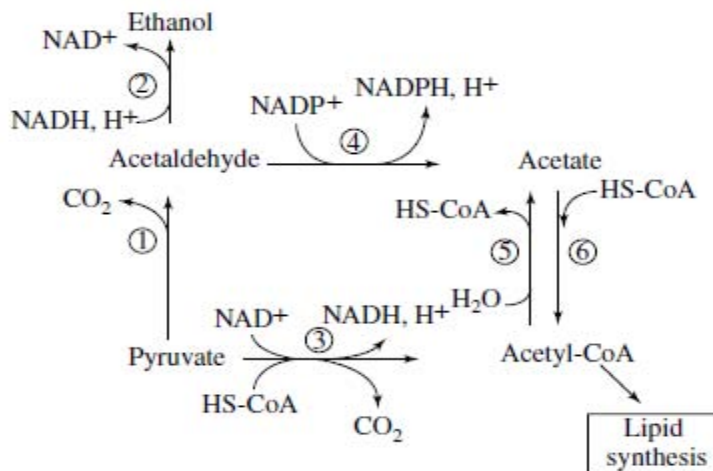


Figure I.4. Acetic acid formation pathways in yeasts (Ribéreau-Gayon et al., 2006)

1-Pyruvate decarboxylase, 2-Alcohol dehydrogenase, 3- Pyruvate dehydrogenase, 4-Aldehyde dehydrogenase, 5- Acetyl-CoA hydrolase, 6- Acetyl-CoA synthase

The higher the sugar content of the must, the more acetic acid the yeast produces during fermentation especially under aerobic conditions. Acetate formation plays an important role in the intracellular redox balance by regenerating reduced equivalents of NADH.

I.3 Ecology of winemaking lactic acid bacteria

Lactic acid bacteria are historically defined as a group of aerotolerant anaerobes, Gram-positive organisms that ferment hexose sugars to produce primarily lactic acid. Today, LAB play a prominent role in the world food supply, identified somehow by RAPD-PCR fingerprinting and 16S-ARDRA (Rodas et al., 2003), performing the main bioconversions in fermented dairy products, meats and vegetables. They are also critical for the production of wine, coffee, silage, coca, sourdough, and numerous indigenous food fermentations (Rossetti and Giraffa, 2005; Makarova et al., 2006).

Lactic acid bacteria are present in all grape musts and wines. Depending on the stage of the winemaking process, environmental conditions determine their ability to multiply. When they develop, they metabolize numerous substrates. Therefore, lactic acid bacteria play an important role in the transformation of grape must into wine. In winemaking, LAB are doubly important as they can both enhance and diminish the quality of wine. Their impact on wine quality depends not only on environmental factors acting at the cellular level but also on the selection of the best adapted species and strains of bacteria. They are responsible for the malolactic fermentation but

they can also cause changes that adversely affect the organoleptic properties of the final product. Shortly after crushing and the start of AF, the LAB population in the grape must generally ranges from 10^3 to 10^4 CFU/ml. During the first days of AF, bacteria are mainly influenced by the original sanitary state of the grapes and the fermentation parameters like temperature. Their amount varies between 10^2 and 10^4 CFU/ml (Ribéreau-Gayon et al., 2006). The major species of LAB present at this stage include *Lactobacillus plantarum*, *Lactobacillus casei*, *Leuconostoc mesenteroides* and *Pediococcus damnosus* as well as *Oenococcus oeni* (*O. oeni*) but to a lesser extent (Wibowo et al., 1985; Lonvaud-Funel et al., 1991). In comparison to the microflora found in the vineyard, the species found in grape must are more diverse and include *L. plantarum*, *L. hilgardii*, *L. brevis*, *P. damnosus*, *P. pentosaceus*, *Ln. mesenteroides* and *O. oeni* (Ribéreau-Gayon et al., 2006). Most of these LAB species generally do not multiply and decline towards the end of alcoholic fermentation, with the exception of *O. oeni* (Wibowo et al., 1985; Lonvaud-Funel et al., 1991; van Vuuren and Dicks 1993; Fugelsang and Edwards 1997; Volschenk et al., 2006). The bacterial population decreases to approximately 10^2 - 10^3 CFU/ml during the active stages of AF and towards its end (Ribéreau-Gayon et al., 2006). The decrease could be attributed to increased ethanol concentrations, high SO_2 concentrations, low pH, low temperatures, the nutritional status and competitive interactions with the yeast culture (Fugelsang and Edwards 1997; Volschenk et al., 2006). During the active stages of alcoholic fermentation, some bacterial species like *O. oeni*, as well as the yeast population multiply, but bacterial growth remains limited. This increase is influenced by the alcohol, the pH and the addition of SO_2 . A maximum population of approximately 10^4 - 10^5 CFU/ml is reached (Ribéreau-Gayon et al., 2006). After the completion of alcoholic fermentation, the surviving bacterial population enters a latent phase followed by the active growth phase. The active growth phase can last for several days, and during this time, the population increases to 10^6 CFU/ml. MLF begins when the total population exceeds 10^6 CFU/ml and a sufficient biomass is achieved (Ribéreau-Gayon et al., 2006; Muñoz et al., 2011).

Besides their morphology in coccid or rod-like forms, the homofermentative or heterofermentative character is a decisive factor in the classification of LAB. Homofermentative bacteria produce more than 85% of lactic acid from glucose. Heterofermentative bacteria produce carbon dioxide, ethanol and/or acetic acid in addition to lactic acid. The main species of

LAB that can survive in grape must and wine despite the hostile conditions are shown in Table I.2.

Table I.2. Main species of lactic acid bacteria in grape must and wine (Ribéreau-Gayon et al., 2006)

| Genus | Metabolism of sugars | Species |
|---|--------------------------------|--|
| <i>Pediococcus</i> | Homofermentative | <i>P. damnosus</i> <i>P. pentosaceus</i> |
| <i>Leuconostoc</i> <i>Oenococcus</i> | Heterofermentative | <i>Ln. Mesenteroïdes</i> <i>O. oeni</i> |
| <i>Lactobacillus</i> | Homofermentative | <i>L. delbrueckii</i> <i>L. mali</i> |
| | Facultative heterofermentative | <i>L. casei</i> <i>L. plantarum</i> |
| | Strict Heterofermentative | <i>L. brevis</i> <i>L. fermentum</i> <i>L. hilgardii</i> |

I.4 Characteristics of *Oenococcus oeni*

Formerly *Leuconostoc oenos*, *Oenococcus oeni* forms now a distinct species that includes genotypically homogeneous Gram-positive cocci (Dicks et al., 1990). The cells of *O. oeni* are non-flagellated, non-sporulating, spherical or slightly elongated and assembled in pairs or small chains (Figure I.5). They are aerotolerant anaerobes and chemo-organotrophic that require a rich medium containing fermentable sugars and vitamins. Their optimum growth temperature is 22 °C and they prefer an initial growth pH around 4.8. They are strict heterofermentative and the fermentation of D (-) glucose produces lactic acid, CO₂ and ethanol or acetic acid. Some strains are able to catabolize arginine. Their growth is not completely inhibited in the presence of 10% ethanol (v/v). They prefer fructose to glucose; grow very well in the presence of glucose and malate or glucose and fructose, and usually ferment trehalose (Liu et al, 1995; Dicks et al., 1995; Ribéreau-Gayon et al., 2006; Terrade et al., 2009; Dicks and Endo, 2009; du Toit et al., 2011). *O. oeni* is remarkable in its ability to tolerate the nutritionally poor and challenging wine

environment such as acidic pH and the presence of 10% ethanol (v/v). The metabolic by-products formed during the growth of *O. oeni* in wine have been shown to positively contribute to the flavor and mouth feel of wines (acetaldehyde, acetoin, acetic acid, diacetyl, 2,3-butanediol) (Versari et al., 1999; Bartowsky, 2005). Ecologically, it has not been possible to find an environmental reservoir of *O. oeni* outside of wine and fermenting grape must. Due to the highly seasonal nature of wine production, it remains mystery as to how this organism is able to rapidly appear in significant numbers in finished wine to undertake the MLF (Wibowo et al., 1985; Bartowsky, 2005).

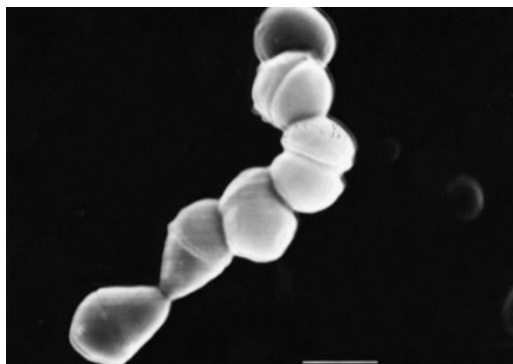


Figure I.5. A chain of *O. oeni* cells (G-Algeria et al., 2004)

The species *Ln. oenos* was considered to form part of the *Leuconostoc* genus until recently, when analysis of the 16S-23S ribosomal rDNA sequence showed that it was different from the other members of the genus. This led to the creation of a new genus, *Oenococcus*, which includes just two species, sharing 96% 16S rRNA sequence homology, the malolactic *O. oeni* (Dicks et al., 1995; Zavaleta et al., 1996) and the non-malolactic *Oenococcus kitaharae* (Endo and Okada, 2006). The first genome sequence of *O. oeni* strain PSU-1, was published by Mills et al., (2005) and showed that the genome was only 1.8 Mb and encoded about 1800 ORFs (Open Reading Frames). This represents a streamlined genome, especially for a free-living bacterium, which is within the lower 10% of all bacterial genome sizes and at the bottom-end of what is observed in other species of LAB. Recently, Marquos et al., (2010) adopted a novel molecular technique to identify *O. oeni* in wine based on the amplification of the 16S rRNA gene with universal primers after restriction with the endonuclease *FseI* generating two fragments of 326 and 1233 bp.

Among wine bacteria, the *FseI* recognition sequence is only found in the 16S rRNA gene of *O. oeni* ensuring the specificity of the method.

I.4.1 Sugar metabolism in *O. oeni*

It has been shown that lactic acid bacteria from wine use sugars as a source of carbon and energy. Wine contains various monosaccharides, mainly pentoses and hexoses as well as disaccharides. The sugars left over by the yeasts after AF are available for fermentation by LAB. The major sugars in wine are represented by glucose and fructose which is the most abundant, then arabinose, ribose and trehalose (Liu et al., 1995; Lonvaud-Funel, 1999; Liu, 2002).

O. oeni uses mainly the pentose phosphate pathway to ferment hexoses and to produce principally but not exclusively lactate. The other molecules produced are essentially CO₂, acetate or ethanol (Figure I.6-B). After being transported into the cell, a glucokinase phosphorylates the glucose into glucose-6-phosphate. Then, two oxidation reactions occur successively: the first leads to the production of gluconate-6-P; the second, accompanied by a decarboxylation, forms the ribulose-5-P. In each of these reactions, a molecule of the coenzyme NAD⁺ or NADP⁺ is reduced (production of 2 reduced coenzymes). The ribulose-5-P is then epimerized into xylulose-5-P. The xylulose-5-P phosphoketolase is the key enzyme of this pathway and the reaction requires phosphate. It catalyzes the cleaving of the xylulose-5-P molecule into acetyl-P and glyceraldehyde-3-P. This latter is metabolized into lactic acid, while the acetyl-P can be reduced to ethanol in a slightly aerated environment or leads to the formation of acetate in an aerated environment with the formation of an ATP molecule. Therefore, changes in conditions not only influence the nature of the products formed but also the energy yield and thus growth (Ribéreau-Gayon et al., 2006; du Toit et al., 2011; Muñoz et al., 2011).

Fructose can also serve as an electron acceptor and is reduced to mannitol to regenerate the NAD⁺ coenzymes necessary for further hexose oxidation (Pilone et al., 1991; Salou et al., 1994). Mannitol is considered a bacterial spoilage product if produced in high amounts and mannitol-tainted wines are described as complex, usually accompanied by high levels of acetic acid, D-lactic acid, n-propanol and 2-butanol. Mannitol wines can also have a slimy texture, vinegar-estery aroma and a slightly sweet taste (Liu, 2002; Bartowsky, 2009).

Some researchers have also reported the production of glycerol, erythritol, and other polyalcohols by *O. oeni* (Liu et al., 1995). According to Veiga-da-Cunha et al., (1993), the pathway responsible for the production of erythritol from glucose under anaerobic conditions involves the isomerization of glucose-6-P followed by cleavage to produce erythrose-4-P and acetyl-P, reduction of erythrose-4-P to erythritol-4-P, and finally hydrolysis of erythritol-4-P to form erythritol. Coenzyme A deficiency appears to be responsible for the formation of erythritol, acetate and glycerol from glucose in the absence of pantothenic acid (Ritcher et al., 2001). In general, the formation of polyalcohols occurs mainly for the reoxidation of NAD (P) H, H⁺ coenzymes (Muñoz et al., 2011).

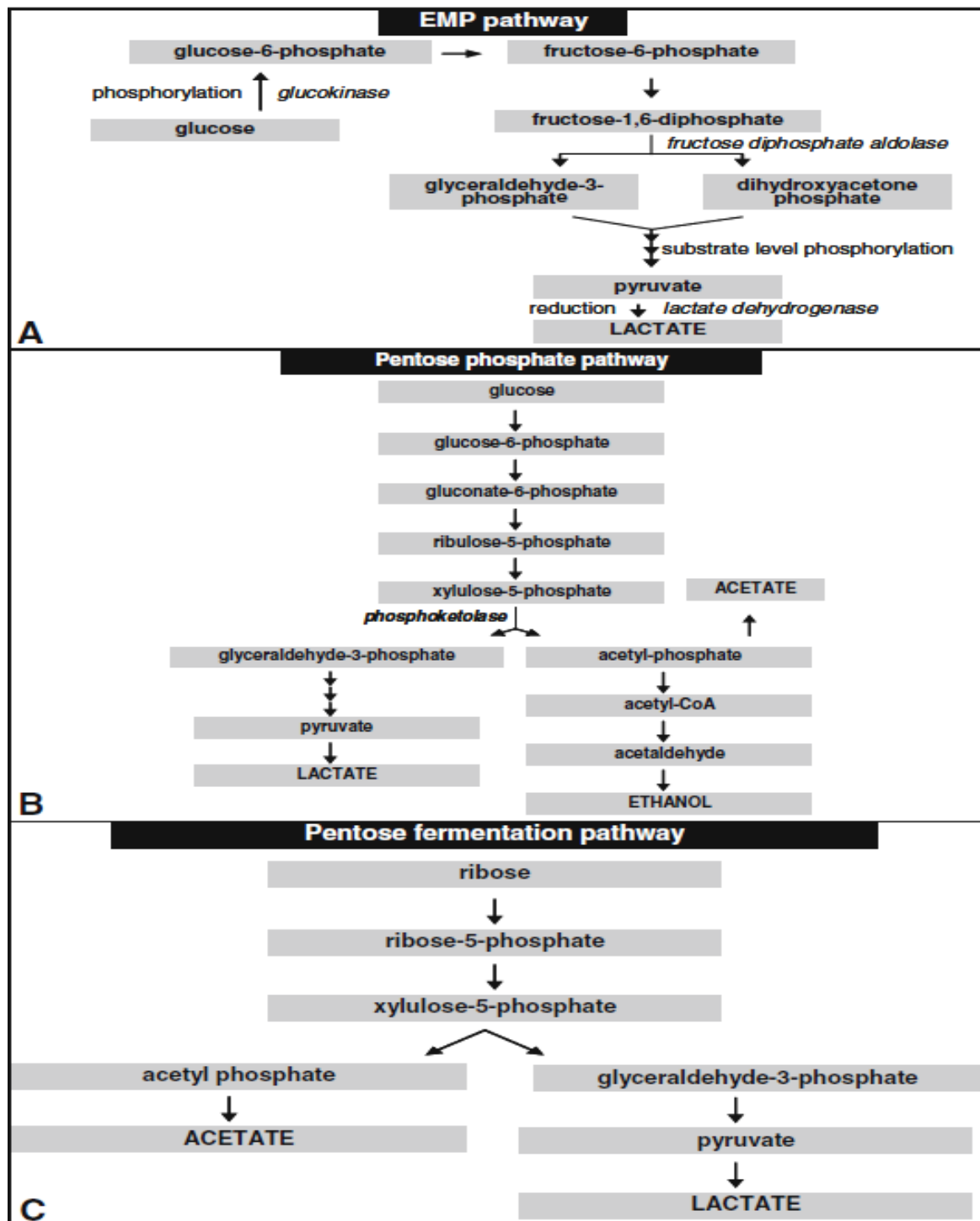


Figure I.6. The three possible carbohydrate fermentation pathways in wine LAB: (a) The EMP pathway (homofermentative) for the fermentation of hexoses, (b) the heterofermentative pentose-phosphate pathway for the fermentation of hexoses, and (c) the pentose-phosphate pathway for the fermentation of pentoses (Fugelsang and Edwards, 1997; Ribéreau-Gayon et al., 2006)

I.4.2 Nutritional requirements of *Oenococcus oeni*

Wine LAB have fastidious nutritional requirements. Carbon essentially comes from sugars and sometimes organic acids. Glucose and fructose are the most represented and the sugar degradation depends on the bacterial species and on environmental factors. *Oenococcus oeni* degrades fructose more easily than glucose and its presence in a mixture with glucose is beneficial to growth. Less than 1 g/l of glucose covers the needs of the bacteria to form biomass necessary for malolactic fermentation. Lactic acid bacteria cannot grow with L-malic acid as a unique carbon source and the role of citric acid should also be taken into account (Hugenholtz, 1993). Substrate co-fermentation by *O. oeni* largely depends on the strain used as well as on the environmental conditions (e.g., substrate, pH, temperature). In a study conducted on Cabernet-Sauvignon, Pinot Noir and Chardonnay wines, using 11 commercial strains of *O. oeni*, malic acid and citric acid were co-metabolized. The rate of malate utilization was dependent on the type of wine (Martineau and Henick-Kling, 1996). When D-glucose, L-malic acid and citric acid were consumed simultaneously at pH 4.8, malic acid was metabolized at a higher rate than with glucose or citric acid alone (Arena et al., 1996; Viljakainen and Laakso, 2000). It appears that when *O. oeni* is allowed to catabolize malic acid before glucose, the consumption rate of sugar increases. In particular, *O. oeni* seems to prefer malate over glucose and citrate as an energy source at low pH (Arninck and Henick-Kling, 1993). Pimentel et al., (1994) reported that three strains of *O. oeni*, isolated from Portuguese wines, metabolized malate before glucose except at high pH (4 and 4.5). In this case, citric acid repressed the consumption of fructose and the consequent formation of mannitol, while stimulating the co-utilization of glucose and the production of acetate. It seems that sugars are not metabolized at low pH (Henick-Kling, 1995). However, the MLF is able to increase the pH to values compatible with sugar consumption and therefore cell's growth (Fourcassie et al., 1992). Mixed-substrates fermentation stimulated cell's growth of *O. oeni* in a model solution at pH 5. The specific growth rate (μ_{\max}) increased from 0.05 to 0.087 and 0.14 h⁻¹, using glucose, glucose-citrate and glucose-fructose substrates, respectively. These results were correlated with an increase in ATP production via the acetate kinase pathway. However, citrate alone did not stimulate microbial growth without the availability of fermentable carbohydrates (Salou et al., 1994). Saguir and Manca de Nadra, (2001) demonstrated that L-malic acid and citric acid favored *O. oeni* growth in nutritional stress

conditions. Citric acid was mainly involved in the biosynthesis of the aspartate-derived essential amino acids and glucose in the cysteine biosynthesis.

Typically, wine contains between 100 and 600 mg/l of total nitrogen mainly in two forms: peptides and free amino acids. Amino acids are essential for protein synthesis and deficiencies in wine contribute to sluggish and stuck MLF even if starter bacteria are inoculated. The presence of several amino acids is essential for bacterial development because of numerous auxotrophies. Compounds found to be essential were valine, isoleucine, leucine, histidine, phenylalanine, tryptophan and arginine. Omission of all amino acids except for arginine, threonine, glycine and proline led to growth failure (Fernandez et al., 2003; Remize et al., 2006; Terrade et al., 2009; Terrade and de Orduña, 2009). In some cases, the uptake of dipeptides like “Gly-Gly” instead of free amino acids, increased the bacterial growth rate of *O. oeni* strain X₂L but not with “Leu-Leu” that required energy (Fernandez et al., 2004). Some studies showed that glutamic acid was not essential for the growth of the MLB (Vasserot et al., 2003; Remize et al., 2006). Depending on the strain, arginine, histidine and tyrosine can be catabolized and serve as energy sources (Ribéreau-Gayon et al., 2006). Notably, *O. oeni* is able to break down arginine secreted by yeasts or released during autolysis, via the arginine deiminase pathway. It is a potential source of energy especially after glucose exhaustion and nutrient starvation and restores the bacterial ability to grow. The synthesis of three enzymes; arginine deiminase, ornithine transcarbamylase and carbamate kinase is induced by the presence of arginine. These enzymes transform stoichiometrically each mole of arginine into one mole of ornithine and two moles of ammonium (Liu et al., 1995; Liu and Pilone, 1998; Tonon and Lonvaud-Funel, 2000; Tonon et al., 2001; Ribéreau-Gayon et al., 2006). Interestingly, arginine in combination with fructose protects bacteria against wine shock and stimulates pH-independent resistance mechanism in bacteria (Bourdineaud, 2006). Somehow, Terrade and de Orduña, (2009), found that arginine and citrulline did not increase the growth of two oenological strains of *O. oeni* in comparison with *L. buchneri* under low pH, but arginine may play a role in microbiological stability.

Among nitrogen compounds, puric and pyrimidic bases play an important role in activating growth but they are not essential. In fact, the needs for adenine, guanine, uracile, thymine, thymidine and xanthine are strain dependent (Ribéreau-Gayon et al., 2006).

Minerals such as Mg^{2+} , Mn^{2+} , K^+ and Na^+ are necessary. The first two are often used as key enzyme cofactors of the metabolism (kinases, malolactic enzyme). Among all metals, the lack of manganese (Mn^{2+}) was found to prevent growth of *O. oeni*. Phosphorus is also required; it is a constituent of nucleic acids, phospholipids and ATP (Ribéreau-Gayon et al., 2006; Terrade and de Orduña, 2009; Theobald et al., 2011).

Vitamins are coenzymes or coenzyme precursors. LAB are incapable of synthesizing B-group vitamins, in particular nicotinic acid, thiamin, biotin and pantothenic acid and their limitations induce growth failure during MLF (Remize et al., 2006; Ribéreau-Gayon et al., 2006; Terrade and de Orduña, 2009).

Some winemakers use inactive dry yeasts that provide amino acids, monosaccharides and fatty acids favorable for the growth of wine lactic acid bacteria. Ageing on lees plays also a crucial role in the improvement of bacterial growth (Patynowski et al., 2002; Andujar-Ortiz et al., 2010).

I.4.3 The malolactic fermentation

Winemaking may involve two fermentation steps. The first one is the alcoholic fermentation (AF) and is conducted by yeasts belonging mainly to the *S. cerevisiae* species. The second one is the malolactic fermentation (MLF) and is performed by lactic acid bacteria belonging mainly to the *O. oeni* species. MLF plays an important role in the determination of the final quality of most red wines, but also certain white wines and classic sparkling wines. Apart from an increase in pH, additional sugars are fermented and aromatic compounds are produced which change the sensorial board of the wine (Liu, 2002; Bauer and Dicks, 2004; Volschenk et al., 2006; Lerm et al., 2010). The cells gain energy from the uniport of the monoanionic L-malate through the generation of a proton gradient across the cell membrane (Salema et al., 1994). Only strains of *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus* resistant to low pH (pH < 3.5), high SO_2 (50 ppm) and 10 % ethanol (v/v), survive in wine (Wibowo et al., 1985; van Vuuren and Dicks, 1993; Lonvaud-Funel, 1999). However, towards the end of the AF, spontaneous MLF is mainly driven by *O. oeni* (van Vuuren and Dicks, 1993; Ribéreau-Gayon et al., 2006). The malic acid concentration differs among grape cultivars and may also differ from year to year in the same cultivar. For this reason, along with other factors, the duration of MLF may differ from one year to the next (Ribéreau-Gayon et al., 2006). It is difficult to induce MLF in wines with malic

acid levels below 0.8 g/l. In this case, using ML starter cultures with a high malate permease activity is recommended (Lallemand reports 1999-2004).

MLF reduces wine acidity by converting the dicarboxylic L-malic acid into the monocarboxylic L-lactic acid and CO₂ by the malolactic enzyme (MW 138 kDa, encoded by *mleA* gene). This enzyme requires NAD⁺ and Mn²⁺ as co-factors (Labarre et al., 1996 b; Strasser de Saad, 1983). The monoanionic L-malate (HM⁻) enters the cell through a membrane malate permease encoded by *mleP* gene, whereas the membrane permeable malate (H₂M) enters the cell by simple diffusion (Labarre et al., 1996a; Bony et al., 1997) (Figure I.7).

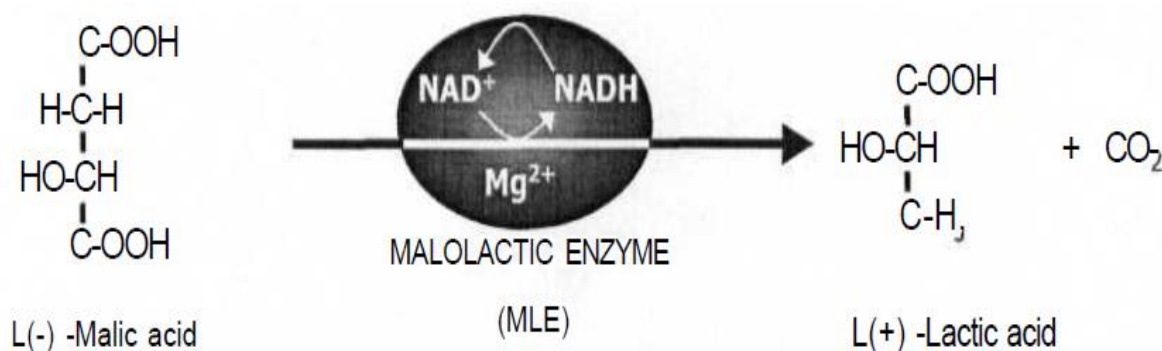


Figure I.7. Malolactic fermentation reaction (Volschenk et al., 2006)

I.4.4 Role of the membrane ATPase of *O. oeni*

Apart from the degradation of carbon substrates in catabolic reactions, the metabolic energy available in bacterial cells can be generated by the ATPase proton pump located in the plasma membrane. Besides, there is a relationship between ATPase activity and adaptation of *O. oeni* to wine conditions (Garbay and Lonvaud-Funel, 1994). ATPase activity depends on the cell's growth phase since it increases during the exponential growth phase reaching a maximum value at its end and decreases during the stationary phase (Carreté et al., 2002). Guzzo et al., (2000) and Carreté et al., (2002) proposed the existence of 2 types of membrane ATPase: H⁺-F₀-F₁ ATPase, Mg²⁺ dependent, having a MW between 300 and 500 kDa, and K⁺-ATPase (P-type). They are distinguished by their susceptibility to inhibitors. H⁺-F₀-F₁ ATPase responsible for 80 % of the ATPase activity in the cell membrane is inhibited by DCCD (N, N-

Dicyclohexylcarbodiimide), while K^+ -ATPase responsible for the remaining 20 % of the ATPase activity is inhibited by vanadate (Guzzo et al., 2000). H^+ -ATPase plays an essential role in the regulation and maintenance of the internal pH. This protein is crucial for acidity tolerance (pH 3.5 or lesser), since mutants of *O. oeni* deficient in H^+ -ATPase were unable to survive in acidic environments (Tourdot-Maréchal, 1993; Tourdot-Maréchal et al., 1999; Galland et al., 2003).

MLF is carried out by some species of the genera *Lactobacillus*, *Lactococcus*, *Oenococcus* and *Pediococcus* (Kunkee, 1991). During MLF, the L-malic acid enters the cells and is subsequently decarboxylated by the malolactic enzyme to yield L-lactic acid and carbon dioxide. The reaction products are then released outside the cell (Figure I.8). The growth and malate consumption in *O. oeni* are not always correlated and the MLF can be successfully carried out without any noticeable growth (Capucho and San Romão, 1994). The malolactic reaction which by itself is not energetic, leads to ATP synthesis via the membrane ATPase. In fact, it generates a proton motive force (PMF) that creates a proton efflux thus activating the ATPase/ATP synthase (Cox and Henick-Kling, 1989; Poolman et al., 1991; Loubiere et al., 1992; Cox and Henick-Kling, 1995; Salema et al., 1996). The PMF is the resultant of the membrane potential $\Delta\phi$ and the internal/external pH difference ΔpH .

Several exchange systems in bacteria mediate the net translocation of charges across the cytoplasmic membrane which results in the generation of a membrane potential. If, in addition, the conversion of a substrate to a product consumes protons, the fermentation will lead to an increase of the internal pH and, consequently, to the formation of a ΔpH (Lolkema et al., 1995).

Under acidic conditions, *O. oeni* maintains a constant internal pH of 5.8-6.3 and involves an electrogenic uniport step. MLF in *O. oeni* proceeds through the uptake of the negatively charged mono-protonated HM^- , which is the prevalent ionic species of malate under acidic conditions. In the cytoplasm, HM^- is rapidly decarboxylated leading to the formation of a membrane permeable lactate (HL) in a proton consuming reaction, the alkalization of the cytoplasm and the generation of a ΔpH ($pH_{in} - pH_{ex}$) (Salema et al., 1994). Therefore, MLF results in the generation of both components of the PMF: the $\Delta\phi$, inside negative, by the inflow of negative charges with $Hmalate^-$ and a ΔpH , inside alkaline, by the internal consumption of protons in the decarboxylation reaction (Ramos et al., 1994). During the growth of *O. oeni*, $\Delta\phi$ increases (negative charges inside the cell) when malate enters the cell (Loubière et al., 1992). According

to Salema et al., (1996), the malolactic enzyme cleaves malic acid, and lactic acid and CO_2 are co-excreted with protons, which causes the pH_{in} to increase. The ΔpH allows the entry of protons and the increase of ATP by the ATP synthase (Cox and Henick-Kling, 1989; 1995) (Figure I.8).

It was recently shown by flow cytometry that the pH_{in} of *O. oeni* during the early stages of MLF drops and becomes equal to the pH_{ex} . For example, it drops to 3.5 if the pH_{ex} is 3.5. Then, it increases to 6 during the exponential growth phase and decreases again to equilibrate with the pH_{ex} in the late stationary phase. The membrane potential increases in early MLF and then decreases. When malic acid is totally exhausted, the pH_{in} along with the $\Delta\phi$ and the vitality decrease. Finally, it was shown that the higher the ΔpH ($\text{pH}_{\text{in}} - \text{pH}_{\text{ext}}$) in *O. oeni* cells is, the shorter the lag phase of the MLF is. The degradation of L-malic acid by non-growing or poorly growing cells of *O. oeni* can be even more rapid at low pH_{ex} values due to an increase in the ΔpH ($\text{pH}_{\text{in}} - \text{pH}_{\text{ex}}$). To better manage the initiation of MLF in wines, the physiological state of *O. oeni* cells must be taken into account (Bouix and Ghorbal, 2015).

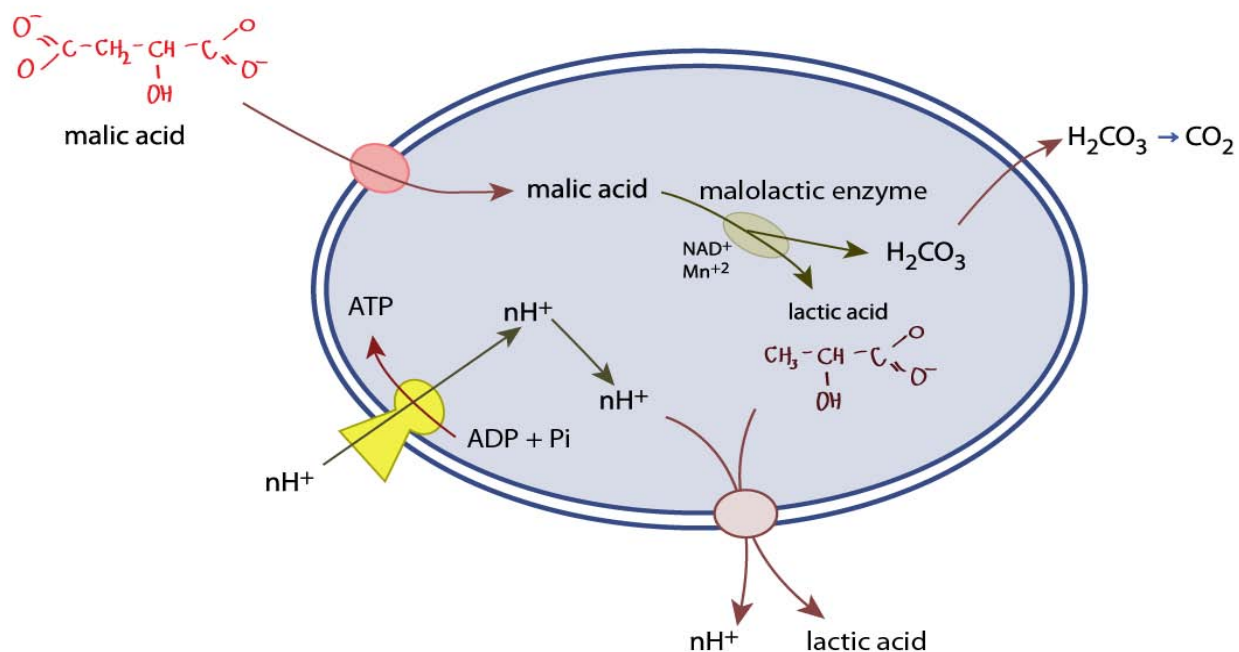


Figure I.8. ATP generation during MLF (Henick-Kling et al., 1991)

I.5 Impact of MLF on the overall wine composition and quality

I.5.1 Loss of acidity and increase in pH

In general, the overall decrease in wine acidity resulting from MLF can vary from 0.1% to 0.3%, and the pH may rise by 0.1 to 0.3 units (Ribéreau-Gayon et al., 2006). Wines produced from grapes cultivated in cool climate viticultural areas naturally contain high concentrations of L-malic acid (up to 8 g/l), and are considered to benefit from such deacidification (Henick-Kling, 1995; Lonvaud-Funel, 1999). Subsequently the rise of pH after MLF, the alcoholic content, the temperature of wines and the interactions of various anions and cations, often promote the precipitation of potassium bitartrate (Beelman and Gallander, 1979; Carré, 1982).

I.5.2 Microbial stability

It is usually believed that a wine that has undergone MLF is more stable from a microbial point of view than the same wine without MLF. This is due to the consumption of nutrients by *O. oeni* that would otherwise be available for the growth of spoilage organisms. Such nutrients include L-malic acid, citric acid, amino acids, nitrogen bases, vitamins and fermentable sugars left after AF (Volschenk et al., 2006). Furthermore, LAB produces antimicrobial compounds such as lactic acid and bacteriocins (nisin, plantaricin) that inhibit the growth of other related bacterial species (Rammelsberg and Radler, 1990; Henick-Kling, 1993). Besides, if MLF occurs prior to bottling it prevents microbial growth in the bottle. There are several reasons why growth in the bottle is undesirable.

I.5.3 Sensorial modifications

Malolactic bacteria can influence the mouth feel of wine. Flavor attributes by MLF (Figure I.9) can be described as buttery, lactic, nutty, yeasty, oaky, sweaty, fruity and earthy (Henick-Kling, 1993; Laurent et al, 1994, Costello et al., 2012). Mechanisms by which MLB can influence wine flavor may include: removal of existing flavor compounds by metabolism and adsorption to the cell wall; production of new bacterial-derived flavor compounds from the metabolism of sugars, amino acids and other substrates; and metabolism or modification of grape-and yeast-derived secondary metabolites to end products having a greater or lesser sensorial impact or masking

other aromatic compounds (Bartowsky and Henschke, 1995; Pozo-Bayon et al., 2005, Izquierdo-Cañas et al., 2008, Bartowsky and Borneman, 2011; Lopez et al., 2011). The net impact of MLF on wine sensorial properties, depends on many factors such as the bacterial strain characteristics, the timing of inoculation (simultaneously or after AF), the varietal aroma intensity of wine and the vinification techniques employed (Henick-Kling, 1995; Semon et al., 2001).

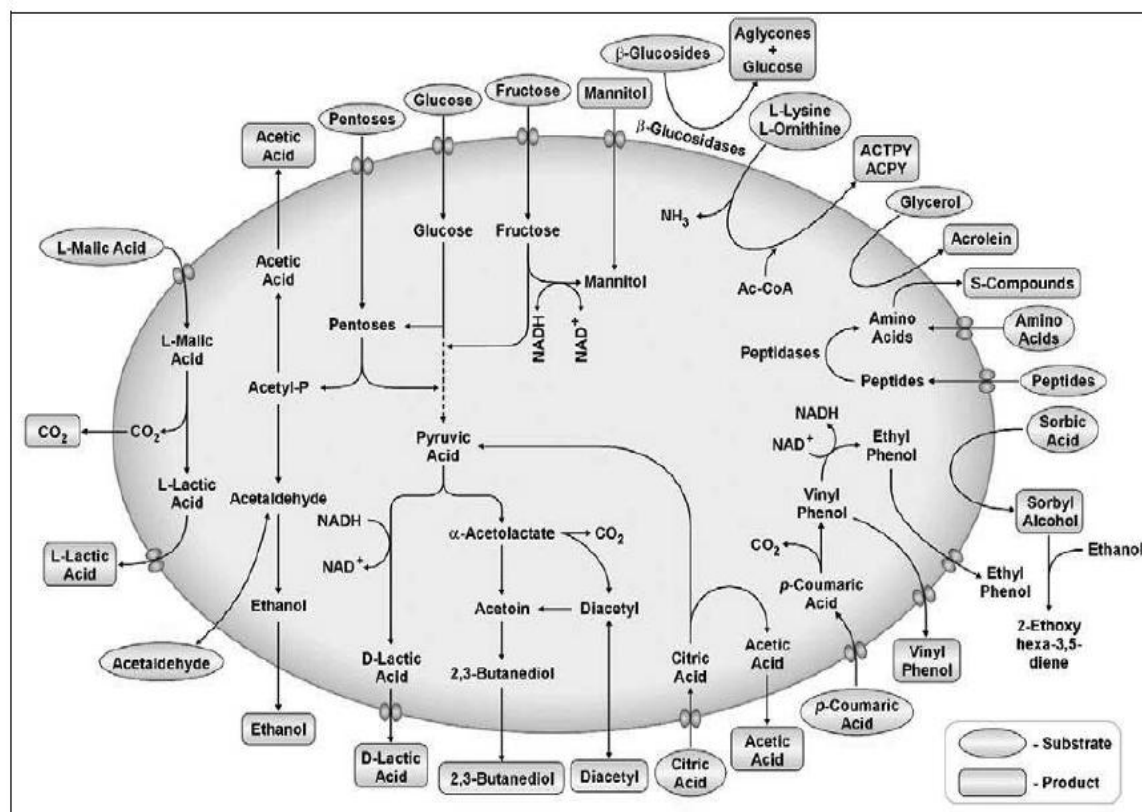


Figure I.9. A schematic representation of the production and modification of flavor-active compounds by LAB (Swiegers et al., 2005)

I.5.4 Impact of MLF on red wine color

MLF in red wine can reduce color intensity. The increase of pH that accompanies MLF can impact the pH-dependent equilibrium of anthocyanin pigments. The glycosidase activity of MLB may also be a potential source of color reduction in red wines (Bartowsky et al., 2002). Glycosylated anthocyanin pigments can be enzymatically attacked by the glycosidase of *O. oeni* and the glucose that is released by this reaction may provide a source of energy for the bacteria, resulting in enhanced growth and MLF. The occurrence of MLF can also affect the condensation

of anthocyanin and tannin, which in turn, has also been shown to have noticeable effect on red wine color and astringency. The metabolism of carbonyls, such as acetaldehyde, by LAB may also have some influence on the equilibrium and stability of red wine color components (Vivas et al., 1997; Osborne et al., 2000; Bartowsky et al., 2002; Bartowsky and Henschke, 2004).

I.6 Biogenic amines

Amines have an important metabolic role in living cells. Polyamines are essential for growth. Amines like histamine and tyramine are involved in the nervous system function and in the control of blood pressure. Biogenic amines (BA) are undesirable in all foods and beverages because if absorbed at high concentrations, they may induce headaches, respiratory distress, heart palpitation, hyper or hypotension, and several allergic disorders (Lonvaud-Funel, 2001). The BA most frequently identified in wine are 1-methylhistamine, putrescine, cadaverine, methylamine, tryptamine, 2-phenylethylamine and ethylamine (Figure I.10). Some of them like histamine and spermidine are normal constituents of the raw materials (Guerrini et al., 2002; Soufleros et al., 1998 and 2007).

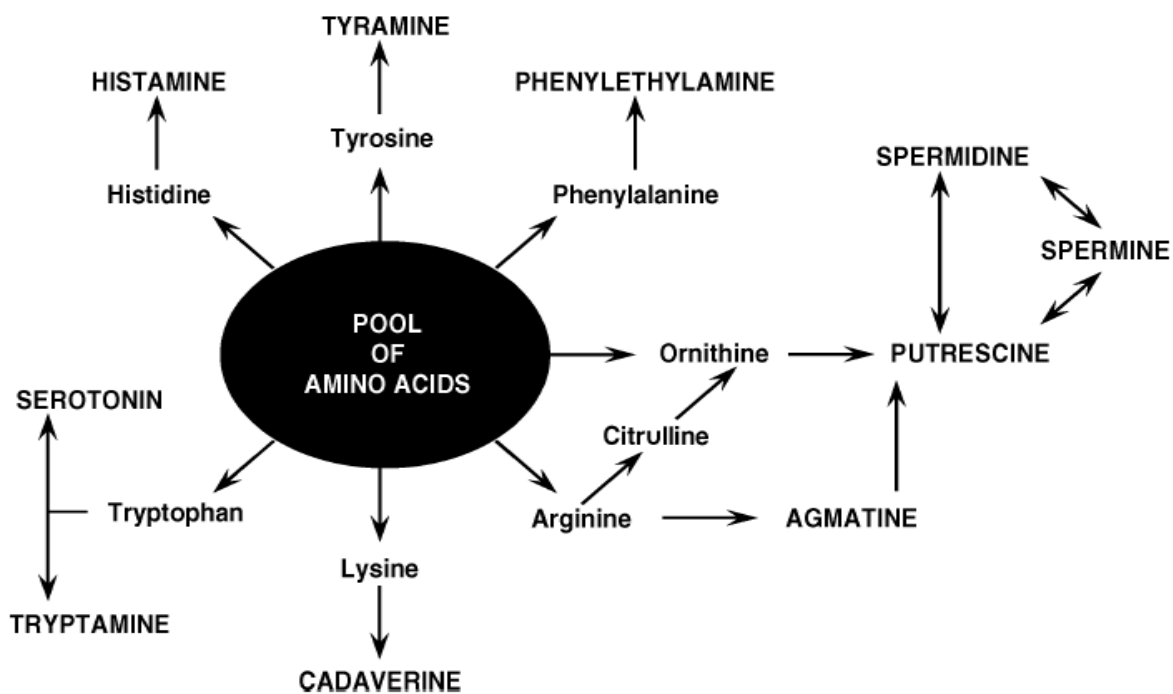


Figure I.10. Precursor amino acids of biogenic amines (Marquos et al., 2008)

The role of wine lactic acid bacteria in amine biogenesis has been studied. It varies according to the bacterial strain and the environmental conditions. When a bacterial cell is stressed, it can activate several metabolic processes for ATP production and survival, one of which is BA production through amino acid decarboxylation (Konings et al., 1995; Lonvaud-Funel, 2001). Nanneli et al., (2008) showed that the use of a selected bacterial strain for the induction of MLF led to the accumulation of putrescine. Therefore, the selected strain possessed the ornithine decarboxylation pathway. In an uncontrolled MLF, some strains of *Lb. hilgardii*, a common spoilage LAB, can grow in wine and increase the levels of histamine and putrescine detected through HPLC and LC-Ion Trap Mass Spectrometry and adversely affect the quality of wine. The pH, temperature, organic acids, amino acids, sugars and ethanol are parameters that influence the production of BA (Arena and Manca de Nadra, 2001; Arena et al., 2007; Pedro et al., 2010). Some studies investigated the ability of some strains of *O. oeni* to produce BA. Depending on the strain studied and the wine type, tyramine, cadaverine and histamine were produced at levels between 1 to 5.3 mg/l (Guerrini et al., 2002; Rosi et al., 2009). Grape varieties or high acidity wines produced in cool climate regions influence the wine amines content. In these regions, some strains of *O. oeni* were able to release phenylethylamine at concentrations up to 47 mg/l and tyramine up to 36 mg/l. These levels are considered to be noxious for consumer's health (Vigentini et al., 2009). Therefore, commercial malolactic starters, after careful selection (e.g., active lyophila), should be added to the vinification process in order to decrease the formation of biogenic amines. The wine storage on lees contributes also to the increase in the biogenic amines level (Lopez et al., 2008; Marquos et al., 2008).

I.7 Inhibitors of malolactic fermentation

Malolactic fermentation is a process influenced by various factors that can inhibit the growth of *O. oeni* and/or stop the malic acid consumption. The inhibitory metabolites released by yeasts during alcoholic fermentation, the grape composition, the physical chemical parameters and the winemaking practices influence the onset and accomplishment of MLF (Bauer and Dicks, 2004; Ciani and Comitini, 2007). The selection of the yeast and bacterial starter strains can be controlled by the winemaker. The interactions that may occur between the yeast and the bacterial strains during winemaking have a direct effect on the bacterial growth and malolactic activity. A yeast strain can be stimulatory, inhibitory or neutral towards a bacterial strain. Various studies

attempted to understand the different types of interactions between yeasts and bacteria and to identify the nature of the metabolites that mediate these interactions (Henick-Kling and Park, 1994; Alexandre et al., 2004; Osborne and Edwards, 2006).

Some of the MLF inhibitory factors are discussed in this section.

I.7.1 Temperature

The optimal temperature for growth of *Oenococcus oeni* is between 27 and 30 °C. But this is not the case in a medium containing a high level of alcohol (13-14% v/v) such as wine. In this case, the optimum growth is between 20 and 23 °C (Britz and Tracey, 1990; Henick-Kling, 1993; Bauer and Dicks, 2004; Ribéreau-Gayon et al., 2006). The optimal temperature for MLF in the presence of ethanol (10-14 % v/v) is between 20 and 25 °C. For a successful MLF, it is recommended to conduct it at a temperature between 18 and 20 °C in order to limit the risks of off-flavors mainly the production of a high volatile acidity (Coucheney et al., 2002). Therefore, the ideal temperature for both bacterial growth and malic acid consumption is around 20 °C (Ribéreau-Gayon et al., 2006). A temperature lower than 18 °C delays the initiation of MLF and its accomplishment, whereas temperatures below 16 °C inhibit the growth of *O. oeni* and decrease the cellular activity (Henick-Kling, 1993; G-Alegria et al., 2004; Ribéreau-Gayon et al., 2006). Chu-Ky et al., (2005) found that cold shocks between 8 and 14 °C affect the plasma membrane but not the cell survival. Temperatures higher than 25 °C disturb the membrane permeability and denature proteins like the membrane ATPase. A heat shock at non-lethal temperatures induces the expression of resistance genes in bacteria like the *hsp 18* gene that encodes the heat shock protein Lo 18. These proteins help maintain the cell's integrity either by repairing the damages caused or by preventing them. Therefore, it could be useful to adapt bacteria to the harsh conditions of wine by heat shocks of the precultures (Chu-Ky et al., 2005).

Tourdot-Maréchal et al., (2000) compared the kinetics of membrane fluidity variation of instantaneously stressed *O. oeni* cells with cells adapted to the stress factor by a pre-incubation in inhibitory growth conditions. Membrane fluidity of heat-adapted cells increased only slightly when exposed to 42 °C and the rate of membrane fluidization was five-fold lower than that of non-adapted cells. Therefore, *O. oeni* was able to express a stress-tolerance mechanism such as the heat shock proteins.

1.7.2 pH

The pH of wine is usually between 3 and 4 but may be lower than 3 in certain wines. The pH is a key factor for the success of MLF. It plays an important role in the determination of the LAB species that will survive and develop as well as their growth rate. At pH values higher than 3.5, a diverse bacterial population is present and *Lactobacillus* and *Pediococcus* are usually the most abundant LAB whereas the more acid wines favor the presence of *O. oeni*. The optimum pH for *O. oeni* growth is between 4.3 and 4.8 and the optimum pH for L-malic acid consumption is 4 (Salou et al., 1991; Bauer and Dicks, 2004; Ribéreau-Gayon et al., 2006). The lower is the pH and the slower is the bacterial growth. The low extracellular pH decreases the intracellular pH of bacteria. Some acids in their dissociated or undissociated forms diffuse inside the cell and affect the proteins (mainly enzymes) and the DNA (Guzzo, 2000). The acidity also affects the selective permeability of the plasma membrane by targeting mainly the membrane proteins (enzymes and transporters). The bacteria can modify the composition of their plasma membrane in response to this stress (fatty acids and proteins).

In wine, the lowest pH at which growth is possible is around 3. But this value depends on the other characteristics of the environment, such as the alcoholic content of wine. If other factors are favorable, then this value is lowered and vice versa. The lag phase is prolonged and the growth is slowed down when the pH is low. To the contrary, if the pH is favorable (pH close to 4), the lag phase goes unnoticed. The growth arrest in an acidic environment happens when the intracellular pH (pH_i) reaches a certain limit (McDonald et al., 1990).

The H^+ -ATPase plays an important role in the maintenance of the intracellular pH in an acidic medium such as wine by extruding protons (refer to paragraph 1.4.4). Fortier et al., (2003) showed that its activity increases at low pH values. It is 1.6 times higher at pH 3.5 than at pH 5.5. This is due to an increase in the expression of the *atp* gene that encodes this enzyme. The lower is the pH and the higher is the expression of the *atp* gene as well as the ATPase activity. Mutants of *O. oeni* lacking the ATPase cannot survive under acidic conditions.

The ability of bacteria to obtain energy from the metabolism of glucose and fructose is inhibited at pH 3.2. The optimum pH for glucose catabolism in *O. oeni* and *Lb. plantarum* is between 4

and 6 (Henick-Kling, 1986; Pan et al., 2010). Finally, the MLF participates to the acid tolerance by increasing the intracellular pH and producing ATP (refer to paragraphs 1.4.3 and 1.4.4).

I.7.3 Oxygen

Malolactic bacteria have been shown to be sensitive to excessive amounts of oxygen. This is the case of *O. oeni*. This means that exposure of the bacteria to undue amounts of oxygen after the completion of alcoholic fermentation should be avoided. Although it has been noted that even low concentrations of oxygen may detrimentally influence MLF, micro-oxygenation may have a positive effect on MLF due to the gentle stirring action associated with the micro-oxygenation process itself. Besides, oxygen reacts with flavoproteins (proteins that contain a nucleic acid derivative of riboflavin such as FAD or Flavin Adenine Dinucleotide and FMN or Flavin Mononucleotide), to maintain a favorable redox equilibrium (Ribéreau-Gayon et al., 1998).

I.7.4 Sulfur dioxide

Sulfur dioxide is an important preservative commonly used in winemaking. Its antimicrobial (Carreté et al., 2002); antioxidant (Danilewicz, 2003) and antienzymatic (Wedzicha et al., 1991) functionalities at low concentrations make it an ideal and cost effective food stabilizer. Besides, SO₂ promotes the extraction and stabilization of phenolic and aromatic compounds due to its effect on membrane permeability. Its effectiveness is directly linked to wine composition and pH and the total concentration found in wine is the sum of added SO₂ (exogenous) and produced SO₂ by *S. cerevisiae* (endogenous). The harvested grapes must be sulfitized with levels between 30 to 50 mg/l for red wines and 60 to 100 mg/l for white wines. The endogenous SO₂ level strongly depends on the yeast strains. *S. cerevisiae* usually produces between 10 to 30mg/l, but some strains can produce more than 100mg/l (Rankine et al., 1970; Suzzi et al., 1985). The production of SO₂ by yeasts depends on the availability of nutrients such as nitrogen (Gyllang et al., 1989; Osborne and Edwards, 2006), pH, temperature (Eschenbruch and Bonish, 1976) and the clarification degree (Liu and Gallander, 1982 and 1983). SO₂ can bind to acetaldehyde and other carbonyl substances in wine.

In wine, the dissociation equilibrium of sulfur dioxide comprises the molecular SO₂, the bisulfite and the sulfite ions according to the following scheme (Figure I.11):

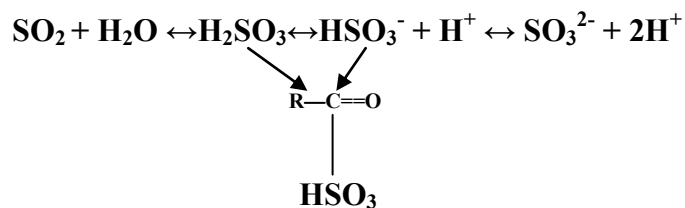


Figure I.11. Different forms of SO₂ in wines (Free form: SO₂, H₂SO₃, HSO₃⁻, SO₃²⁻; Active forms: SO₂, H₂SO₃ Combined form: RCOHSO₃)

SO₂ is a major factor in MLF inhibition (Henick-kling and Park, 1994). At pH 3.5, a concentration of 15 mg/l or more of free SO₂ is lethal for *O. oeni*. The malolactic activity is very sensitive to sulfur dioxide. 20 mg/l of combined SO₂ reduce the malolactic activity by 13 %, 50 mg/l by 50 % and 110 mg/l totally inhibit it (Lafon-lafourcade, 1970). 61 mg/l of total SO₂ at pH between 3.5 and 3.8, and temperature between 15 and 20 °C in Cinsault wine caused a loss of wine color (Van der Westhuizen and Loos, 1981). Lafon-Lafourcade and Peynaud (1974); Rose and Phil-Kington (1989); Fugelsang and Edwards (2007), showed that the bound form is 5 to 10 times less effective or has much weaker antimicrobial properties than the free form. However numbers of researchers have suggested that bound SO₂ may be more antimicrobial than previously believed (Larsen et al., 2003; Osborne and Edwards, 2006) particularly with regard to the more SO₂ sensitive wine LAB. The major form of bound SO₂ in wine is typically acetaldehyde-bound SO₂ as acetaldehyde is produced by yeasts during AF (Romano et al., 1994). The next most important SO₂ binder found in wines is pyruvic acid, which is also a product of yeast metabolism. Wells and Osborne (2011) and Osborne et al., (2006) showed that SO₂ bound to acetaldehyde and pyruvic acid was inhibitory to wine LAB growth (*Lb. hilgardii* WS-7, *P. parvulus* WW-1, *P. damnosus* ATCC 43013 and *O. oeni* Viniflora oenos) at concentrations as low as 5 mg/l. At concentrations higher than 50 mg/l, the growth was totally arrested (Wibow et al., 1985). Wine LAB can release the SO₂ bound to acetaldehyde and pyruvic acid at pH 3.3 and 3.6. Bound SO₂ has a bacteriostatic rather than a bactericidal action and causes a sluggish bacterial growth and MLF.

The availability of the molecular SO₂ which is the active form depends on the concentration of free SO₂ and the pH. According to Romano and Suzzi (1992), SO₂ penetrates in its molecular form within the cell by diffusion. At a high cytosolic pH nearly 6.5, it gives the HSO₃⁻ followed by an accumulation of charged sulfite ions (SO₃²⁻) that react with biological molecules such as

essential vitamins, cofactors, proteins, nucleic acids and enzymes. These reactions lead to the bacterial growth inhibition and cell's death (Gunnison et al., 1981). Delfini and Morsiani (1992) found that a concentration of molecular SO₂ higher than 0.5 mg/l was able to completely inhibit 10 strains of *Leuconostoc* and 4 strains of *Lactobacillus*. Moreover, a population of 2 x 10⁶ cells/ml of *Leuconostoc* was totally killed 22 h after exposure to 0.84 mg/l of molecular SO₂.

Carreté et al., (2002) showed that 40 mg/l of total SO₂ at pH 5.5 and at 27 °C were able to reduce about 63% of the H⁺-ATPase specific activity and consequently reduced the bacterial viability and malolactic activity. They also showed that SO₂ worked synergistically with decanoic acid.

At the end of MLF, the wine must be sulfitized again in order to reduce the risk of post-MLF spoilage by *O. oeni* responsible for the production of acetic acid and biogenic amines and to control the growth of spoilage bacteria such as *Pediococcus* and *Lactobacillus* (Jackowetz and de Ordña, 2012). Finally, a low pH and a high ethanol level act synergistically with sulfur dioxide.

I.7.5 Ethanol

Ethanol is the main metabolite released by *S. cerevisiae* during the AF and exerts a direct effect on the growth and metabolic activities of MLB. It plays an essential role in their survival in wine and the fulfillment of the MLF. The ability of LAB to tolerate elevated concentrations of ethanol is dependent on a number of factors including the strain selected, the fermentation temperature and the shock duration and its severity (Bauer and Dicks, 2004; Lerm et al., 2010). Ingram et al., (1984) and Rosa and Sa-Correia, (1992) argued that the ethanol inhibition is due to the incorporation of this small amphiphilic molecule to the polar lipid-water interface of the plasma membrane. The barrier will be ruptured which will increase the membrane polarity and allow the passage of other polar molecules. Indeed, this can lead to an increase of the diffusion of H⁺ ions inside the cell and a simultaneous loss of certain intracellular compounds (Da Silveira et al., 2002; 2003). Furthermore, ethanol will affect the hydrophobic interactions between the hydrocarbon chains of phospholipids and the intrinsic proteins of the membrane. The positioning and the configuration of membrane proteins will be also affected as well as their activity, notably the ATPase activity.

A rate of 10-14% ethanol (v/v) combined with an acid shock, induces an instantaneous membrane fluidization which reduces bacterial viability (Chu-Ky et al., 2005). Capucho and San

Romaô, (1994) showed that the malolactic activity was not inhibited with an alcoholic degree of more than 12% (v/v). However, they demonstrated along with other authors an inverse correlation between growth and high levels of ethanol (Davis et al, 1988; Henick-Kling, 1993; Alexander et al., 2004; Bauer and Dicks, 2004). Generally, strains of *O. oeni* are able to survive and proliferate in 10% ethanol (v/v) at pH 4.7 (Tracey and Britz, 1990). Some strains of *O. oeni* and *L. plantarum* can even grow at 13% ethanol (v/v) (G-Algeria et al., 2004). Temperatures of 25 °C and above combined with ethanol levels of 10 to 14 % cause a total cessation of growth (Henick-Kling, 1993). Therefore, ethanol and temperature work synergistically. Zapparoli et al., (2009) investigated a possible strategy to conduct MLF in wines that generally do not support MLF due to high ethanol concentrations. The study was performed in Amerone wines with an alcohol content of up to 16% (v/v) and both co-inoculation and sequential inoculation strategies were investigated. Complete degradation of L-malic acid was observed with the use of a starter preparation consisting of bacterial cells that were treated in a wine/water mixture for 48 hours prior to inoculation in the wine. Despite the fact that complete MLF occurred under both inoculation scenarios, the sequential inoculated wine took 112 days to complete MLF, compared to 70 days for co-inoculation. Co-inoculation of high alcohol wines with acclimatized bacterial cells could be a valid strategy for conducting complete MLF in potential high alcohol wines, especially in warm regions like South Africa where grapes are harvested with a high sugar concentration. Ethanol in high levels (118.3 g/l) at pH 3.2, may affect the volatile aroma profile of some white Riesling and Chardonnay wines (Knoll et al., 2011).

O. oeni cells adapted to ethanol are capable of responding to its fluidizing effect by increasing the rate of lipids at the lipid-water interface and decreasing permeability to keep cell integrity (Couto et al., 1996). It was demonstrated that adapted bacteria have an adjustment mechanism which allows compensating the accumulation effect of toxic substances in their membrane (Couto et al., 1996; Tourdot-Maréchal et al., 2000; Teixeira et al., 2002). Tourdot-Maréchal et al., (2000) showed that the rate of membrane fluidization was threefold lower in bacterial cells pre-incubated in ethanol than with non-adapted cells. Pre-incubation in ethanol induced a rapid increase in membrane rigidity. This response involved the synthesis of stress proteins called small heat shock proteins or smHsp like the Lo18 produced by *O. oeni* (Jobin et al., 1997; Coucheney et al., 2005). These chaperones-like proteins interact to stabilize the phospholipid

bilayer (Becker and Craig, 1994, Tsvetkova et al., 2002; Coucheney et al., 2005 and Maitre et al., 2014).

I.7.6 Medium chain fatty acids (MCFA)

Medium chain fatty acids are part of the aroma compounds in fermented beverages and their production with other major volatiles during AF is dependent on must composition, grape cultivar, microbial strains, nitrogen availability, fermentation temperature, aeration, wine pH and winemaking practices (Houtmann, 1980; Kunkee, 1988; Lonvaud-Funel et al., 1988; Capucho and San Ramaô, 1994; Nygaard and Prahl, 1997; Bardi et al., 1999; Torjia et al., 2003a and b; Alexandre et al., 2004).

Lonvaud-Funel et al., (1988) identified medium chain fatty acids (hexanoic, octanoic, decanoic and dodecanoic acids) as one of the metabolites produced by yeasts during AF and that exert an inhibitory effect on both yeasts and bacteria. They mainly found that the hexanoic, octanoic and decanoic acids together exert a stronger inhibition than each one alone. 4 mg/l of decanoic acid alone and 0.5 mg/l of dodecanoic acid alone were able to inhibit MLF. Curiously, Viegas et al., (1985; 1989) demonstrated that these by-products of AF, especially octanoic and decanoic acids in their undissociated forms act in synergy with ethanol decreasing the maximum specific growth rate and biomass yield of *S. cerevisiae* and *Kluveyromyces marxianus* and increasing the duration of the AF lag phase. Edwards et al., (1990) suggested that concentrations of decanoic acid between 5 and 10 mg/l inhibited *O. oeni* growth and MLF while 30 mg/l were lethal. Capucho and San Ramaô (1994) found that concentrations of decanoic and dodecanoic acids lower than 12.5 and 2.5 mg/l respectively stimulated the growth of MLB and MLF while higher concentrations were inhibitory.

The inhibition of the bacterial growth and the malolactic activity by these acids depends on their concentration and on the medium pH. For example, the inhibition of the malolactic activity of *O. oeni* by the decanoic acid is more important at pH 3 than at pH 6. In fact, the pK_a of the decanoic acid is 4.9. Therefore, the major form of this acid at pH 3 is the undissociated form which is the toxic or active form of all MCFA. This form is able to passively diffuse inside the cell and at the pH_i which is around 6.5, will deprotonate leading to a decrease of the pH_i and a dissipation of the proton gradient, thus inhibiting the intracellular enzymes and the cell transport system (Capucho

et San Romaô, 1994). Carreté et al., (2002) demonstrated that the fatty acids mainly the decanoic and dodecanoic acids act in synergy with ethanol since all of them target the cell membrane. They also found that the decanoic acid works in synergy with low pH and/or ethanol to inhibit the membrane ATPase which activity is closely related to MLF. This can be explained by the capacity of these acids to solubilize in the hydrophobic part of the membrane modifying its composition and permeability, thus leading to protons diffusion inside the cell and to the loss of the intracellular amino acids (Stevens and Hofemye, 1993). The position, the structure and the function of membrane proteins is also affected by these acids (e.g., ATPase).

Not all the yeast strains have the ability to produce these fatty acids. In a study conducted by Nehme et al., (2008), none of the four *S. cerevisiae* strains tested were able to produce significant levels of medium chain fatty acids. The highest concentrations produced were 0.7 mg/l of octanoic acid, 2.9 mg/l of decanoic acid and 0.2 mg/l of dodecanoic acid. These concentrations are far below the inhibitory concentrations reported by the previous studies.

I.7.7 Antimicrobial peptides (AMP)

The role of antimicrobial peptides in the inhibition of MLF is mentioned and described in paragraph I.9 of this chapter.

I.7.8 Nutrient depletion

Besides the physical chemical parameters and yeast metabolites, the nutritional status of wine is crucial for the survival and growth of LAB as well as their ability to carry out MLF. The availability of certain nutrients is therefore imperative (Fugelsang and Edwards, 1997). The lack of essential nutrients such as some free amino acids and vitamins due to their consumption by yeasts during AF can be detrimental (Nygaard and Prah, 1996; Guilloux-Benatier et al., 2006). During winemaking, sugars are mainly consumed by yeasts. However, the remaining sugars (few g/l) after AF including hexoses and pentoses cover the bacterial needs. Beltran et al., (2008), demonstrated that the consumption of some amino acids like arginine by yeasts during AF reduces their concentration to levels that are insufficient for bacterial growth and MLF. Pinotage, a well-known South African variety, was inoculated with a commercial malolactic culture

following the completion of AF. 110 days later, only 40% of the initial concentration of malic acid was metabolized. The “health status” of the wine was examined by microscope analysis and the volatile acidity was determined. A good and healthy population of *O. oeni* was found and the volatile acidity levels were also reasonably low. On the basis of these analyses, a specific malolactic bacteria nutrient, OptiMalo Plus[®], was added. Eleven days later, the MLF was completed (Lallemand report, 2005). OptiMalo Plus[®] is a natural nutrient developed by Lallemand Inc. specifically for MLF and is commercialized in international markets to overcome potentially difficult circumstances. It is a blend of inactive yeasts rich in amino acids, mineral cofactors, vitamins, cell wall polysaccharides and cellulose. The cellulose provides a surface area to help keep the bacteria in suspension and to help adsorb toxic compounds that may be present at the end of primary fermentation (e.g., medium chain fatty acids).

1.7.9 Lees compaction and residual lysozyme activity

As a result of the hydrostatic pressure, the lees found at the bottom of a tank can be compacted to such an extent that yeasts, bacteria and nutrients are “captured” and cannot function properly. It has recently been observed that larger tank sizes may correlate with increasing delays in the initiation of the MLF. The inhibition of the start of the MLF in larger tanks can be overcome by pumping over either on the day of inoculation or on the second day after inoculation with the bacteria. A general recommendation would be to stir the lees regularly (at least weekly) to ensure that bacteria and nutrients are kept in suspension (Lallemand, 2005). In most cases, racking the wine off the gross lees is recommended (Lallemand reports, 1999-2004).

Lysozyme is an enzyme proposed to substitute SO₂ for the control of spoilage LAB and acetic bacteria and to delay the MLF (Bartowsky 2003a, b). This bacteriolytic enzyme proved to be active against *O. oeni* in white must, red must and in synthetic media (Delfini et al., 2004). A dose of 200 mg/l has a similar effect to sulfiting at 50 mg/l. A 200-300 mg/l dose of lysozyme provides a useful complementary treatment, especially in white wines, where lysozyme is more stable than in red wines. The latter contain a high level of tannins that interact with lysozyme and precipitate it. An addition of 500 mg/l of lysozyme inhibited MLF and an addition of 250 mg/l promoted microbial stability in red wine after AF was complete (Lerm et al., 2010). If lysozyme is used during the production of wine, residual levels of this enzyme may impact the time

required for the onset of MLF. Lysozyme can react with phenolics and precipitate but in general, stability was shown in bottled wines of Riesling, Shiraz and Cabernet Sauvignon (Bartowsky, 2003b). Care must be taken to follow the supplier's recommendations with regard to the required time between the addition of lysozyme and the inoculation of the commercial MLF culture.

I.7.10 Pesticides

The occurrence and concentration of pesticides residues in grapes and consequently in wines depend on grapevine pests characteristics of each vine-growing region, type of grape growing, pesticide concentrations and climatic conditions from the last spraying until harvest (Čuš et al., 2010). The use of pesticides to control pests and diseases is a common practice in vineyards to increase crop yield. Pesticides are usually detected by fast low pressure gas chromatography coupled with a mass spectrometry (Cuñha et al., 2009). In the presence of pesticides, MLF sometimes progresses more slowly than desired (Rueidiger et al., 2005). Cabras et al., (1994) reported that the malolactic activity of *O. oeni* was affected by the presence of certain pesticides. Carreté et al., (2002) found that 20 mg/l of copper reduced the F₀-F₁ ATPase activity of approx. 25 %. Copper, is a well-known competitor of Mg²⁺ cofactor. Vidal et al., (2001) examined the inhibitory effect of two commonly used pesticides, copper and dichlofluanid, on several strains of *O. oeni* and on MLF in wine. Sensitivity to these pesticides varied and was enhanced by the presence of ethanol. A decrease in cell number was detected and not a decrease in malolactic activity.

I.7.11 Phenolic compounds

The different concentrations of polyphenols in wines have an important impact on consumers with the increase of wine commercialization (Rodriguez Vaquero et al., 2007). The major phenolic compounds present in grapes and wine include the non-flavonoids and flavonoids. The non-flavonoids consist of stilbenes (e.g., resveratrol) and phenolic acids (hydroxycinnamic and hydroxybenzoic acids). The flavonoids include the anthocyanins, flavan-3-ols (tannins) and flavonols (Cheyner et al., 2006).

The influence of phenolic compounds on lactic acid bacteria is not very clear. While some may stimulate the bacterial growth and metabolic activity, others can be inhibitory (Rodriguez et al.,

2009). The amount of phenolics present in wine is cultivar specific and dependent upon the vinification procedures applied by the winemaker (Rozès et al., 2003). The interaction between LAB and phenolic compounds is influenced by various factors including the strain of LAB (Hernández et al., 2007; García-Ruiz et al., 2008) and the type and concentration of phenolic compounds present in wine (Stead, 1993; Reguant et al., 2000; García-Ruiz et al., 2008). Phenolic compounds can affect the occurrence and the rate of MLF (Vivas et al., 1997). They can be transformed by LAB and detectable differences in the phenolic content after MLF have been reported (Hernández et al., 2007). The main compounds that can be transformed by LAB include hydroxycinnamic acids and their derivatives, flavonols and their glycosides, flavan-3-ol monomers and oligomers, as well as *trans*-resveratrol and its glucoside (Hernández et al., 2007). Reguant et al., (2000) and Alberto et al., (2001), showed that catechin stimulates the growth of *O. oeni* and *Lb. hilgardii* as well as the MLF. Field and Latenga, (1992); Vivas et al., (2000); Campos et al., (2003a) showed that procyanidins formed by epicatechin and catechin and some phenolic acids negatively affect the growth and viability of LAB, including *O. oeni*. Phenolic aldehydes exert a toxic effect on microorganisms but there is little knowledge on their activity against wine bacteria (Gill and Holley, 2004). Garcia-Ruiz et al., (2011) compared the inhibitory potential of 18 phenolics to that of potassium metabisulfite and lysozyme, on three LAB isolated from wine: *O. oeni*, *Lb. hilgardii* and *P. pentosaceus*. Stilbenes and flavonols showed the greatest inhibitory effects on the growth, hydroxycinnamic and hydroxybenzoic acids showed a moderate effect, while flavan-3-ols and phenolic alcohols had a negligible effect.

Figueiredo et al., (2007) tested various phenolic aldehydes (cinnamaldehydes: coniferaldehyde and sinapaldehyde and benzaldehydes: vanillin, syringaldehyde, p-hydroxybenzaldehyde, 3, 4-dihydroxybenzaldehyde, and 3,4,5-trihydroxybenzaldehyde) and found that most of them had an inhibitory effect. The cinnamaldehydes, especially sinapaldehyde, were the most inhibitory for bacterial growth. The weak polar nature of cinnamaldehydes may give them a greater affinity to the lipid content of the cell membrane (O'Connor and Rubino, 1991). Concerning benzaldehydes, the inhibitory effect was less marked and the number of hydroxyl groups of the benzaldehyde derivatives was linked to their inhibitory power. Thus, 3,4-dihydroxybenzaldehyde and 3,4,5-trihydroxybenzaldehyde had a greater inhibitory effect than the p-hydroxybenzaldehyde. These molecules act on the outer surface of cells by combining with SH groups of proteins (Ramos-Nino et al., 1998). Vanillin showed no inhibitory effect. Finally, for these phenolic aldehydes,

the concentrations normally found in wine (1-2 mg/l) are not inhibitory and higher concentrations such as those used by Figueiredo et al., (2007) are needed to show inhibitory effects.

García-Ruiz et al., (2008) reported the metabolism by LAB of 100 to 250 mg/l of phenolic compounds and inhibition by concentrations exceeding 500 mg/l. Reguant et al., (2000) found hydroxycinnamic acids to be inhibitory at high concentrations. MLF was delayed by *p*-coumaric acid at concentrations of more than 100 mg/l and by ferulic acid at concentrations of more than 500 mg/l. Similarly, García-Ruiz et al., (2008) and Silva et al., (2011) reported the use of free hydroxycinnamic acids as a way of controlling *Lb. plantarum* growth and found ferulic acid to be more inhibitory than *p*-coumaric acid, while the esters of ferulic acid did not affect growth. Vivas et al., (1997) found a slight inhibitory effect of vanillic acid on *O. oeni* IOB-8413, while protocatechuic acid had no effect.

Although the mechanisms by which phenolic compounds inhibit LAB are not entirely clear, some possible explanations can be given. Possible mechanisms are based on the interactions of phenolic compounds with cellular enzymes (Campos et al., 2003b; García-Ruiz et al., 2008) and the adsorption of phenols to cell walls (Campos et al., 2003b). Phenolic compounds could lead to a loss in potassium ions, glutamic acid and intracellular RNA, as well as causing a change in the composition of fatty acids (Rozès et al., 1988; García-Ruiz et al., 2008; 2011). Certain characteristics of wine LAB, like the production of volatile acids and the malolactic activity, are differently affected by the presence of phenolics, and this is dependent on the bacterial strain (Campos et al., 2009). Among the flavonols, kaempferol and quercetin strongly inhibited *O. oeni* Viniflora Oenos. Quercetin inhibits the replication of DNA and acts on the cell membrane (Cuhsnie and Lambert 2005).

Flavanols (catechin and epicatechin) do not inhibit the growth of *O. oeni* at the concentrations normally found in wine (10-200 mg/l) (Goldberg et al., 1998). Furthermore, catechin, epigallocatechin gallate and quercetin stimulated the bacterial growth of *O. oeni* strain B2 and this stimulation increased with the concentration (Reguant et al., 2000; Theobald et al., 2008).

Free anthocyanins and other phenolic compounds like gallic acid, are able to stimulate the cell growth and malic acid degradation of LAB. Phenol carboxylic acids and catechin stimulated the

growth of *O. oeni* strains ATCC23279 and IB8413, probably by enhancing the metabolism of citric acid and reducing the initial lag phase of LAB (Vivas et al., 1997; Rozès et al., 2003). Rozès et al., (2003) studied the effect of phenolic compounds (the phenolic acids: *p*-coumaric, ferulic, caffeic and gallic acids as well as catechin and the anthocyanin malvidin-3-diglucoside) in a synthetic medium on the growth of *O. oeni*. A concentration of 50 mg/l of phenolic compounds was stimulatory to *O. oeni* growth. This stimulatory effect could be attributed to the role that phenolic compounds play in protecting bacterial cells from ethanol as well as the fact that phenolic compounds reduce the redox potential of the wine which promotes cell growth.

The presence of phenolic compounds has also the potential to influence certain quality parameters in wine. Cavin et al., (1993) reported the ability of LAB to metabolize hydroxycinnamic acids which results in the formation of volatile phenols with the potential to produce off-flavors. A strain of *O. oeni* studied by Campos et al., (2009) was able to produce higher concentrations of acetate in the presence of phenolic acids. This could be due to enhanced citric acid metabolism at the expense of sugar consumption as claimed by Rozès et al., (2003). It was also found that this phenomenon is strain dependent. In contrast, Reguant et al., (2000), found that gallic acid was able to delay or totally inhibit the formation of acetic acid from citric acid.

I.7.12 Bacteriophages

Bacteriophages are viruses capable of massively destroying cultures of sensitive bacterial strains. Inside the bacterial cell, it uses its own genome and the enzyme equipment of the cell to ensure the necessary synthesis. Phages can be moderate or virulent. The infection of *O. oeni* by phages was firstly reported by Sozzi et al., (1976; 1982). Abrupt stoppages of MLF were caused by phages that destroyed *O. oeni* population at low pH (Henick-Kling et al., 1986). A large number of strains of *O. oeni* isolated from wines were checked for lysogeny with mitomycin C as inducer. As a result of these tests, 45 to 90% of the strains proved to be lysogenic, suggesting that lysogeny is widespread among bacteria isolated from wines during malolactic fermentation. The sensitivity of bacteria to phages varied widely and was strain dependent. All the lysogenic strains were resistant to infection by the temperate phage they released. Some phages infected none of the strains (Poblet and Lonvaud-Funel, 1996; Poblet-Icart et al., 1998).

I.8 Stress resistance mechanisms of LAB during MLF

Living in wine can be very stressful for a cell; it requires a great deal of resilience to tolerate, amongst other things, high levels of ethanol and a low pH. *O. oeni* is the best LAB adapted to the aggressive ecological medium of wine and involves sophisticated mechanisms that enable it to survive and grow (Bartowsky, 2009). The cytoplasmic membrane is the first sensitive target of damage in cells, as demonstrated by the leakage of intracellular substances and variation in membrane fluidity (Da Silveira et al., 2003). It is likely that there are at least four mechanisms adopted by *O. oeni* to survive and function in wine: stress protein synthesis (Guzzo et al., 2000), activation of a proton-extruding ATPase (Carreté et al., 2002), proton motive force generated by MLF (Drici-Cachon et al., 1996; Salema et al., 1996) and modifications in membrane fluidity (Tourdot-Maréchal et al., 2000; Da Silveira et al., 2003). Granvalet et al., (2008) showed a decrease in oleic acid and an increase in cyclopropane fatty acid in an-ethanol stressed cell adapted to acidity during stationary phase. Numerous proteins are produced when a cell first encounters a stress (Sico et al., 2009; Olguin et al., 2010). In this context, the FtsH protein is a universal molecular device belonging to the AAA protein family that protects MLB against wine toxicity when the bacteria are subjected to alcohol, sulfite and ethidium bromide. It is also over expressed with high fermentation temperatures and osmotic shocks (Bourdineaud et al., 2003). Besides, heat shock proteins or HSP (e.g., Lo 18 of *O. oeni*) are usually associated with cellular membrane fractions (Jobin et al., 1997). HSP are chaperone-like proteins that either prevent or repair damages in cells. The *O. oeni* small heat shock protein Lo18, has a MW of 18 kDa and is rich in alanine, serine and tyrosine (Weidmann et al., 2010) and is identical to the α -crystallin family (Becker and Craig, 1994). It is regulated by the stress gene repressor CtsR (Grandvalet et al., 2005) and induced by multiple stresses such as a low pH of 3, a heat shock at 42 °C and the presence of 12% ethanol (v/v) as well as the presence of benzyl alcohol. It acts as a lipochaperone on phospholipids (increases the molecular order of phospholipids and regulates membrane fluidity) and as a molecular chaperone on damaged proteins (prevents aggregation of proteins during stress conditions) (Coucheney et al., 2005; Darsonval et al., 2016)). During the stationary growth phase, it also acts like chaperones by binding to partially unfolded proteins in an ATP-independent manner (Guzzo et al., 1997), and prevents their irreversible aggregation under heat shock (Craig et al., 1993). It also stabilizes some enzymes such as lactate dehydrogenase and citrate synthase even at 60 °C (Delmas et al., 2001) and stabilizes the plasma

membrane (Jobin et al., 1997; Guzzo et al., 1997; Tsetkova et al., 2002; Török et al., 2002; Coucheney et al., 2005). Other HSP are studied in *O. oeni* including clpL and clpP that enable microbial development under stress conditions by managing the life of regulatory proteins (Beltramo et al., 2006). In addition, clpX, a heat inducible protein, is preferentially expressed at the beginning of the exponential growth phase (Jobin et al., 1999b, Guzzo et al., 2000).

Many other stress related proteins play a role in bacterial resistance under wine conditions, such as thioredoxin or trxA protein produced in response to oxidative stress and heat shock. Its expression is not related to growth (Jobin et al., 1999a). Another protein playing a key role in acid tolerance is the H⁺-ATPase which is overexpressed at low pH (e.g., pH 3.5). It is essential for malic acid metabolism and regulation of the intracellular pH in winemaking conditions (Fortier et al., 2003). OmrA, a bacterial homologue dependent multidrug resistance protein, enables bacterial growth in high salt media and procures resistance to ethanol and sodium laurate, a substance released by yeasts during AF (Bourdineaud et al., 2002; 2004). The ability of *O. oeni* to survive in wine is detailed in Figure I.12 below.

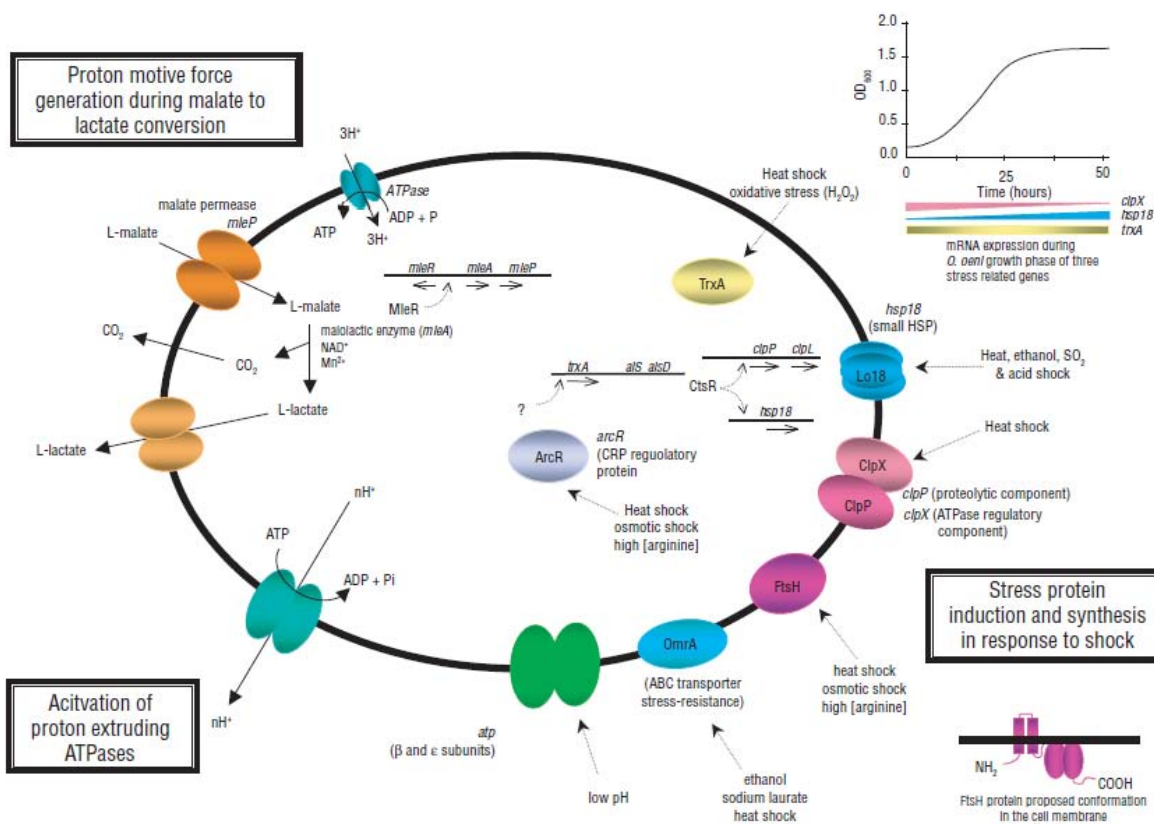


Figure I.12. Three mechanisms that are important in conferring, in *O. oeni*, the ability to survive in wine: (1) the proton motive force generated by malic acid metabolism, (2) the activation of proton-extruding ATPase, and (3) the stress protein induction and synthesis in response to shock. MLF is involved in proton motive force generation and the maintenance of internal pH by proton consumption during the L-malate decarboxylation step. The ATPase systems that function as proton extruding pumps are suggested to provide the means for acid tolerance by regulating the intracellular pH. Stress protein synthesis occurs typically in response to an environmental shock triggered by the wine medium. Many of these stress proteins function as molecular chaperones or proteases that may participate in their folding or degradation processes of denatured proteins in the cell (Bartowsky, 2005)

Another challenge in wine for MLB is the starvation due to the lack of essential nutrients. However, *O. oeni* found a way to resist this fact; the exoprotease activity. *O. oeni* possesses an active EprA protease that is able to hydrolyze several proteins (Folio et al., 2008) including proteins in grape juice at a significantly high rate (Farias et al., 1996; Farias and Manca de Nadra, 2000). Moreover, the strain X₂L showed the highest extracellular proteolytic activity in the presence of 60 mg/l SO₂ and 10 to 12% ethanol (v/v) (Rollán et al., 1998).

I.9 Wine microbiome, a dynamic environment of microbial interactions

Microbes coexist and interact in many environments, and this is of practical relevance in various fields. Indeed, microbial interactions occur in bioremediation of pollutants, agriculture, forestry, environmental protection, food processing, biotechnology and medicine (Liu et al., 2015).

In winemaking, grape must and wine constitute a complex microbial ecosystem containing a mixture of different species and strains. Individual microorganisms interact, and the types of interaction found in mixed population of microorganisms are generally classified as direct or indirect.

Neutralism, mutualism, symbiosis, competition, commensalism and amensalism (antagonism) are considered to be indirect interactions; direct interactions, such as parasitism, predation, quorum sensing, cell-to-cell contact and horizontal gene transfer, may also occur during fermentation (Alexandre et al., 2004; Strehaiano et al., 2010; Ivey et al., 2013). Classical methods (e.g. interactions on culture medium) and recently the omics approaches (genomics, proteomics, metabolomics) play a major role in clarifying the interaction modalities.

A brief description of each type of interaction is given below:

-Neutralism means that there is no change in the growth rate of either microorganism due to the presence of the other and thus, there is no observable interaction.

-Mutualism is much more common than neutralism and involves several different mechanisms. The exchange of nutrients and growth factors (e.g. phenylalanine or folic acid) are one of these mechanisms. Microbiologically, the mixed culture grows well, while pure cultures exhibit almost no growth.

-Very close mutualistic ties, such that the partnership is necessary for the survival of one or both species are often termed symbiosis.

-Competition refers to the dependence of two species on a common factor such as food supply, light, space or some other limiting resource. Consumption of this common factor by each species limits its availability to the other, so that the growth rates of both organisms are affected negatively.

-In commensalism, the first microorganism gain benefits from the second with no significant effect on the latter. In this version, one species removes a toxin for the second species, but, in contrast to mutualism, the latter organism provides no special benefits for the detoxifier. The opposite occurs in amensalism or antagonism, where the second species exerts a negative effect during the interaction with the first species (Bailey and Ollis, 1986).

-Quorum sensing is a term used to describe cell-to-cell communication. This sensing mechanism is based on the production, secretion, and detection of small signaling molecules, whose concentration correlates with the abundance of secreting microorganisms in the medium (Choudhary and Schmidt-Dannert, 2010).

-Nissen et al., (2003) proposed that the early growth arrests of *Hanseniaspora uvarum*, *Kluyveromyces thermotolerans* and *Torulaspora delbrueckii* in co-culture with *S. cerevisiae* is not due to a quorum sensing effect or other yeast metabolites such as acetaldehyde (Cheirati et al., 2005) as well as the nutrient depletion (Taillandier et al., 2014), but rather to a cell-cell contact mechanism regulating their growth in mixed cultures. Evidence of a cell contact mechanism regulating *Torulaspora delbrueckii* cell density in co-culture with *S. cerevisiae* has been reported. Renault et al., (2013) observed a much higher viability of *Torulaspora delbrueckii* when physically separated from *S. cerevisiae* (co cultures of the two yeasts in double fermenters) than in standard mixed co-culture. Nissen and Arneborg (2003) and Arneborg et al., (2005 a, b) demonstrated through an interactive optical trapping that *S. cerevisiae* at high cell densities gave a lesser ability for others *NS* to compete for space. Kemsawasd et al., (2015a) showed a combined effect of cell-to-cell contact and AMP released by *S. cerevisiae* in the death of *Lachencea thermotolerans* (*NS* wine yeast) during mixed-cultures AF.

-Predation and parasitism: In this type of relationship, one species lives totally dependent on the other. The victim becomes a substrate and is completely digested in the case of predation or a portion of its tissues is consumed as in the case of parasitism. Thus bacteria may be parasitized by viruses (bacteriophages) as the P58 phage in *O. oeni* (Arendt et al., 1991).

-Horizontal gene transfer: the potential of microbes to exchange genetic information through gene transfer is a major factor in their genetic adaptation and evolution. Diverse bacteria and

yeast species are in close contact on grapes, during AF and MLF, and this might promote the horizontal gene transfer (Liu et al., 2015). The *S. cerevisiae* EC1118 genome sequence contains 3 gene clusters resulting from horizontal transfers. Genes in these clusters encode key functions linked to the winemaking process, such as carbon and nitrogen metabolism, cellular transport and the stress response (Novo et al., 2009).

Wine is a complex microbial ecosystem containing mixtures of diverse microorganisms favoring interactions: there are yeast-yeast interactions, bacteria-yeast interactions, bacteria-bacteria interactions and filamentous fungi-yeast interactions.

1. Yeast-yeast interactions:

Fermentations involving added or natural complex yeast consortia exhibit numerous kinds of interactions. These interactions entail negative or positive effects.

a- Negative effect: Killer toxins produced by *Saccharomyces* and non-*Saccharomyces* with activity against sensitive yeasts

Microbes competing for limited resources have established a broad arsenal of lethal compounds aiming at the inhibition or destruction of competitors. In addition to the low oxygen availability that can be detrimental for *non-Saccharomyces*, killer toxins (fungicidal proteins) also contribute to the removal of these yeasts (Hansen et al., 2001). They are released by different strains of *S. cerevisiae* and bind to the mannoproteins or glucan units of the cell wall of sensitive yeasts (Meinhardt and Klassen, 2009). Many types of these killer factors are well identified such as K₁, K₂ and K₂₈ encoded by double stranded RNA (dsRNA) of virus-like cytoplasmic particles (VLPs), respectively M₁, M₂ and M₂₈. Only one type named KHS (killer of heat sensitive) is a chromosomally-encoded killer. K₁ and K₂ disrupt cytoplasmic membrane functions, while K₂₈ is a unique virus of *S. cerevisiae* strain 28 known to be unstable and disrupts DNA synthesis in sensitive cells (Schmitt and Tipper, 1990). Sensitive cells such as *NS* strains are most susceptible to the action of the killer factor when in log phase (Woods and Bevan, 1968; Carrau et al., 1993; Alexandre et al., 2004; Pérez-Nevado et al., 2006; Albergaria et al., 2010, Mostert and Divol, 2014).

Many other *NS* yeast strains are extensively studied and used to prevent food and beverages spoilage as they also produce killer toxins. *Kluyveromyces phaffii* liberate a killer toxin used as a potential biopreservative agent to control apiculate wine yeasts, such as *Hanseniaspora uvarum*. It can be fungistatic or fungicidal depending on the concentration. Its inhibitory activity in grape juice is comparable to SO₂ and is active in a pH range between 3 and 5 and at a temperature lower than 40 °C (Ciani and Fatichenti, 2001). *Kluyveromyces wickerhamii* secretes another killer toxin (KwKt) active against *Brettanomyces/Dekkera* in grape must at a concentration between 40 and 80 mg/l. Antispoilage effects prevent “Bret” character of wines and so, ethyl phenols are not produced. It is a suitable biological strategy to control *Dekkera* yeasts during fermentation, wine ageing and storage (Comitini and Ciani, 2011).

Schawnniomyces occidentalis produces a killer toxin lethal to sensitive strains of *S. cerevisiae*. The toxin has a protein composition sensitive to pepsin and papain and similar to the chromosomally encoded killer toxin and may reduce the risk of contamination by undesirable yeast strains during commercial fermentations (Chen et al., 2000). Interestingly, HMK mycocin produced by *Williopsis mrakii*, controls both silage and yoghurt spoilage by yeasts (Lowe et al., 2000). *Pichia anomala* WC 65 produces a toxin effective against opportunistic *Candida albicans* at a pH value comparable to those of wine ferments such as pH 3.5 (Sawant et al., 1988; 1989 and 1990; Yap et al., 2000).

b- Positive effect: *Saccharomyces* and non-*Saccharomyces* (NS)

A good example of a synergetic interaction has been reported in a *Kloeckera apiculata/S. cerevisiae* co-culture. The apiculate cells remained viable for longer than in pure culture (Mendoza et al., 2007). Commensalism between *Saccharomyces* and *NS* has been also evidenced. For example, the high extracellular proteolytic activity of some *NS* yeasts (Charoenchai et al., 1997; Dizey and Bisson, 2000) causes the release of amino acids from proteins present in the medium, and these amino acids are then used by *S. cerevisiae* (Fleet, 2003). The early death of non-*Saccharomyces* yeasts after the early stages of AF can also provide nutrients for *S. cerevisiae* thanks to their passive release during autolysis. Conversely, *S. cerevisiae* autolysis after AF may be a significant source of micronutrients for the growth of spoilage species, especially those of *Dekkera/Brettanomyces* (Guilloux-Benatier et al., 2001).

Among the non-*Saccharomyces* yeast species, *Brettanomyces bruxellensis* is better adapted than other wild yeasts to persist during AF thanks to its ethanol tolerance (Renouf et al., 2007).

2. Yeast-bacteria interactions:

The interactions between bacteria and yeasts during AF and MLF have a direct effect on induction and completion of MLF, which is an important factor for wine quality. Liu et al., (2016) studied the exometabolomic profile of *S. cerevisiae* strains and related that these substances can have a negative (MLF-) or positive effect (MLF+) on the MLF.

a- Negative effect: AMP produced by *S. cerevisiae* with activity against *O. oeni*

The nature of the toxic compounds produced by wine yeasts and that inhibit the bacterial growth and MLF was investigated by many authors as an amensalism mechanism (Taillandier et al., 2002; Strehaiano et al., 2010). It has been suspected that the reason why MLF cannot be started sometimes is due to antimicrobial peptides (AMP) produced by yeasts (Fornachon, 1968; Lafon-Lafourcade, 1973; King and Beelman, 1986; Lemaesquier, 1987; Wibowo et al., 1988). It is known that Gram positive bacteria including LAB have specific ligand binding proteins or receptors to capture these AMP, identified to be an ATP binding cassette (ABC transporters) anchored to the cell membrane. The Opp system of *Lc. lactis* serves as a good example. They do not only play a role in cell's nutrition, but besides, they participate in various signaling processes that allow them to monitor the local environment (Detmers et al., 2000).

Large numbers of wine yeast strains have been screened for antibacterial activity. Parfentjev (1958) found that a strain of *S. cerevisiae* was able to produce an antibacterial protein with an isoelectric point of 2 whereas Motzel and Cook (1958) and Robinson et al., (1962) found two antibacterial cyclic peptides.

Dick et al., (1992) identified two cationic proteinaceous substances with an antibacterial activity released by *S. cerevisiae* R107 presenting a MW ≤ 14 kDa; one with the characteristics of lysozyme and the other a small protein with a high isoelectric point.

Comitini et al., (2005) found that *S. cerevisiae* F63 was able to produce a proteinaceous compound with a MW greater than 10 kDa that strongly inhibited the growth of *O. oeni* CHR as well as its ability to consume L-malic acid. The proteinaceous compound was dose dependent and was able to reduce the bacterial growth with a typical saturation kinetic thus suggesting the presence of a receptor on the bacterial cell. Therefore its bacteriostatic or bactericidal effect depended on its concentration and the incomplete MLF was correlated to its bactericidal effect.

Osborne and Edwards (2007) identified a 5.9 kDa peptide produced by *S. cerevisiae* RUBY. ferm and inhibiting *O. oeni* Viniflora oenos. They suggested that the peptide inhibited the bacteria along with SO₂. The mechanism proposed was that of bacteriocins forming membrane pores and facilitating the entry of SO₂ inside the cells thus leading to the bacterial death and arrest of MLF. They also showed that wine samples taken at different intervals of the AF (days 9, 16 and 23) were able to inhibit *O. oeni* Viniflora oenos.

Nehme et al., (2010) were able to identify a peptidic fraction with a MW between 5 and 10 kDa released by *S. cerevisiae* strain D and that strongly inhibited *O. oeni* strain X. The fraction exerted a bacteriostatic effect and strongly inhibited the malate consumption.

Mendoza et al., (2010) showed that *S. cerevisiae* mc2 released a proteinaceous compound presenting a MW between 3 and 10 kDa that inhibited the growth of *O. oeni* X₂L and *Lactobacillus hilgardii* 5W but not their ability to consume L-malic acid. The bacterial growth was inhibited with a typical saturation kinetic similar to that suggested by Comitini et al., (2005).

Albergaria et al., (2010) showed that *S. cerevisiae* CCMI 885 started to secrete three antifungal peptides presenting an apparent MW of about 6, 4.5 and 4 kDa at the end of the AF exponential growth phase (day 2) with a gradual increase of their concentration during the stationary growth phase (days 4 and 7). Later on, Branco et al., (2014) demonstrated that these peptides were active against a wide variety of wine-related yeasts such as *Hanseniaspora guilliermondii* and *Dekkera bruxellensis* but also against the growth of *O. oeni*. The peptides corresponded to different fragments of the C-terminal amino acid sequence of the *S. cerevisiae* glyceraldehyde 3-phosphate dehydrogenase (GAPDH) enzyme. Two main fragments of 1.638 and 1.622 kDa were involved in the inhibition. They suggested that the peptides were released by apoptotic yeast cells of *S. cerevisiae* during the stationary phase. Previously, Silva et al., (2011) showed

that GAPDH is presumably a substrate of metacaspases during apoptosis. Branco et al., (2014) evaluated only the effect of these peptides on the growth of *O. oeni* and no data concerning the malate consumption was shown. Interestingly, Branco et al., (2015), found that these AMP compromise the plasma membrane integrity of *H. guilliermondii* cells, disturbing the pH homeostasis and inducing a loss of culturability.

Beside its glycolytic role, GAPDH displays several other activities in different subcellular locations (membrane, cytosol, nucleus), including a primary role in apoptosis and in a variety of critical nuclear pathways (Silva et al., 2011). Regarding these findings, GAPDH plays an important role in the defense system of different microorganisms. In *S. cerevisiae*, three related but not identical GAPDH isoenzymes with different specific activities are encoded by unlinked genes noted *TDH1*, *TDH2* and *TDH3*. Mutation of *TDH2* and *TDH3* affects negatively the growth (McAlister and Holland. 1985b). Delgado et al., (2001; 2003) found that each of the three encoded polypeptides by the *TDH1-3* genes is associated with the cell wall of the wine yeast in response to starvation and temperature upshift. In humans, GAPDH aggregates, participate in oxidative stress and induce cell death (Nakajima et al., 2009). Branco et al., (2014) reported that the use of a *S. cerevisiae* strain with a deleted metacaspase (*YCA1* gene) significantly prevented the death of *H. guilliermondii* during AF. Taken together, all these findings suggest that the presence of extracellular GAPDH-derived peptides at the end of the AF exponential growth phase is probably due to apoptotic cells of *S. cerevisiae* cleaving GAPDH with their metacaspases.

b- Positive effect: stimulation of *O. oeni* by *S. cerevisiae*

The stimulation of ML bacteria by yeasts has been studied in far less details. Guilloux-Benatier et al., (1985) showed that yeast autolysates prepared with different levels of proteolysis stimulated the growth and malolactic activity of different LAB. The same authors demonstrated that yeast mannoproteins, which constitute a major component of the macromolecules produced by yeasts, have also been associated with stimulation of bacterial growth in wine. In addition, nitrogen components, especially those with a MW<1 kDa, are the most effective for the stimulation of the bacterial growth. These components include important amino acids such as arginine, isoleucine, glutamic acid and tryptophan (Alexandre et al., 2004).

Some cellar practices participate in the activation of wine lactic acid bacteria. Maceration, carbon dioxide, ageing on lees and settling are among these positive factors (Ribéreau-Gayon et al., 2006).

3. Bacteria-bacteria interactions:

The negative activity between wine related LAB is in most cases related to the presence of bacteriocins. Bacteriocins are peptides produced by Gram + bacteria that inhibit closely related species (Jack et al., 1995). Their size is normally lesser than 10 kDa and they form pores which destruct the cell membranes of sensitive bacteria. The study of bacteriocins, such as nisin, plantaricin and kappacin is widely developed in different food sectors especially in milk and dairy industries since they are active against a wide range of pathogenic and alteration microorganisms (Delves-Broughton et al., 1996; van Reenen et al., 1998; Verellen et al., 1998; Malkoski et al., 2001 and Nel et al., 2002). In addition, they are qualified as potential therapeutic agents like antibiotics (Matsuzaki, 2009). Search for bacteriocins is being launched in the wine sector as a control of MLF and spoilage microorganisms, but till now their use is prohibited. Green et al., (1997), Nel et al., (2001; 2002) and Diez et al., (2012), showed a bacteriostatic effect of pediocin PA-1 and a bactericidal effect of pediocin PD-1 from *P. acidilactici* and *P. damnosus* NCFB 1832 against *O. oeni* and other *Pediococcus* in winemaking. Pediocin PA-1 may be useful as a biopreservative against unwanted bacterial microflora that affect wine quality. Nel et al., (2002), have shown that pediocin PD-1, when compared with nisin and plantaricin 423 is the most effective in the removal of an established biofilm of *O. oeni* on stainless steel surfaces in Chardonnay wines. Adherence of MLB to surfaces may have a pronounced effect on the ability of MLB to survive during AF and conduct spontaneous MLF.

I.10 Aims of the study

The current project is a continuation of the work already initiated by Nehme et al., (2008; 2010). They have already studied the interactions between different strains of *S. cerevisiae* and *O. oeni*. *S. cerevisiae* strain D strongly inhibited the growth of *O. oeni* strain X and its ability to consume L-malic acid during sequential fermentations performed in synthetic grape juice media. An

extracellular yeast proteinaceous compound was involved in the MLF inhibition and was found to work synergically with ethanol. The present project aimed at revalidating the MLF inhibition with the same pair and further characterizing the yeast inhibitory proteinaceous compound. After interrupting the work for almost six years, the first concern was to demonstrate that neither the yeast strain D lost its ability to release the proteinaceous compound nor did the bacteria acquire resistance. Then, the compound was further characterized by determining its MW, the timing of its release during AF and its mechanism of action with an attempt to purify it and sequence it. The interactions were also studied in natural grape musts in order to evaluate their reproducibility in real winemaking conditions.

Chapter II. Materials and Methods

II.1. Materials

II.1.1 Microorganisms

Three strains of *Saccharomyces cerevisiae* (A, D and R) and one strain of *Oenococcus oeni* (X) were used in this work and were kept anonymous as requested by the providers. The strains A and D were produced and marketed by Lallemand Inc. (Blagnac, France) whereas the strain R was provided by Laffort (Bordeaux, France).

II.1.2 Chemicals

All culture media components were supplied by Sigma-Aldrich (Taufkirchen, Germany) except for the yeast extract and the peptone that were supplied by Oxoid (Hampshire, UK). The chemicals used for SDS-PAGE were purchased from Bio-Rad (California, USA). The enzymatic kits for the determination of the L-malic acid, L-lactic acid, D-lactic acid, acetic acid, citric acid and ethanol concentrations were all purchased from Boehringer Mannheim/R-Biopharm (Darmstadt, Germany).

II.1.3 Culture media

II.1.3.1 Conservation and reactivation media for yeasts

Yeast strains were maintained at 4 °C on YEPD agar slants presenting the following composition (Table II.1):

Table II.1. Composition of the YEPD medium

| Components | Amount (g/l) |
|---------------|--------------|
| Yeast extract | 10 |
| Peptone | 20 |
| D-Glucose | 20 |
| Agar | 20 |

The same medium composition without agar was used for the reactivation of the yeast stock culture in order to prepare the preculture. The media were autoclaved at 121 °C for 20 min before use.

II.1.3.2 Conservation and reactivation media for bacteria

The bacteria were stored at -20 °C in Nalgene cryogenic tubes (Thermo Fisher Scientific, Waltham, MA-USA) in MRS broth (*De Man, Rogosa and Sharpe*) containing 20 % glycerol (v/v). They were reactivated in the same MRS medium with 3 % ethanol (v/v) added to prepare the preculture. After autoclaving (121 °C, 15 min), the ethanol was added aseptically through sterile filter membranes of 0.22 µm cutoff (Elvetec services, Meyzieu, France). The MRS broth presented the following composition (Table II. 2):

Table II.2. Composition of the MRS broth

| Components | Amount (g/l) |
|--------------------------------|--------------|
| Peptone | 10 |
| Meat extract | 8 |
| Yeast extract | 4 |
| D (+) – Glucose | 20 |
| Dipotassium hydrogen phosphate | 2 |
| Sodium acetate trihydrate | 5 |
| Triammonium citrate | 2 |
| Magnesium sulfate heptahydrate | 0.2 |
| Manganous sulfate heptahydrate | 0.05 |

The MRS medium was supplemented with 1 ml of Tween 80 (Sigma P8074). The pH of this medium was 6.2 at 25 °C.

II.1.3.3 Synthetic medium for the preparation of yeast and bacterial starter cultures

The yeast and bacterial starter cultures (inocula) for alcoholic fermentations, malolactic fermentations and sequential fermentations were prepared in a synthetic medium presenting the following composition (Table II.3):

Table II.3. Composition of the synthetic medium used for yeast and bacterial starter cultures

| Components | Amount (g/l) |
|--------------------------------|--------------|
| D-Glucose | 50 |
| Yeast extract | 1 |
| Ammonium sulfate | 2 |
| Citric acid | 0.3 |
| L-malic acid | 5 |
| L-tartaric acid | 5 |
| Magnesium sulfate | 0.4 |
| Potassium dihydrogen phosphate | 5 |

The pH of this medium was adjusted to 3.5 using a 10 M NaOH solution. After autoclaving at 121 °C for 20 min, 6 % ethanol (v/v) were added aseptically only to the medium intended for the preparation of the bacterial starter culture. In this case, the pH was readjusted to 3.5 using an 85 % orthophosphoric acid solution.

II.1.3.4 Synthetic media used for yeast and bacterial cultures

a. Synthetic grape juice medium (SGJ)

The SGJ medium was used to perform yeast and bacterial pure cultures (alcoholic and malolactic fermentations respectively). The medium composition that simulated the natural grape juice consisted of (Table II.4):

Table II.4. Composition of the synthetic grape juice medium

| Components | Amount (g/l) |
|--------------------------------|--------------|
| D-Glucose | 100 |
| D-Fructose | 100 |
| Yeast extract | 1 |
| Ammonium sulfate | 2 |
| Citric acid | 0.3 |
| L-malic acid | 3 |
| L-tartaric acid | 5 |
| Magnesium sulfate | 0.4 |
| Potassium dihydrogen phosphate | 5 |

The pH of this medium was adjusted to 3.5 with 10 M NaOH solution. The medium was autoclaved at 121 °C for 20 min before use.

b. Synthetic wine medium (SWM)

The medium composition simulated wine, yet it was lacking yeast metabolites with the exception of ethanol. It was used to perform malolactic fermentations with the bacterial strain tested and served as a control for sequential fermentations. The medium composition consisted of (Table II.5):

Table II.5. Composition of the synthetic wine medium

| Components | Amount (g/l) |
|--------------------------------|---------------------|
| D-Glucose | 0.5 |
| D-Fructose | 0.5 |
| Yeast extract | 0.5 |
| Ammonium sulfate | 0.2 |
| Citric acid | 0.3 |
| L-malic acid | 3 |
| L-tartaric acid | 5 |
| Magnesium sulfate | 0.2 |
| Potassium dihydrogen phosphate | 2 |

The pH of this medium was adjusted to 3.5 using a 10 M NaOH solution before autoclaving at 121 °C for 20 min. After autoclaving, 10 % ethanol (v/v) corresponding to 80 g/l were added aseptically through sterile filter membranes of 0.22 µm cutoff (Elvetec services). The pH was readjusted to 3.5 using an 85 % orthophosphoric acid solution.

c. Natural grape juice medium (NGJ medium)

A marketed natural red grape juice obtained from a mix of cultivars was used for alcoholic fermentations. The sugar and the L-malic acid concentrations were respectively 200 g/l and 3 g/l. SO₂ was absent and the pH was adjusted to 3.5 using an 85 % orthophosphoric acid solution.

d. Cabernet Sauvignon and Syrah musts

Two grape cultivars of Cabernet Sauvignon and Syrah provided by Clos Saint-Thomas (Kab Elias, Lebanon) were destemmed, crushed and macerated at 10, 60, 70 and 80 °C for 48 h before

alcoholic fermentation. The sugar and the L-malic acid concentrations in the musts were adjusted to 200 g/l and 3 g/l respectively. The pH was adjusted to 3.5 using an 85 % orthophosphoric acid solution. The musts were sulfitized with 100 mg/l of sodium bisulfite.

e. Modified MRS broth

This medium was used to evaluate the inhibitory effect on bacterial growth and L-malic acid consumption of different proteinaceous fractions obtained by ultrafiltration and ammonium sulfate precipitation of the SGJ media fermented by *S. cerevisiae* strains A and D.

3 g/l of L-malic acid were added to the MRS medium described in paragraph II.1.3.2. The medium was autoclaved at 121 °C for 15 min. After autoclaving, 10 % ethanol (v/v) and the proteinaceous fraction to be tested were aseptically added to the medium. The pH was then adjusted to 3.5 using an 85 % orthophosphoric acid solution. The medium was finally filtered through sterile membranes of 0.22 µm cutoff (Elvetec services). The filtered media were collected in sterile Erlenmeyer flasks.

In a separate experiment, 0.1 g/l of L-arginine and/or 3 g/l of D-Fructose were added to the modified MRS media containing the inhibitory proteinaceous fractions to evaluate their impact on the bacterial growth and the malate consumption.

f. SGJ medium with yeast nitrogen base instead of yeast extract

The alcoholic fermentations (AF) were carried out by the different yeast strains in the SGJ media, the NGJ media and the Cabernet Sauvignon and Syrah musts described in paragraphs II.1.3.4 a, c and d. The AF with *S. cerevisiae* strain D was also conducted in the same SGJ medium once by replacing the yeast extract by a yeast nitrogen base without peptides but with amino acids (Fluka-Buchs, Switzerland) and another time by a yeast nitrogen base lacking both peptides and amino acids (Sigma-Aldrich). The composition of both types of yeast nitrogen base is presented in Table II.6:

Table II.6. Composition of both types of yeast nitrogen base

| Components | Amount (l) | |
|-------------------------------|---|--|
| | Yeast nitrogen base without peptides but with amino acids | Yeast nitrogen base lacking peptides and amino acids |
| Ammonium sulfate | 5 g | 5 g |
| L-Histidine HCl | 10 mg | - |
| DL-Methionine | 20 mg | - |
| DL-Tryptophan | 20 mg | - |
| Biotin | 2 µg | 2 µg |
| Calcium pantothenate | 400 µg | 400 µg |
| Folic acid | 2 µg | 2 µg |
| Niacin | 400 µg | 400 µg |
| p-aminobenzoic acid | 200 µg | 200 µg |
| Pyridoxine HCl | 400 µg | 400 µg |
| Riboflavin | 200 µg | 200 µg |
| Thiamine HCl | 400 µg | 400 µg |
| Inositol | 2 mg | 2 mg |
| Citric acid | - | 0.1 g |
| Boric acid | 500 µg | 500 µg |
| Copper sulfate | 40 µg | 40 µg |
| Potassium iodide | 100 µg | 100 µg |
| Ferric chloride | 200 µg | 200 µg |
| Manganese sulfate | 400 µg | 400 µg |
| Sodium molybdate | 200 µg | 200 µg |
| Zinc sulfate | 400 µg | 400 µg |
| Potassium phosphate monobasic | 1 g | 1 g |
| Magnesium sulfate | 0.5 g | 0.5 g |
| Sodium chloride | 0.1 g | 0.1 g |
| Calcium chloride | 0.1 g | 0.1 g |

The amino acid's composition of the yeast extract (Oxoïd) was as follows: valine (1 %), tyrosine (4.95 %), tryptophan (0.85 %), threonine (2.73 %), serine (3.42 %), proline (0.88 %), phenylalanine (3.78 %), methionine (0.8 %), lysine (5.4 %), leucine (6.04 %), isoleucine (4.81

%), glycine (5.95 %), glutamate (13.49 %), cystine (0.76 %), aspartate (7.07 %), arginine (3.31 %), alanine (0.91 %).

II.2 Methods

II.2.1 Fermentations

II.2.1.1 Alcoholic fermentation (AF)

The synthetic grape juice (SGJ) and the natural grape juice (NGJ) media as well as the Cabernet Sauvignon and the Syrah musts were inoculated by the yeasts at an initial concentration of 3×10^6 cells/ml (Thoma counting chamber) and the AF was carried out until total or cessation of sugar consumption (< 2 g/l). The yeast inoculum was beforehand prepared in two steps. First, a preculture of the yeast strain was obtained by reactivating the stock culture in YEPD broth for 24 h. Second, 2 ml of the preculture were used to inoculate 50 ml of a low sugar concentration synthetic grape juice medium: D-Glucose 50 g/l and absence of D-Fructose (starter culture, paragraph II.1.3.3). This step was carried out for 24 h and provided the yeast inoculum. All the fermentation steps were carried out in a multi stack shaking incubator (Labtech, Italy) at 22 °C with stirring at 150 rpm in Erlenmeyer flasks. The yeast growth kinetic and metabolism were monitored by collecting two samples per day. After determination of kinetic parameters, samples were centrifuged before initiating the biochemical tests and filtered before HPLC analysis.

All the AF steps were performed in three replicates.

II.2.1.2 Malolactic fermentation (MLF)

The synthetic grape juice (SGJ) and the synthetic wine medium (SWM) were inoculated by the bacteria at an initial concentration of 2×10^6 cells/ml (Petroff-Hausser counting chamber) and the MLF was carried out until total or cessation of L-malic acid consumption. The bacterial inoculum was beforehand prepared in two steps. First, a preculture of the bacterial strain was obtained by reactivating the stock culture in MRS broth for 24 h. Second, 2 ml of the preculture were used to inoculate 50 ml of a low sugar concentration synthetic grape juice medium: D-Glucose 50 g/l and absence of D-Fructose (starter culture, paragraph II.1.3.3). This step was

carried out for 24 h and provided the bacterial inoculum. All the fermentation steps were carried out in a multi stack shaking incubator (Labtech, Italy) at 22 °C with stirring at 150 rpm in Erlenmeyer flasks. The bacterial growth kinetic and metabolism were monitored by collecting one sample per day. 3 % ethanol (v/v) and 6 % ethanol (v/v) were added respectively to the preculture and starter culture steps when the MLF was performed in the SWM.

All the MLF steps were performed in three replicates.

II.2.1.3 Sequential fermentations

After completion of the AF, the yeast fermented media (SGJ, NGJ and Cabernet Sauvignon and Syrah musts) were subjected to different steps before inoculation of the lactic acid bacteria. First, yeast cells were removed by centrifugation (3500 rpm for 20 min at 4 °C) and the supernatant was recovered. Then, the L-malic acid concentration was measured and readjusted to 3 g/l. Next, the pH was adjusted to 3.5 using a 10 M NaOH solution. Finally, the yeast fermented media were filtered aseptically through 0.22 µm membranes (Elvetec services) and the sterile yeast-free fermented media were recuperated in autoclaved Erlenmeyer flasks.

Afterward, the sterile yeast-free fermented media were inoculated by the malolactic bacteria at an initial concentration of 2×10^6 cells/ml and the MLF was followed by regular sampling until the cessation of L-malic acid consumption. All fermentation steps for both AF and MLF were carried out at 22 °C with stirring at 150 rpm in Erlenmeyer flasks. The sequential fermentations were performed in three replicates and three yeast/bacteria pairs were tested:

S. cerevisiae strain A/*O. oeni* strain X

S. cerevisiae strain D/*O. oeni* strain X

S. cerevisiae strain R/*O. oeni* strain X

II.2.2 Analytical methods

II.2.2.1 Cell enumeration

For the enumeration of the microbial cells, two different cell counting chambers were used: Thoma counting chamber for yeasts (0.1 mm depth) and Petroff-Hausser counting chamber for bacteria (0.02 mm depth).

The cell concentration was calculated by the following formulas:

- For yeasts:

$$X \text{ (cells/ml)} = [(\text{number of cells counted}) / (\text{number of large squares counted})] \times D \times 0.25 \times 10^6$$

- For bacteria:

$$X \text{ (cells/ml)} = [(\text{number of cells counted}) / (\text{number of large squares counted})] \times D \times 125 \times 10^4$$

D = dilution factor

II.2.2.2 Yeast viability

The viability was evaluated by counting yeast cells using the Thoma counting chamber after staining the sample (v/v) with methylene blue for 10 min. The methylene blue dye was prepared by dissolving 0.1 g of methylene blue into one liter of 2 % sodium citrate solution. Blue cells were considered as dead while the colorless ones were living cells (Lange et al., 1993).

The percentage of living cells was then calculated.

II.2.2.3 Optical density (OD) or absorbance measurement

The OD of the microbial samples (yeasts and bacteria) was measured with an UV/Vis Spectrophotometer (Shimadzu, Japan) at 620 nm in plastic cuvettes of 1 cm width. To maintain a linear correlation between the cell concentration and the OD, the absorbance should remain less than or equal to 0.8, otherwise the sample should be diluted.

II.2.2.4 Biomass or dry weight measurement

The dry weight measurement of yeasts and bacteria was performed using a moisture analyzer (Ohaus, UK). The sample was centrifuged in order to remove the supernatant. The pellet was recovered and washed twice with distilled water to remove any residual substrate. It was finally suspended in a small volume of distilled water and introduced into the analyzer. The sample was heated until total evaporation of water and stabilization of the dry weight. The results were expressed in g/l.

II.2.2.5 DNS method for the determination of the sugar concentration

The sugar consumption during the alcoholic fermentation was followed using the dinitrosalicylic (DNS) colorimetric method (Miller, 1959) based on the presence of free carbonyl groups (C=O) and the results were expressed in g/l. The main reducing sugars in the synthetic and natural grape juices are D-Glucose and D-Fructose. This method involves mixing DNS reagent with a sample, heating to catalyze the reaction and measuring the visible absorbance of the products (particularly 3-amino-5-nitrosalicylic acid).



The DNS reagent was conserved in amber bottles at 4 °C for 15 days and was prepared as following:

- 3, 5-Dinitrosalicylic acid (2.5 g)
- Sodium potassium tartrate (75 g)
- NaOH (4 g)
- Distilled Water (250 ml)

A stock solution of 2 g/l of sugars composed of 50 % D-Glucose and 50 % D-Fructose was prepared and used to create a calibration curve covering a range of concentrations of: 0, 0.5, 1, 1.5 and 2 g/l. Since the sugar concentration at the start of the alcoholic fermentation was 200 g/l, the samples taken should be diluted so as not to exceed the upper limit of the calibration curve (2 g/l). After dilution, 1 ml of DNS was added to 1 ml of each sample and mixed thoroughly before incubating the tubes in a water bath for 10 minutes at 100 °C. The tubes were then cooled on ice and 10 ml of distilled water was added. Next, the samples were mixed and kept in a dark place for 15 min. The OD was measured using a spectrophotometer at 540 nm and the unknown concentrations of the samples were determined from the calibration curve.

II.2.2.6 Glycerol and ethanol measurement by HPLC

Glycerol and ethanol produced by yeasts during the AF were measured using the HPLC method. The column used was an Aminex[®] HPX-87H Bio-Rad (30 cm x 7.8 mm) presenting a cationic

H⁺ coverage, thermostated at 40 °C, and coupled to a Biorad guard column (H⁺). The solvent used was a 5 x 10⁻³ M sulfuric acid solution eluted at a flow rate of 0.4 ml/min with a pump P1000XR Thermo Separation Products. The HPLC was coupled to an automate injector AS100 with an injection clasp of 20 µl, a refractive index detector IV and a Borwin V 1, 2 software for data analysis. The results were calculated according to glycerol and ethanol standard scales and expressed in g/l.

II.2.2.7 Fatty acids measurement by GC

The medium chain fatty acids produced by yeasts during the AF were measured using the Gas Chromatography (Hewlett Packard HP 5890) with hydrogen as vector gas and a capillary column SGE FFAP. The detector was FID and the split less mode was used. Octan-3-ol was used as internal standard. Results were contracted out in the ISVV, University of Bordeaux and were expressed in mg/l.

II.2.2.8 Determination of the SO₂ concentration

The concentration of SO₂ produced by yeasts during the AF was evaluated using the Ripper iodometric method (Recueil des méthodes analytiques de l'OIV, 1974). The results were expressed in mg/l.

Reagents:

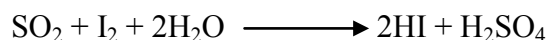
- Iodide iodate solution 0.1N (Fluka 3805)
- Starch suspension (100 ml, 5 g of starch, 10 g of NaCl)
- H₂SO₄ 98 %, 1/3 diluted
- KOH 1N
- Determination of free SO₂:

First, 2 ml of sulfuric acid and 2 ml of the starch suspension were added to 50 ml of the fermented SGJ. Then, a volume V of iodide was added until the appearance of a blue-violet color at equivalence.

- Determination of total SO₂:

15 ml of KOH 1N were added to 50 ml of the fermented SGJ for 10 minutes. The KOH was used to release the sulfur dioxide combined to aldehydes. Then, 5 ml of sulfuric acid and 2 ml of starch suspension were added respectively. Then, a volume V' of iodide was added until the appearance of a blue-violet color added at equivalence.

Based on the following reaction:



At equivalence:

$$\begin{aligned} n(\text{I}_2) &= n(\text{SO}_2) && n = \text{number of moles} \\ \text{CV}(\text{I}_2) &= \text{CV}(\text{SO}_2) && C = \text{molarity (mol/l)} \\ &&& V = \text{volume (ml)} \end{aligned}$$

- Determination of the molecular toxic form of free SO₂:

The molecular toxic form of free SO₂ was determined by the following formula (www.itv-midipyrenees.com):

$$\text{Molecular SO}_2 \text{ (mg/l)} = \text{Free SO}_2 \text{ (mg/l)} / [10^{(\text{pH}-\text{pKa})} + 1]$$

The pH value was 3.5 and corresponded to the initial pH of all the culture media used in this study. The pKa of SO₂ at 22 °C and in the presence of ethanol between 9 and 11 % (v/v) is 2.

II.2.2.9 Measurement of polysaccharides

Principle

The assay consists in precipitating the polysaccharides contained in the fermented medium by adding hydrochloric acid and ethanol. The supernatant is then filtered through filtration membranes and the retentate is weighed (Vinidea.net-Wine Internet Technical Journal, 2002).

Reagents:

-HCl 36 %

-Ethanol 95 %

Experimental procedure:

- Centrifuge 20 ml of the medium at 12,000 x g for 10 minutes

- Recover the supernatant in an Erlenmeyer flask of 100 ml
- Precipitate the polysaccharides by adding 4 ml of HCl 36 % diluted 1:1 and 80 ml of ethanol 95 %
- Stir at 4 °C for 24-48 h
- Dry acetate cellulose filters of 0.45 µm cut-off (Sartorius, Goettingen-Germany) in an oven at 40 °C for 30 minutes and weigh (P₁)
- Use the membranes for vacuum filtration of the medium to be analyzed after polysaccharides precipitation
- Dry again in an oven at 40 °C for 30 minutes and weigh the dried filters (P₂)

$$[\text{Total Polysaccharides (mg/l)}] = (P_2 - P_1) / V_{\text{medium}}$$

II.2.2.10 Determination of the protein concentration by the Lowry method

Principle

The Lowry method is based on the formation of a complex between the peptide bonds and the copper (II) sulfate under alkaline conditions. This complex reduces the phosphomolybdic and phosphotungstic acids of the Phenol Folin-Ciocalteu reagent to give a blue complex quantified by spectrophotometry (Frolund et al., 1995).

Reagents

Reagent A: Na₂CO₃ (20 g/l) + NaOH (0.1 N) + Potassium Sodium Tartrate (1 g/l)

Reagent B: CuSO₄.5H₂O (5 g/l)

Lowry reagent: 50 ml A + 5 ml B

Phenol Folin-Ciocalteu reagent: ready to use, ½ diluted, freshly prepared

The standard curve was prepared with a 500 mg/l BSA (bovine serum albumin) stock solution to cover the following range of concentrations: 0, 50, 125, 250, 375, 500 mg/l.

Experimental procedure:

Add to each tube:

- 0.5 ml of each sample or standard solution (distilled water was used for the blank)
- 2 ml of the Lowry reagent. Mix well and let stand at room temperature for 10 min

- 0.2 ml of the Phenol Folin-Ciocalteu reagent. Mix well and let stand away from light at room temperature for 30 min
- Measure the OD at 691 nm

The results were determined according to the standard curve and expressed in mg/l.

II.2.2.11 Determination of the protein concentration at 280 nm

The OD of the purified or the fractionated proteins solubilized in a phosphate buffer (0.1 M, pH 6.7) was estimated by spectrophotometry at 280 nm (Thermo Scientific NanoDrop 2000/2000c TM-USA). A volume of 1 μ l was poured on the pedestal. BSA standard solutions ranging from 0.1 mg/ml to 10 mg/ml were prepared and used for calibration. The blank was read against the same buffer used to suspend the protein pellets.

In order to obtain a linear correlation between the OD and the protein concentration, the absorbance should remain below 1. Otherwise the sample must be diluted. This rapid and sensitive method requires a protein amount of 100 μ g or more in each sample.

II.2.2.12 Determination of total polyphenols

Principle

The phenolic compounds in musts and wines are oxidized by the Folin-Ciocalteu reagent. The latter is composed of phosphotungstic ($H_3PW_{12}O_{40}$) and phosphomolybdic ($H_3PW_{12}O_{40}$) acids that are reduced during the oxidation of phenols into a blue complex of tungsten and molybdenum oxides quantified at 750 nm.

Experimental procedure as described by Ribéreau-Gayon et al., (2006)

1 ml of diluted must or wine (1:5) was first introduced into a volumetric flask of 100 ml. 50 ml of distilled water, 5 ml of the Folin-Ciocalteu reagent and 20 ml of a 20 % sodium carbonate solution were then added to the flask. Finally, the volume was completed to 100 ml with distilled water and well mixed. After an incubation period of 30 min at room temperature, the absorbance was measured at 750 nm using distilled water as blank. A reference sample was prepared by replacing must/wine with distilled water and the same procedure was followed. The OD

difference between the must/wine and the reference sample was calculated and used to determine the concentration of total polyphenols by referring to a calibration curve prepared with gallic acid. The result was multiplied by the dilution factor and expressed in mg/l.

II.2.2.13 Enzymatic assays for the determination of the L-malic acid, L-lactic acid, D-lactic acid, acetic acid, citric acid and ethanol concentrations

The enzymatic assays (Boehringer Mannheim/R-Biopharm, Darmstadt-Germany) of L-malic acid (Kit. No. 10139068035), L-lactic acid (Kit. No. 10139084035), D-lactic acid (Kit. No. 11112821035), acetic acid (Kit. No. 10148261035), citric acid (Kit. No. 1013976035) and ethanol (Kit. No 10176290035) were performed at room temperature (20-25 °C). An UV/visible spectrophotometer (Thermoscientific, USA) was used for the determination of the OD at 340 nm except for ethanol at 334 nm. Cuvettes of 1 cm light path were used. The results were expressed in g/l.

II.2.3 Characterization of the yeast anti-MLF proteinaceous compounds

II.2.3.1 Heat and protease treatments of the SGJ media fermented by *S. cerevisiae* strain D

At the completion of AF (120 h), the SGJ medium fermented by *S. cerevisiae* strain D, was first centrifuged (3000 rpm for 20 min at 4 °C) and the supernatant was recovered and submitted to the following treatments:

- Heat treatments at 100 °C for 30 min and at 121 °C for 20 min.
- Enzymatic treatments with 10 µg/µl of 3 different proteases for 1h at 37 °C after adjusting the medium pH to the optimum pH (pH_{opt}) for activity of each enzyme: pH_{opt} of pepsin (Sigma P7125) = 2, pH_{opt} of trypsin (Sigma 93613) = 7.6, pH_{opt} of papain (Sigma R3375) = 6.7. To increase the pH, a 10 M NaOH solution was used and to lower it an 85 % orthophosphoric acid solution was used.

At the end of these treatments, the L-malic acid concentration was readjusted to 3 g/l and the pH was brought back to 3.5 before bacterial inoculation.

Then, 250 ml of the untreated and treated yeast fermented media were inoculated with *O. oeni* strain X at an initial concentration of 2×10^6 cells/ml and the MLF was conducted in Erlenmeyer

flasks at 22 °C with stirring at 150 rpm. The same bacterial preculture steps described in paragraph II.2.1.2 were used for the preparation of the bacterial inoculum. The MLF was followed by regular sampling until the cessation of L-malic acid consumption.

As a reference for bacterial growth and malic acid degradation kinetics during the sequential fermentations performed in the untreated and treated yeast fermented media, two control cultures of *O. oeni* strain X were carried out. The first one was performed in 250 ml of the SGJ medium and the second one in 250 ml of the SWM. These control cultures were grown at 22 °C with stirring at 150 rpm starting with an initial concentration of 2×10^6 cells/ml.

All the treatments and the MLF steps were performed in three replicates.

II.2.3.2 Fractionation of the yeast fermented media by ultrafiltration

Principle

The ultrafiltration of the yeast fermented media separates the different components according to their MW by using membranes presenting different cut-offs. It also leads to the concentration of the retentate. The concentration factor depends on the initial volume filtered and the final volume retained by the membrane. The diffusion of the components from one side of the membrane to another is the result of the pressure difference. Ultrafiltration is generally tangential and the fluid flows parallel to the membrane unlike the frontal conventional filtration. Cross flow filtration reduces membrane fouling problems. In the following experiment, several ultrafiltration modules containing membranes with different cut-offs were used. The separation of the components according to their MW was due to the tangential force created by centrifugation.

Technique

In order to determine the apparent molecular weight of the anti-MLF proteinaceous compounds, the yeast fermented medium was fractionated by ultrafiltration through centrifugal filter units (3,500 xg, 45 min, 4 °C) having cut-offs of 10 kDa (Amicon® Ultra-15 with ultracel-10 membrane), 5 kDa (Corning® Spin-X UF 20 Sigma-Aldrich) and 3 kDa (Amicon® Ultra-15 with ultracel-3 membrane). First, a volume of 210 ml of the fermented SGJ medium was distributed in the ultrafiltration units presenting a cut-off of 10 kDa. Each unit can initially contain a volume of 15 ml. 14 units were filled and at the end of the ultrafiltration a volume of 0.2 ml was retained by the membrane of each unit. A total retentate of 2.8 ml with a MW \geq 10 kDa and 75 times

concentrated was obtained. The filtrate ≤ 10 kDa was ultrafiltered again using the units of 5 kDa cut-off. The principle was the same and 2.8 ml of a fraction with a MW between 5 and 10 kDa 75 times concentrated was obtained. The filtrate lower than 5 kDa was ultrafiltered using the units of 3 kDa cut-off and a fraction of a MW between 3 and 5 kDa 75 times concentrated was obtained. This work yielded 3 retentates of 2.8 ml each, 75 times concentrated containing compounds ≥ 10 kDa, between 5 and 10 kDa and between 3 and 5 kDa and a non-concentrated filtrate ≤ 3 kDa.

Each retentate (2.8 ml) was added to 20 ml of a modified MRS medium and was finally 10.5 times concentrated whereas the non-concentrated filtrate ≤ 3 kDa replaced the distilled water for the preparation of the corresponding modified MRS medium (Figure II.1).

O. oeni strain X was then inoculated into these four media at an initial concentration of 2×10^6 cells/ml and the MLF was carried out at 22 °C with stirring at 150 rpm in Erlenmeyer flasks. The same modified MRS medium without the fractions of the yeast fermented medium was used as a control for this experiment. The MLF was followed by regular sampling until the cessation of L-malic acid consumption.

All the fractionation steps and the MLF steps in the modified MRS media with or without the proteinaceous fractions were performed in triplicate.

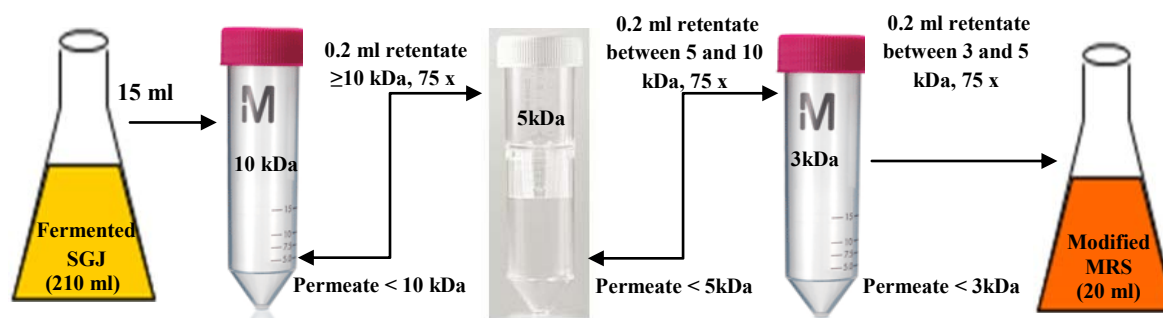


Figure II.1. Ultrafiltration of the yeast fermented SGJ medium for harvesting different peptidic fractions

II.2.3.3 Timing of the release of the 5-10 kDa inhibitory fractions by *S. cerevisiae* strain D

The AF was carried out for 5 days (120 h) in 2 l of the SGJ medium. Each 24 h, a supernatant of 210 ml was collected and fractionated by ultrafiltration in order to isolate the corresponding 5-10 kDa peptidic fraction. Five fractions were finally collected and were referred to as D1 (24 h), D2

(48 h), D3 (72 h), D4 (96 h) and D5 (120 h). The fraction from each day (2.8 ml, 75 times concentrated) was added to 20 ml of a modified MRS medium and was finally 10.5 times concentrated.

O. oeni strain X was then inoculated into these media at an initial concentration of 2×10^6 cells/ml and the MLF was carried out at 22 °C with stirring at 150 rpm in Erlenmeyer flasks. The same modified MRS medium without the 5-10 kDa fractions was used as a control for this experiment. The MLF was followed by regular sampling until the cessation of the L-malic acid consumption.

All the fractionation steps and the MLF steps in the modified MRS media were performed in three replicates.

II.2.3.4 Fractionation of the yeast fermented media by ammonium sulfate precipitation

Principle

Proteins have unique solubility profiles in neutral salt solutions. Low concentrations of neutral salts usually increase the solubility of proteins; however, proteins are precipitated from solution as ionic strength is increased, it's the *Salting Out* phenomenon. This property can be used to precipitate a protein from a complex mixture. Ammonium sulfate $[(\text{NH}_4)_2\text{SO}_4]$ is commonly used because it is highly soluble and does not denature the proteins. An online web browser was used to determine the proper amount of ammonium sulfate to achieve a specific concentration:

(www.encorbio.com/protocols/AM_SO4.htm)

Technique

The SGJ media fermented by yeasts were centrifuged to recuperate 500 ml of the supernatant (3500 rpm, 20 min, 4 °C). The residual $(\text{NH}_4)_2\text{SO}_4$ in the yeast fermented media was negligible. Therefore the starting percentage saturation of ammonium sulfate in these media was almost 0. The program on [www.encorbio.com/protocols/AM_SO4.htm] enables us to calculate how much solid ammonium sulfate we need to add to a specific volume of a solution to get specific percentage saturation at a specific temperature. The different amounts of $(\text{NH}_4)_2\text{SO}_4$ added were therefore calculated to reach the following saturation degrees: A: 0-20 %, B: 20-40 %, C: 40-60 %, D: 60-80 % and E: 80-100 %.

For example, in order to reach a saturation degree of 20 %, 55 g of $(\text{NH}_4)_2\text{SO}_4$ should be added. The salt was added gradually for one hour while shaking at 4 °C. Then, after 90 min, the medium was centrifuged at 5000 rpm for 30 min at 4 °C. Next, the protein pellet was suspended in 10 ml of a phosphate buffer (0.1M, pH 6.7), thus our sample was finally 50 times concentrated. Finally, the sample was desalted using an ultrafiltration unit of 5 kDa cut-off. 0.2 ml of a protein retentate with a MW \geq 5 kDa was obtained. The ultrafiltration added a new concentration factor of 50 X. The retentate which was then 2500 times concentrated (50 x 50) was added to 20 ml of a modified MRS medium in order to evaluate its inhibitory effect. It was 100 times diluted and was finally 25 times concentrated. The remaining supernatant was used again to reach a new saturation level and the same procedure was repeated.

The inhibitory effect of the five precipitated fractions (A, B, C, D and E) was evaluated separately in modified MRS media inoculated by 2×10^6 cells/ml of *O. oeni* strain X. The MLF was followed by regular sampling until cessation of malate consumption and compared to that of a modified MRS control.

All the experiments were performed in three replicates.

II.2.3.5 Protein purification by Ion Exchange Chromatography

Principle

Adsorption chromatography is defined as the separation of compounds by adsorption to, or desorption from, the surface of a solid support by an eluting solvent. Separation is based on differential affinity of the protein for the adsorbent or eluting buffer. The most commonly used protein separation is Ion Exchange Chromatography (IEXC) defined as the reversible adsorption between charged molecules and ions in solution and a charged solid support matrix. A positively charged matrix is called an anionic exchanger (AEX) because it binds negatively charged ions or molecules in solution. A negatively charged matrix is called a cationic exchanger (CEX) because it binds positively charged ions or molecules. The protein of interest is first adsorbed to the IECX under buffer conditions (ionic strength and pH) that maximize the affinity of the protein for the matrix. Contaminating proteins of different charges pass through the exchanger unadsorbed. Proteins bound to the exchangers are selectively eluted from the column by gradually changing the ionic strength and/or pH of the eluting solution. As the composition of

the eluting buffer changes, the charges of the proteins change and/or their affinity for the ion-exchange matrix is decreased.

Besides, ion exchange chromatography is used to concentrate and purify proteins based on their ionic charge or isoelectric point (pI) at a given pH. At the isoelectric point of a protein, its net charge is zero. At a pH higher than the pI of the protein, the protein will be negatively charged and will bind to an AEX resin. At a pH lower than the pI , the protein will be positively charged and will bind to a CEX resin. When an ion is applied to an ion exchange of opposite charge, it is adsorbed to the resin, while neutral ions or ions of the same charge are eluted in the flow-through fraction. Binding of the ions is reversible, and adsorbed molecules are commonly eluted with salt or a change of pH.

Technique

The nature of the fractionated proteins/peptides obtained by ultrafiltration and ammonium sulfate precipitation was unknown. Therefore, they were further purified through cationic and anionic exchange chromatography.

The columns used were Bio-Scale™ Mini cartridges of 5 ml; UNO sphere Q [$-N^+(CH_3)_3$] as strong anion exchanger and UNO sphere S [$-SO_3^-$] as strong cation exchanger (Bio-Rad). First, the pump flow rate was set to 6 ml/min. The cartridge was washed for 2 min with a degassed low-salt buffer (corresponding binding buffer + NaCl 0.5 M) and for 5 min with a degassed high-salt buffer (corresponding binding buffer + NaCl 1 M). The degassing was performed in an ultrasonic bath (Thermo scientific-USA). The cartridge was then equilibrated with the same low-salt buffer for 5 min. Next, the flow rate was reduced to 1 ml/min for the purification protocol. At the end of each run, the cartridge can be regenerated with 1 M NaCl followed by starting buffer.

Sample preparation

1 ml of the 5-10 kDa fraction of the 60-80 % ammonium sulfate precipitate was used for purification. The samples were degassed and sterile filtered through 0.45 μ m filters (Sartorius, Goettingen- Germany) before use.

First, 500 ml of the SGJ fermented by *S. cerevisiae* strain D were used to recuperate the 60-80 % precipitate. The precipitate was then suspended in 10 ml of the corresponding binding buffer (50 X) and submitted to ultrafiltration in the 10 kDa unit. The filtrate lower than 10 kDa was

ultrafiltered again using the 5 kDa unit. The desalted retentate between 5 and 10 kDa was recuperated (0.2 ml, 2500 X). 1 ml of this retentate was collected for each analysis.

Binding and elution buffers

Binding buffers: 1 M Tris buffer pH 8.3 was used for the anionic exchanger. 10 mM phosphate buffer pH 6.7 or citric acid buffer pH 5.2 were used for the cationic exchanger.

Elution buffers: For both anionic and cationic exchange chromatography, increasing salt gradients were adopted for elution. The elution buffers were therefore composed of binding buffers coupled to increasing concentrations of NaCl (0.1, 0.5 and 1 M). The eluted samples were concentrated by the speed-vacuum-concentrator (each 1 ml of eluate gave a final volume of 0.5 ml) and used to test their inhibitory effect on the malolactic enzyme activity of the enzymatic extract *in vitro* (paragraph II.2.4).

II.2.3.6 Protein purification by Aurum™ Ion Exchange Mini Kits and Columns

Principle

Aurum AEX and CEX columns allow rapid fractionation of complex protein mixtures in only a few steps using the common technique of ion exchange chromatography. Each Aurum AEX (732-6706) or CEX (732-6703) column contained 0.2 ml of UNO sphere™ Q [$-N^+(CH_3)_3$] or S [$-SO_3^-$] support. The buffers selected were the following:

- Binding buffers: 20 mM phosphate pH 6.7 for CEX and 20 mM Tris pH 8.3 for AEX
- Elution buffers: binding buffer + 0.5 M NaCl, binding buffer + 1 M NaCl

Table II.13 gives the common buffers used for ion exchange chromatography.

Table II.7. Common buffers used for ion exchange chromatography

| Cation | Buffering Range | Anion | Buffering Range |
|-------------|-----------------|-----------------|-----------------|
| Acetic acid | 4.8–5.2 | Bicine | 7.6–9.0 |
| Citric acid | 4.2–5.2 | Bis-Tris | 5.8–7.2 |
| HEPES | 7.6–8.2 | Diethanolamine | 8.4–8.8 |
| MES | 5.5–6.7 | Diethylamine | 9.5–11.5 |
| MOPSO | 6.5–7.9 | L-histidine | 5.5–6.0 |
| Phosphate | 6.7–7.6 | Imidazole | 6.6–7.1 |
| PIPES | 6.1–7.5 | Pyridine | 4.9–5.6 |
| TES | 7.2–7.8 | Tricine | 7.4–8.8 |
| Tricine | 7.8–8.9 | Triethanolamine | 7.3–8.0 |
| | | Tris | 7.5–8.0 |

Technique

All samples should be filtered before application to Aurum ion exchange columns. This can be accomplished using a 0.45 μm syringe filter or by centrifuging the sample at 10,000 x g for 5 min. The same 5-10 kDa peptidic fractions of the 60-80 % ammonium sulfate precipitates previously described in paragraph II.2.3.5, were used for further purification by the Aurum kit.

1 ml of these fractions 2500 X were collected for each analysis.

The following steps describe the procedure adopted (Figure II.2):

- 1- Place an ion exchange column in a 12 x 75 mm test tube and allow the resin to settle for at least 5 min
- 2- Remove the cap and break off the tip from the bottom of the ion exchange column. Return column to test tube
- 3- Start gravity flow in the column and allow residual buffer to drain from the column (approximately 2 min). If the column does not begin to flow, push the cap back on the column and then remove it again to start the flow
- 4- Once the residual buffer has drained, wash the column with 2 x 1 ml of the appropriate Aurum AEX or CEX binding buffer using gravity flow. Allow each wash to pass fully through the column and drain
- 5- After the last wash, place the column in an empty 2 ml collection tube and centrifuge for 10 sec at 1,000 x g in a microcentrifuge to dry resin bed and frit. Discard the collection tube
- 6- Place the column in a clean 2 ml collection tube and label it “**unbound**”

- 7- Load 1 ml of the sample with its appropriate buffer onto the column and allow the sample to gravity filter through the column, collecting in the collection tube. If the column does not begin to flow, push the cap back on the column and remove it again to start the flow
- 8- Place the column in another clean 2 ml collection tube
- 9- Wash the column with 0.3 ml of Aurum AEX or CEX binding buffer. Unbound fractions can be combined for further analysis if desired. Otherwise, unbound fractions can be discarded
- 10- Place the column in a 2 ml collection tube and wash with 0.6 ml of the appropriate Aurum AEX or CEX binding buffer. Centrifuge column for 20 sec at 1,000 x g in a microcentrifuge, collecting the eluate in the tube. This wash can be discarded
- 11- Place the column in a new 2 ml collection tube and label it “**bound #1**”
- 12- Add 0.3 ml of the appropriate Aurum AEX or CEX elution buffer that contains 0.5 M NaCl to the top of the column and centrifuge for 10 sec at 1,000 x g
- 13- Wash with an additional 0.3 ml of the Aurum AEX or CEX elution buffer into the same “bound #1” tube. Centrifuge for 10 sec at 1,000 x g and recuperate the first eluate
- 14- Place the column in a new 2 ml collection tube and label it “**bound #2**”
- 15- Wash with 0.3 ml of the Aurum AEX or CEX elution buffer that contains 1 M NaCl. Centrifuge for 10 sec at 1,000 x g and recuperate the second eluate

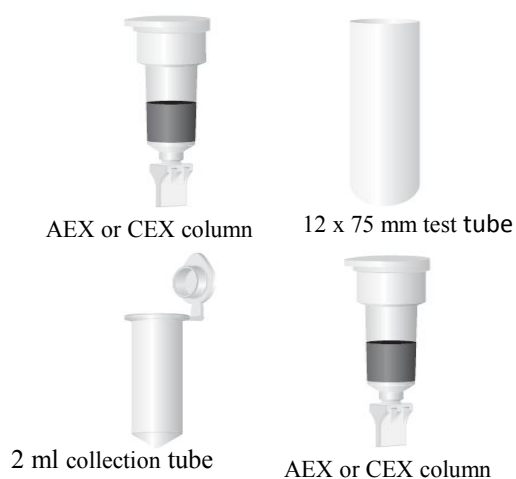


Figure II.2. Aurum ion exchange mini kit components

II.2.3.7 Protein purification by Tricine SDS-PAGE

Sample preparation

The proteinaceous fractions were analyzed by Tricine SDS-PAGE as described by Schägger and Jagow (1987) and Schägger (2006), to separate proteins on the basis of their sizes.

500 ml of the SGJ fermented by *S. cerevisiae* strain A (reference strain) and *S. cerevisiae* strain D (inhibitory strain) were used for this experiment. The 60-80 % ammonium sulfate precipitate from each medium was suspended in 10 ml of phosphate buffer (0.1 M, pH 6.7). The proteins were therefore 50 times concentrated. Then, the 10 ml were ultrafiltered using a 10 kDa unit and the filtrate lower than 10 kDa was ultrafiltered again in the 5 kDa unit. 0.2 ml of a desalted retentate with a MW between 5 and 10 kDa was recuperated. An additional concentration factor of 50 X was added and the sample was finally 2500 times concentrated (2500 X).

A protein precipitation solution or TCA solution (Table II.14) was used for further concentration of the samples which were then dried in a dry heat block (Major Science, USA) for 15 min (Sanchez, 2001). 1 volume (250 μ l) of TCA is needed for each 4 volumes of concentrated proteins (1 ml, 2500 X). Proteins were pelleted (14,000 x g, 4 °C, 15 min), washed twice in 200 μ l of ice-cold acetone (14,000 x g, 4 °C, 5 min) and suspended in 100 μ l Triton X-100 buffer (2 % v/v Triton X-100, 1 % v/v dithiothreitol, Sigma-Aldrich). The proteins were finally 25 000 times concentrated (25 000 X).

Table II.8. Protein precipitation solution or TCA solution composition

| Components | | Amount |
|--------------------|-----------|---------------|
| TCA | | 20 g |
| DTT | | 0.2 g |
| Acetone | | 80 ml |
| diH ₂ O | | 6 ml |
| Acetone | to | 100 ml |

Reagents and Technique

Loading sample buffer: 2X Laemmli buffer (30 ml)

The composition of the loading buffer (Table II.15) was the following: 62.5 mM Tris-HCl pH 6.8, 2 % SDS, 25 % Glycerol, 0.01 % Bromophenol Blue, 5 % β -mercaptoethanol (added fresh). The peptidic samples were mixed with the buffer (1:1) and set at 75 °C for 10 min in a dry heat block prior to electrophoresis. Therefore, the loading buffer was finally reduced to 1X before use.

Table II.9. Laemmli sample buffer composition

| Components | | Volume (ml) |
|-------------------------|-----------|-------------|
| 0.5 M Tris-HCl pH 6.8 | | 3.75 |
| 50 % Glycerol | | 15 |
| 1 % Bromophenol Blue | | 0.3 |
| 10 % SDS | | 6 |
| diH₂O | to | 30 |

β -mercaptoethanol (50 μ l to 950 μ l sample buffer) added before use

Running buffer: 10 X TrisTricine SDS (1 l)

The composition of the running buffer (Table II.16) was the following: 1 M Tris, 1 M Tricine, 1 % SDS, pH 8.3. The running buffer was reduced to 1X with distilled water before use.

Table II.10. Running buffer composition

| Components | | Amount (g) |
|-------------------------|-----------|------------|
| Tris base | | 121.10 |
| Tricine | | 179.20 |
| SDS | | 10 |
| diH₂O | to | 1 l |

Electrophoresis was carried out using a ready-to-use [8-16 %] Mini-PROTEAN[®] TGX[™] Precast gels composed of 12 wells in a Mini-PROTEAN[®] Tetracell (Bio-Rad Laboratories, Richmond-CA, USA). 20 µl of concentrated samples were run against 10 µl precision plus protein Dual-Xtra standard (2-250 kDa, criterion 10-20 % Tris-Tricine) composed of 12 recombinant proteins (Bio-Rad) at 100 V for 15 min. Gels were stained using Bio-safe[™] Coomassie G-250 stain or Silver stain kit (Bio-Rad). Imaging and documentation were done by using the Gel Doc XR⁺ System, controlled by Image Lab[™] software (Bio-Rad).

II.2.4 Peptides/Proteins sequencing by LC1D-nanoESI- LTQ-Velos Orbitrap

The sequencing was contracted out in PAIB2 (Plate-forme d'Analyse Intégrative des Biomolécules Phénomiques des Animaux d'Intérêt Bio-agronomique), Laboratoire de Spectrométrie de Masse, Centre de Recherches INRA Val de Loire-Tours.

The protein bands excised from SDS-PAGE were submitted to in-gel tryptic digestion. The peptidic extract was then dehydrated and analyzed by high resolution mass spectrometry. The peptides derived from the digestion of the bands were analyzed by a nanoUHPLC Ultimate 3000 RSLC system (Thermo Electron, US) coupled to a high resolution mass spectrometer LTQ Orbitrap Velos ETD (Thermo Electron, US).

The samples were desalted and concentrated in line by a precolumn Acclaim PepMap100 C18 trap (3 µm, 100 µm x 2 cm) (Dionex), then separated on an analytical reverse phase column Acclaim PepMAP C18 (2 µm, 75µm x 50 cm) (Dionex) with a flow rate of 300 nl/min.

The profile of the gradient was as follows:

- Equilibration of the column with 96 % of solvent A (0.1 % formic acid/2 % acetonitrile) and 4 % of solvent B (80 % acetonitrile/15.9 % H₂O/0.1 % formic acid, v/v)
- Gradient of 4 to 55 % B in 90 min
- 99 % B for 10 min

Data acquisition was done automatically between high resolution MS modes (R60 000) and MS/MS (low resolution fragmentation): an enhanced centroid full scan (m/z 400-1800) was followed by MS/MS centroid scans of the 20 most intense peaks detected (0.25 Qz, 10 ms activation time, collision energy 35). The dynamic exclusion was activated for 30 sec with a "repeat count" of 1.

The data was transformed into "mascot generic file" (.mgf) using the Proteome discoverer 1.3 software. It was then confronted with the database via MASCOT and via PD Deamon (local server version 2.3) by considering the following criteria:

- Indexed data bank: nr NCBI
- Trypsin and no enzyme
- 2 miss cleavages
- 5 ppm mass accuracy for MS and 0.8 Da for MS/MS
- Peptide load 2 and 3+
- Carbamidomethylation, N-terminal acetylation and oxidation of methionines in variable modifications
- Data Format: mgf
- Decoy database
- Instrument: ESI-FTICR
- Taxonomy: *_saccharomyces cerevisiae_*

The peptides and proteins identified by the Mascot search engine were then validated by the algorithms "Peptide Prophet" and "Protein Prophet" using Scaffold 4 software.

From the GeLC-MS/MS analyzes, interrogations were conducted from the NCBI bank against the taxonomy *_saccharomyces cerevisiae_*.

II.2.5 *In vitro* evaluation of the inhibitory effect of the yeast peptidic fractions on the malolactic enzyme activity

II.2.5.1 Preparation of the cell-free bacterial enzymatic extract

The modified MRS medium was inoculated with *O. oeni* strain X at an initial concentration of 2×10^6 cells/ml and the bacterial culture was followed for 5 days at 22 °C with stirring at 150 rpm. After a centrifugation at 3500 rpm for 20 min at 4 °C, the supernatant was removed and 0.4 g of the bacterial pellet were weighed and suspended in 800 µl of a specific buffer (citrate buffer pH 3.5 and 5.5, phosphate buffer pH 6 and 6.7, buffer concentration 0.1 M). The bacterial cells were then lysed by disrupting them with glass beads of 300 µm diameter (Sigma-Aldrich) in a FastPrep® FP120 bead-beater device (BIO 1010/Savant) at 6 m s^{-1} for 3 x 45 s (Mehmeti et al., 2011). Between 2 consecutive runs, the cells were kept on ice for 1 min. Cell debris were

removed by centrifugation at 14,000 x g for 10 min at 4 °C (Larsen et al., 2006). The supernatant containing the cell-free enzymatic extract was recovered and the volume completed to 2 ml using the same buffer.

II.2.5.2 Monitoring of the L-malic acid consumption *in vitro*

The 2 ml of the enzymatic extract were divided into 2 aliquots of 1 ml each. One was used as a control and the other as a test medium to which the peptidic fractions were added. The reaction mixture is given in Table II.17. The reagents were added to each aliquot in the order listed in the Table II.17. The enzymatic reaction was performed at 30 °C for 30 min at pH 5.5 and 6, 40 min at pH 6.7 and 70 min at pH 3.5.

At the end of each assay, the concentrations of the L-malic acid consumed and the L-lactic acid produced were measured in both aliquots (control and test) and compared.

The preparation of the enzymatic extract and the evaluation of the inhibitory effect of each peptidic fraction on the malolactic enzyme activity were performed in three replicates.

Table II.11. Reaction mixture for the monitoring of the L-malic acid consumption *in vitro*

| Aliquot 1 (Control) | Aliquot 2 (Test) | Amounts |
|--|--|----------------|
| Enzymatic extract | Enzymatic extract | 1 ml |
| Mn ²⁺ | Mn ²⁺ | 0.1 mg |
| NAD ⁺ (35 g l ⁻¹) | NAD ⁺ (35 g l ⁻¹) | 0.1 ml |
| ^a buffer | ^b ABP | 0.14 ml |
| L-malic acid (1 g l ⁻¹) | L-malic acid (1 g l ⁻¹) | 1 mg |

^a buffer: 0.1 M of either citrate or phosphate buffer depending on the chosen pH for the enzymatic reaction

^b ABP: antibacterial peptides. The ABP tested were those of the 5-10 kDa fractions isolated at different intervals of the AF, the 5-10 kDa fractions taken from the ammonium sulfate precipitates of 60-80 % saturation level, the ammonium sulfate precipitates of 0-60 % saturation level, the eluates from the IEXC and the bands of migrated peptides from Tricine SDS-PAGE.

II.2.6 Well plate test

Principle

This test aimed at evaluating on agar plates the inhibitory effect of the peptidic fractions obtained by ultrafiltration of the SGJ medium fermented by *S. cerevisiae* strain D. These fractions were introduced in wells dug inside the agar medium already containing the sensitive bacteria (*O. oeni* strain X). Inhibitory molecules diffuse radially around the wells and the inhibition appears as clear zones around them (the inhibition halo). The diameter of these zones is measured (cm) and used as the measurement unit for this test.

Technique

O. oeni strain X was grown overnight in MRS broth at 22 °C with stirring at 150 rpm. A modified MRS agar medium was prepared as follows:

- MRS 55.3 g/l
- Agar 12 g/l
- L-malic acid 5 g/l
- D-Fructose 3 g/l
- L-arginine 0.1 g/l

After autoclaving at 121 °C for 15 min, the modified MRS agar medium was cooled in a water bath at 45 °C before adding the bacterial strain in question so as to have an initial concentration of 2×10^6 cells/ml. The medium was then poured into Petri dishes and wells of 5 mm diameter were subsequently cut into the agar (3 wells per plate).

One of these wells was filled with the 5-10 kDa peptidic fractions obtained by ultrafiltration of the SGJ medium fermented by *S. cerevisiae* strain D (inhibitory fraction). Different volumes were tested: 10, 25, 50, 75, 100, 125, 150, 175 and 200 μ l. The other wells served as controls and contained non inhibitory fractions (fraction < 3 kDa, between 3 and 5 kDa and \geq 10 kDa released by yeast strain D or a 5-10 kDa peptidic fraction released by the reference yeast strain or strain A).

The same experiment was conducted by introducing fractions of 5-10 kDa taken from the 60-80 % ammonium sulfate precipitates of the medium fermented by strain D. The control used in this experiment was precipitated fractions from the medium fermented by strain A.

The plates were then incubated for approximately 12 h at 4 °C. This step slows down the bacterial growth and gives enough time for the proteins to diffuse into the agar prior to obtaining a high bacterial growth. Finally the plates were incubated at 30 °C for 72 h before measuring the diameter of the clear zones around the wells.

II.2.7 Statistical analyses

Means and standard deviations of the assays were calculated using conventional statistical methods. Each experiment was performed in three replicates. Statistical analysis (ANOVA) was applied to the data to determine differences ($p < 0.05$). Means differences were made by using Tukey's HSD test. The statistical analysis was carried out using Stat graphics XV.I for windows.

Chapter III. Results and Discussion

PART A- Determination of the kinetic parameters and the biochemical profiles of the oenological strains used during the alcoholic and malolactic fermentations

I. Introduction

In the first part of this study, pure cultures of two strains of *S. cerevisiae* (A and D) were carried out in synthetic grape juices to achieve alcoholic fermentations (AF). AF were followed until total or cessation of sugar consumption (≤ 2 g/l). Then, the yeast fermented media were used to carry out malolactic fermentations (MLF) after bacterial inoculation. MLF were followed until complete or cessation of malate consumption. This strategy was referred to as sequential fermentation. *O. oeni* strain X was the only bacterial strain used in this study. Pure cultures of this strain were performed in the synthetic grape juice (SGJ) and the synthetic wine (SW) media. These pure cultures or bacterial controls were used as a reference for bacterial growth and malate consumption kinetics during sequential fermentations.

Sequential fermentations were also carried out in commercial natural red grape juices (NGJ) and in Syrah and Cabernet Sauvignon musts used for red winemaking. The latter simulated the natural winemaking conditions and were referred to as microvinifications. An additional yeast strain (strain R) was tested in NGJ. The kinetics of the AF performed with the strain R as well as the kinetics of the MLF performed in the NGJ fermented by strain R (sequential fermentations) are given in appendix A-IX. This strain was tested because it is one of the most strains used in Lebanon.

In order to monitor the AF and MLF, kinetic and biochemical parameters were measured by regular sampling. The kinetic parameters evaluated were the OD, the cell concentration, the viability, and the biomass for both AF and MLF. The biochemical parameters measured during AF were the sugar, L-malic acid and citric acid consumed as well as the ethanol, glycerol, acetic acid, SO₂ and proteins produced. For MLF, the L-malic acid, citric acid and sugars consumed and the L-lactic acid, D-lactic acid and acetic acid produced were measured.

II. Alcoholic fermentations (AF)

II.1. Kinetic parameters of the AF carried out by *S. cerevisiae* strains D and A in SGJ media

The kinetic parameters of the AF carried out by *S. cerevisiae* strains D and A in SGJ media are given in tables III.1 and III.2 and in figures III.1, III.2 and III.3 and then discussed.

Table III.1. Growth and sugar consumption kinetics of *S. cerevisiae* strain D during AF in SGJ media

| Time (h) | Cell concentration $\times 10^6$ (cells/ml) | Viability (%) | OD | Biomass (g/l) | Sugar (g/l) |
|----------|--|---------------|-------|----------------------|-----------------------|
| 0 | 2.88 (± 0.32) | - | 0.031 | 0.062 (± 0.01) | 200.44 (± 0.12) |
| 18 | 35 (± 1.41) | 96 | 0.3 | 0.66 (± 0.52) | 167.62 (± 1) |
| 26 | 141 (± 0.82) | 87.45 | 1.27 | 2.61 (± 0.005) | 138.41 (± 2.31) |
| 42 | 367 (± 8.22) | 89 | 3.3 | 6.75 (± 0.089) | 126.87 (± 2.3) |
| 50 | 396 (± 0.06) | 93.21 | 3.37 | 7.15 (± 0.009) | 65.75 (± 0.97) |
| 71 | 390 (± 1.25) | 91 | 4.2 | 7.06 (± 0.005) | 15.95 (± 1.26) |
| 92 | 385 (± 2.5) | 92.64 | 4.4 | 7.09 (± 0.02) | 1.87 (± 0.04) |
| 115 | 379 (± 4.11) | 92.5 | 3.8 | 7.04 (± 0.03) | 0.66 (± 0.03) |
| 122 | 373 (± 4.78) | 92 | 3.76 | 6.99 (± 0.009) | 0.55 (± 0.04) |

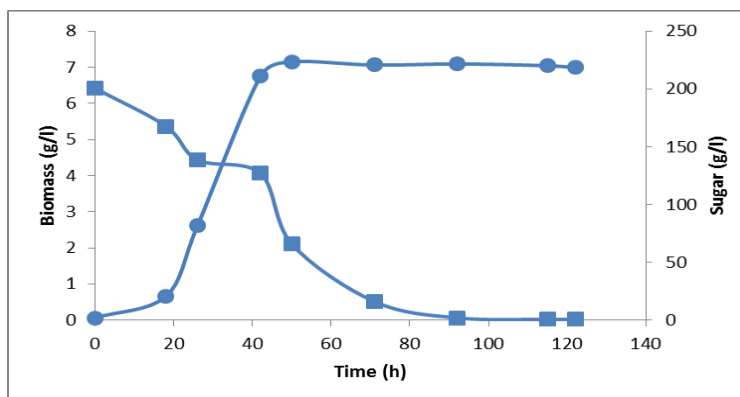


Figure III.1. Growth of *S. cerevisiae* strain D (●) and sugar consumption (■) during AF in SGJ media. Each value is the mean of triplicate experiments \pm SD

Table III.2. Growth and sugar consumption kinetics of *S. cerevisiae* strain A during AF in SGJ media

| Time (h) | Cell concentration $\times 10^6$ (cells/ml) | Viability (%) | OD | Biomass (g/l) | Sugar (g/l) |
|----------|---|---------------|-------|---------------------|-----------------------|
| 0 | 3.02 (± 0.21) | - | 0.091 | 0.06 (± 0.01) | 192.27 (± 6.95) |
| 8 | 4 (± 0.16) | 95.17 | 0.113 | 0.83 (± 0.52) | 186.17 (± 6.72) |
| 24 | 61 (± 3.56) | 87 | 0.124 | 3.03 (± 0.33) | 129.30 (± 4.78) |
| 32 | 94 (± 4.19) | 85 | 0.184 | 3.64 (± 0.37) | 124.07 (± 4.54) |
| 48 | 221 (± 16.39) | 83 | 0.230 | 4.43 (± 0.21) | 74.33 (± 2.62) |
| 56 | 249 (± 18.79) | 81 | 0.283 | 4.93 (± 0.29) | 66.20 (± 2.36) |
| 72 | 318 (± 12.23) | 79 | 0.344 | 5.62 (± 0.31) | 52.37 (± 1.88) |
| 80 | 239 (± 33.48) | 78 | 0.255 | 5.27 (± 0.29) | 46.71 (± 1.66) |
| 96 | 224 (± 1.63) | 82 | 0.239 | 4.84 (± 0.39) | 33.63 (± 1.19) |
| 104 | 170 (± 35.05) | 80 | 0.204 | 4.4 (± 0.50) | 30.97 (± 1.10) |
| 120 | 248 (± 33.74) | 87.6 | 0.154 | 4.94 (± 0.49) | 10.53 (± 0.38) |
| 128 | 247 (± 3.30) | 85.40 | 0.134 | 4.91 (± 0.13) | 2.37 (± 0.31) |

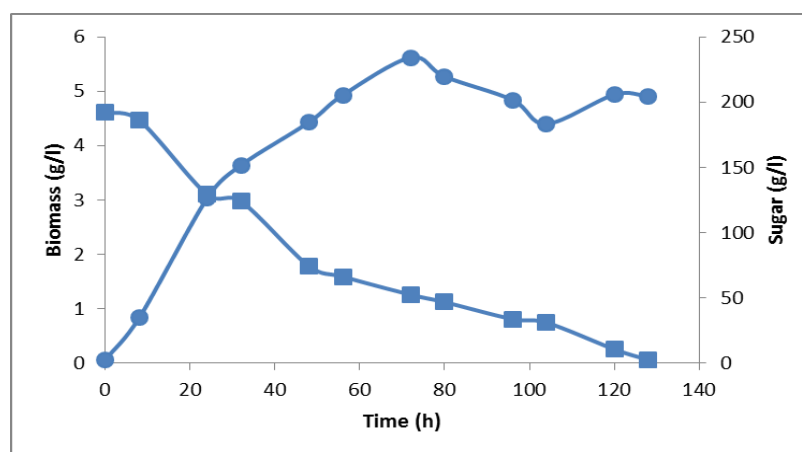


Figure III.2. Growth of *S. cerevisiae* strain A (●) and sugar consumption (■) during AF in SGJ media. Each value is the mean of triplicate experiments \pm SD

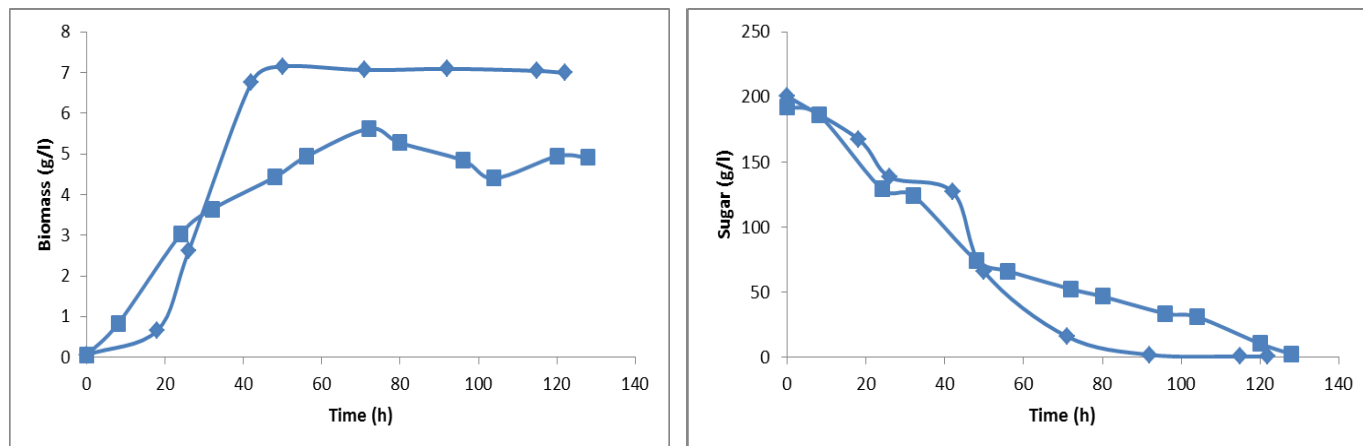


Figure III.3. Comparison of the growth kinetics of *S. cerevisiae* strain D (◆) and strain A (■) and the sugar consumption kinetics during AF in SGJ media. Each value is the mean of triplicate experiments \pm SD

Figures III.1, III.2 and III.3 allow us to follow the growth kinetics of *S. cerevisiae* strains A and D during alcoholic fermentations as well as the kinetics of the sugar consumption. Both growth curves are characterized by the absence of a lag phase and the presence of an active growth phase followed by a stationary phase. The yeasts quickly adapted to the SGJ media because they were previously submitted to two preculture steps. With the strain D, an acceleration growth phase is observed from the beginning of the AF followed by the exponential growth phase (log phase) which is linear and short ($t_0 = 20$ h and $t_f = 40$ h). The specific growth rate during the log phase is constant and maximal and corresponds to $\mu_{\max} = 0.07$ h⁻¹. Then, the growth rate slows down leading to the stationary phase that starts at $t = 50$ h. The AF with the strain A starts directly with the log phase which lasts 72 h and presents a $\mu_{\max} = 0.03$ h⁻¹. Starting with identical inocula (around 3×10^6 cells/ml), the two yeast strains have reached different maximal populations (396×10^6 cells/ml for D and 318×10^6 cells/ml for A). Along with the growth, we note that the sugar consumption is rather continuous throughout the alcoholic fermentation. For both strains, the stationary phases are reached before total sugar consumption which is conventional in winemaking (66 g/l of remaining sugar with the strain D and 52 g/l with the strain A). During the stationary phase, the sugar consumption and the ethanol production are achieved. Both alcoholic fermentations were followed until cessation of sugar consumption (0.55 g/l and 2.37 g/l of remaining sugars after 122 h and 128 h of AF for the strains D and A

respectively). No decline phase was detected and the yeasts kept a high percentage of viability between 85 and 92 % (Tables III.1 and III.2).

It can be concluded that despite the kinetic differences already mentioned, the two strains have a similar overall behavior.

The linear correlations between the biomass and the cell concentration, the biomass and the OD and the cell concentration and the OD for each yeast strain are given in appendices A-I and A-IV. AF trials were performed with strain D in SGJ media by replacing the yeast extract by two types of yeast nitrogen base. The first one lacked both amino acids and peptides and the second contained amino acids but not peptides. In both cases, AF couldn't be completed. Their kinetics are presented in appendices A-II and A-III.

The standard curve used for sugar analysis by the DNS method is also given in the appendix.

II.2. Biochemical parameters of the AF carried out by *S. cerevisiae* strains D and A in SGJ media

Some biochemical parameters were evaluated during the AF carried out by *S. cerevisiae* strains D and A in SGJ media and are given in table III.3 and then discussed.

Table III.3. The biochemical profile of *S. cerevisiae* strains D and A during AF in SGJ media

| Biochemical parameters | <i>S. cerevisiae</i> strain D | <i>S. cerevisiae</i> strain A |
|----------------------------------|-------------------------------|-------------------------------|
| Ethanol (g/l) | 88 (±0.35) | 86 (±0.73) |
| Glycerol (g/l) | 8.7 (±0.141) | 6.5 (±0.23) |
| Acetate (g/l) | 0.24 (±0.04) | 0.15 (±0.23) |
| Consumed citrate (g/l) | 0 (±0) | 0 (±0) |
| Free SO ₂ (mg/l) | 0.1 (±0.009) | 2.6 (±0.6) |
| Total SO ₂ (mg/l) | 0.28 (±0.05) | 7.61 (±2) |
| Molecular SO ₂ (mg/l) | 0.0032 (±0.0002) | 0.08 (±0.008) |
| Octanoic acid (mg/l) | 0,7 | 16,7 |
| Decanoic acid (mg/l) | <0,1 | 1,9 |
| Dodecanoic acid (mg/l) | <0,1 | 0,2 |
| Initial proteins (mg/l) | 483.6 (±0.44) | 483.6 (±0.44) |
| Final proteins (mg/l) | 1453 (±12.67) | 906 (±0.17) |
| Initial L-malic acid (g/l) | 3.06 (±0.029) | 3.01 (± 0.26) |
| Final L-malic acid (g/l) | 2.21 (±0.069) | 2.17 (±0.13) |
| Consumed L-malic acid (g/l) | 0.85 (±0.014) | 0.84 (±0.018) |
| Mannoproteins (mg/l) | 200 (±0.061) | 285 (± 0.26) |
| Initial /Final pH | 3.5/3.25 (±0.1) | 3.5/3.04 (±0.13) |

Tables III.1, III.2 and III.3 enable us to calculate the biomass yield (1) and the ethanol yield (2) for both yeast strains.

$$(1) Y_{(X/S)} \text{ (g/g)} = (X_f - X_0) / (S_0 - S_f);$$

X_f: final biomass (g/l), X₀: initial biomass (g/l), S₀: initial sugar concentration (g/l), S_f: final sugar concentration (g/l)

$$(2) Y_{(P/S)} \text{ (g/g)} = (P_f - P_0) / (S_0 - S_f)$$

P_f: final ethanol concentration (g/l), P₀: initial ethanol concentration (g/l), S₀: initial sugar concentration (g/l), S_f: final sugar concentration (g/l)

The biomass yield is 0.035 g/g and 0.026 g/g for the strains D and A respectively. The ethanol yield is 0.44 g/g and 0.45 g/g for the strains D and A respectively.

The biomass yield in fermentation conditions varies usually between 0.05 and 0.1 g/g indicating that the biomass yields obtained are rather weak. The ethanol yield varies usually between 0.45 and 0.47 g/g which is close to the values obtained. The weak biomass yields are probably due to the production of secondary metabolites mainly glycerol and acetic acid (Table III.3).

Glycerol is the most important by-product of alcoholic fermentation after ethanol and carbon dioxide. It is produced in wine at concentrations of 2–11 g/l, depending on yeast strain; grape must composition and fermentation conditions. In *S. cerevisiae*, this polyol plays two major roles in physiological processes: it controls intracellular redox balance and combats osmotic stress. Glycerol is a non-volatile compound which has no aromatic properties, but which significantly contributes to wine quality by providing sweetness and fullness (Pretorius 2000, Remize et al., 2003, Ribéreau-Gayon et al., 2006).

In opposite, the formation of acetic acid is undesirable since it increases the volatile acidity of wine. In healthy grape must with a moderate sugar concentration, *S. cerevisiae* produces relatively small quantities (100-300 mg/l), varying according to the strain (Ribéreau-Gayon et al., 2006). The concentrations obtained with both strains are low and acceptable from an organoleptic and oenological point of view since they are lower than 1.1 g/l (Table III.3).

The final pH of the SGJ media fermented by *S. cerevisiae* strain A was remarkably low although this strain produced a lower amount of acetic acid than strain D and consumed the same amount of malate as strain D (Table III.3). The consumption of malate normally increases the pH. The low final pH value (3.04) could therefore be explained by the release of organic acids by strain A during AF that was not measured. One probable example could be succinic acid (Henick-Kling, 1993; Liu et al., 2015).

Both yeast strains consumed low amounts of L-malic acid which is common in *S. cerevisiae* strains (Taillandier and Strehaiano, 1991). Indeed, at the pH of the SGJ media (pH 3.5), only 43 % of the malate present (pKa 3.4) are in their non-dissociated form and can passively diffuse across the cell membrane (Bony et al., 1997). This is the only way used by *S. cerevisiae* to transport malate because they lack the malate permease. However, only 0.28 % of the initial malate was consumed by both yeast strains (Table III.3). This can be explained by the inability of *S. cerevisiae* to degrade high amounts of L-malic acid. Indeed, malic acid is mainly catabolized in *S. cerevisiae* by oxidative decarboxylation catalyzed by the malic enzyme (Boles et al., 1998). This enzyme has a low affinity to the substrate ($K_M= 50$ mM) and is localized in the mitochondria, which are few and dysfunctional during AF (Osothsilp, 1987).

Some metabolites produced by yeasts during the AF can have a great influence on the occurrence and achievement of MLF conducted later on by lactic acid bacteria. Among these metabolites,

we have measured ethanol, SO₂ and medium chain saturated fatty acids which are capable of acting synergistically to inhibit bacterial growth and/or malate consumption.

The inhibitory role of ethanol was described in paragraph I.7.5 of chapter I and its contribution to the MLF inhibition will be described in paragraph III of this chapter.

The endogenous SO₂ level produced depends strongly on the yeast strain, the availability of nutrients such as nitrogen, the pH and the temperature (Eschenbruch and Bonish, 1976; Gyllang et al., 1989; Osborne and Edwards, 2006). *S. cerevisiae* strains usually produce between 10 and 30 mg/l of SO₂ (Dittrich, 1987) but in some cases more than 100 mg/l can be detected (Henick-Kling et Park, 1994). SO₂ can be found in its free or combined form (combination to carbonyl groups such as acetaldehyde). The free SO₂ may be in its ionic form and/or in its molecular active form depending on the pH of the medium. The most active form against microorganisms is the molecular form as described in paragraph I.7.4 of chapter I. The amount of molecular SO₂ produced by strains A and D are far lower than the inhibitory concentrations found in the literature. Indeed, Delfini and Morsiani (1992) found that a concentration of molecular SO₂ greater than 0.5 mg/l was able to completely inhibit the cell growth of 10 strains of *Leuconostoc oenos* (currently named *O. oeni*) and 4 strains of *Lactobacillus*. In addition, a *Leuconostoc* population of 2×10^6 cells/ml died off 22 h after the addition of 0.84 mg/l of molecular SO₂. Therefore, we can conclude that the molecular SO₂ produced by the yeast strains A and D cannot be considered as an inhibiting factor of MLF during the sequential fermentations conducted later on in the media fermented by these yeast strains.

The medium chain saturated fatty acids (C₈ to C₁₂) produced by yeasts may also have an influence on MLF. The inhibitory activity of these metabolites has been described in paragraph I.7.6 of chapter I of this manuscript. It is mainly due to the non-dissociated form of these acids which is the predominant form in our case because the pH of the media is below the pK_a of these acids (pK_a_{oct}=4.89, pK_a_{dec}=4.9, pK_a_{dod}=4.8). Although strain A produced higher amounts of these acids (Table III.3), the amounts produced are still far below the inhibitory concentrations found in the literature and cannot significantly participate to the MLF inhibition observed during sequential fermentations. Indeed, Edwards and Beelman (1987) demonstrated that 5 to 10 mg/l of decanoic acid should be added to the grape juice in order to inhibit the bacterial growth and the MLF. Lonvaud-Funel et al., (1988) also showed that either 4 mg/l of decanoic acid or 0.5 mg/l of dodecanoic acid were necessary to inhibit MLF in wine.

In a previous study, Nehme et al., (2008) showed that the yeast strains A and D completely consumed the ammonium nitrogen but partially consumed the alpha amino nitrogen during AF. The influence of the nutritional behavior of yeasts on the MLF during sequential fermentations will be discussed in paragraph III.

Table III.3 shows a protein production by both yeast strains during the AF. These proteins may include peptidic fractions of low molecular weight stimulatory of the MLF and macromolecules including proteins and mannoproteins also stimulatory of the MLF (Guilloux-Benatier et al., 1993; 1995). The amount of mannoproteins produced by both yeast strains is mentioned in table III.3. Besides these stimulatory molecules, the SGJ media fermented by yeasts may also contain proteinaceous compounds that inhibit MLF. The latter will be studied in part B of this chapter.

III. Malolactic fermentations

III.1. Pure cultures of *O. oeni* strain X in synthetic grape juice and synthetic wine media

Pure cultures of *O. oeni* strain X were carried out in synthetic grape juice (SGJ) and synthetic wine (SW) media and were used later on as bacterial controls during sequential fermentations in order to quantify the inhibition or stimulation caused by *S. cerevisiae* strains D or A on the bacterial growth and the malate consumption kinetics. The composition of the SW medium simulated the natural wine, yet it was lacking the yeast metabolites to the exception of ethanol. In fact, the SO₂ and medium chain fatty acids amounts produced by both yeast strains were negligible (paragraph II.2, Table III.3) and far below the inhibitory concentrations found in the literature to cease MLF. Therefore the only yeast metabolite tested was ethanol and the SW medium was designed to quantify the inhibitory effect of ethanol on the bacterial growth and the malate consumption. This helped us determine the contribution of other yeast metabolites (proteinaceous compounds) to the MLF inhibition observed during sequential fermentations. The nutrient content of the SW medium was reduced by 50 % in comparison to the SGJ medium.

The average specific growth rate μ (h⁻¹) and the average specific rate of malate consumption Q_s (g g⁻¹ h⁻¹) were calculated by applying the following formulas:

$$-\mu \text{ (h}^{-1}\text{)} = (dX/dt_1) \cdot 1/X \text{ with } dX = (X_f - X_0) \text{ and } X = (X_f + X_0)/2$$

X_f : final bacterial biomass; X_0 : initial bacterial biomass; dt_1 : growth duration (h)

$$-Q_s \text{ (g g}^{-1} \text{ h}^{-1}) = (dS/dt_2) \cdot 1/X \text{ with } dS = (S_0 - S_f) \text{ and } X = (X_f + X_0)/2$$

S_0 : initial malate concentration; S_f : final malate concentration; dt_2 : duration of MLF (h)

The kinetic and biochemical parameters of the pure cultures of *O. oeni* strain X in synthetic grape juice and synthetic wine media are presented in tables III.4 and III.5 and in figure III.4.

Table III.4. Kinetic parameters of the pure cultures of *O. oeni* strain X in synthetic grape juice and synthetic wine media

| Time $\pm(3h)$ | Cell concentration $\times 10^6(\text{cells/ml})$ | | Biomass (g/l) | | OD | | L-malic acid (g/l) | |
|-------------------|--|-------------------------|----------------------------|----------------------------|------|------|------------------------|-------------------------|
| | SGJ | SWM | SGJ | SWM | SGJ | SWM | SGJ | SWM |
| 0 | 2 (± 0.25) | 2 (± 0.25) | 0.0016 (± 0.0002) | 0.0016 (± 0.0002) | 0.02 | 0.02 | 3 (± 0.06) | 3 (± 0.06) |
| 48 | 3 (± 0.25) | 2.25 (± 0.25) | 0.0024 (± 0.0002) | 0.0018 (± 0.0002) | 0.03 | 0.02 | *- | - |
| 96 | 4.25 (± 0.38) | 2.75 (± 0.25) | 0.0034 (± 0.0003) | 0.0022 (± 0.0002) | 0.04 | 0.03 | - | - |
| 168 | 75 (± 7.5) | 5 (± 0.5) | 0.06 (± 0.006) | 0.004 (± 0.0004) | 0.7 | 0.05 | - | - |
| 240 | 176.25 (± 8.8) | 10 (± 1) | 0.141 (± 0.007) | 0.008 (± 0.0008) | 1.6 | 0.09 | 1.98 (± 0.04) | 2.6 (± 0.05) |
| 336 | 180 (± 9.4) | 12.5 (± 1.25) | 0.144 (± 0.0075) | 0.01 (± 0.001) | 1.7 | 0.12 | - | - |
| 408 | 176.25 (± 8.8) | 10 (± 1) | 0.141 (± 0.007) | 0.008 (± 0.0008) | 1.6 | 0.12 | 0.26 (± 0) | 2.4 (± 0.05) |
| 446 | | 10.75 (± 1.13) | | 0.0086 (± 0.0009) | | 0.12 | 0 (± 0) | 2.27 (± 0.045) |
| 576 | | 10.5 (± 1) | | 0.0084 (± 0.0008) | | 0.12 | | - |
| 672 | | 15.75 (± 1.63) | | 0.0126 (± 0.0013) | | 0.15 | | - |
| 744 | | 65 (± 6.25) | | 0.052 (± 0.005) | | 0.6 | | - |
| 840 | | 72.5 (± 7.5) | | 0.058 (± 0.006) | | 0.7 | | 1.08 (± 0.022) |
| 912 | | 66.25 (± 6.25) | | 0.053 (± 0.005) | | 0.7 | | - |
| 1000 | | 67.5 (± 6.25) | | 0.054 (± 0.005) | | 0.7 | | 0.5 (± 0) |
| 1200 | | 67.5 (± 6.25) | | 0.054 (± 0.005) | | 0.7 | | 0 (± 0) |

* The dashes in empty boxes correspond to samples where the L-malic acid concentration was not measured

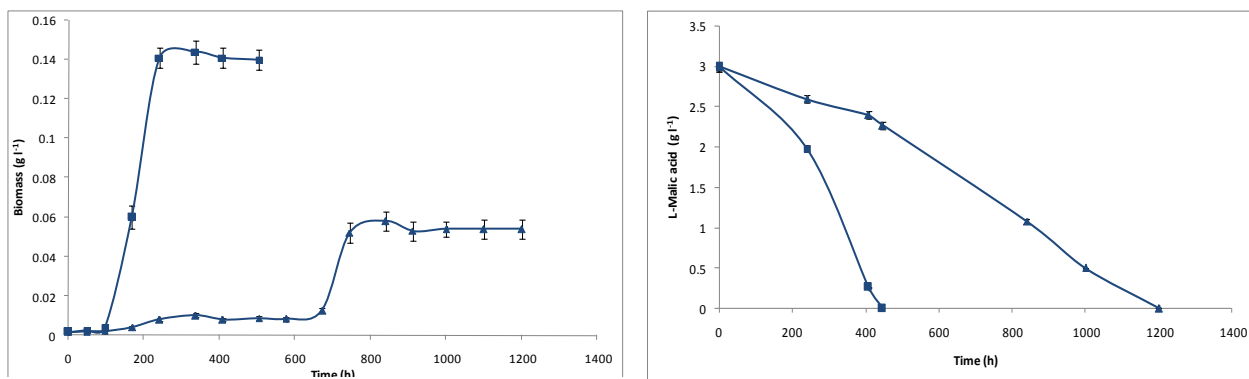


Figure III.4. Growth of *O. oeni* strain X in SGJ media (■) and in SW media (▲) and corresponding malate consumption. Each value is the mean of triplicate experiments \pm SD

Table III.5. Some biochemical parameters of *O. oeni* strain X during its pure cultures in the SGJ and the SW media

| Biochemical parameters | SGJ medium | SW medium |
|------------------------------|------------------------|----------------------|
| Consumed L-malic acid (g/l) | 3.00 (\pm 0.06) | 3.00 (\pm 0.06) |
| Produced L-lactic acid (g/l) | 2.00 (\pm 0.04) | 2.00 (\pm 0.04) |
| Produced D-lactic acid (g/l) | 0.160 (\pm 0.007) | 0.100 (\pm 0.006) |
| Produced acetic acid (g/l) | 0.011 (\pm 0.002) | 0.006 (\pm 0.002) |
| Consumed sugar (g/l) | 1.080 (\pm 0.002) | 0.540 (\pm 0.002) |
| Consumed citric acid (g/l) | 0.0036 (\pm 0.0032) | 0.005 (\pm 0.001) |

In the SGJ media, the MLF lasted 446 h and the final bacterial biomass reached was 0.14 g/l. In the SW media, the MLF was 2.7 times longer (1200 h) and the final biomass was 2.6 times lesser (0.054 g/l) than in the SGJ media. Moreover a long lag phase of about 670 h was observed in this case. The specific growth rate in the SW media (0.006 h^{-1}) was reduced by 25 % compared to the one obtained in the SGJ media (0.008 h^{-1}). However, the specific rate of malate consumption remained the same ($0.1 \text{ g g}^{-1} \text{ h}^{-1}$). The longer duration required for total malate consumption in the SW media was a consequence of the reduced bacterial biomass formed. Therefore, we can conclude that the 10 % ethanol (v/v) along with the reduced amount of certain nutrients in the SW media limited the bacterial growth rather than the ability of each cell to consume L-malic acid. Capucho and San Romão (1994) also showed that the malolactic activity was not inhibited with an alcoholic degree of more than 12 % (v/v) unlike the bacterial growth which was strongly affected. In the SW medium, the malate consumption started before any noticeable bacterial growth. At the end of the lag phase which lasted 670 h, about half of the malate was already

consumed. Thus, the malate consumption and the bacterial growth are not necessarily correlated (Capucho and San Romão, 1994).

The nutrient content of the SW medium was reduced by 50 % in comparison to the SGJ medium. However, Nehme et al., (2008) previously demonstrated that the nutritional requirements of *O. oeni* strain X, especially the alpha-amino nitrogen were still largely covered in the SW medium. They also demonstrated that *O. oeni* did not consume the ammoniacal nitrogen.

Table III.5 shows that *O. oeni* strain X consumed very low amounts of sugars in order to ensure growth and malate consumption. *O. oeni* is a strict heterofermentative bacterium that usually ferments hexoses through the pentose phosphate pathway producing in addition to D-lactate, CO₂, acetate and/or ethanol. Only 1.08 g/l of sugars were consumed in the SGJ media and 0.54 g/l in the SW media. As a consequence, the D-lactic acid and acetic acid produced by this strain were also very low and no ethanol was detected. Besides, it did not consume a significant amount of citrate. The acetate produced may also derive from citrate metabolism.

The linear correlations for *O. oeni* strain X between the biomass and the OD, the cell concentration and the OD and the biomass and the cell concentration are given in appendix A-VI.

III.2 Sequential fermentations with the pairs *S. cerevisiae* strain A/*O. oeni* strain X and *S. cerevisiae* strain D/*O. oeni* strain X in synthetic and natural grape juices

At the end of the AF, yeasts were removed by centrifugation; the malate was readjusted to 3 g/l and the pH to 3.5. The yeast fermented media were then sterile filtered and recuperated in autoclaved Erlenmeyer flasks before inoculation with *O. oeni* strain X. The MLF were followed by regular sampling until total or cessation of malate consumption. The detailed kinetic parameters of the AF carried out in natural grape juices (NGJ) are given in appendix A-IX. Moreover, those of the MLF performed in NGJ and SGJ fermented by yeasts are given in appendices A-VII, A-VIII and A-IX.

Figure III.5 and table III.6 describe the growth of *O. oeni* strain X and the malate consumption during the sequential fermentations.

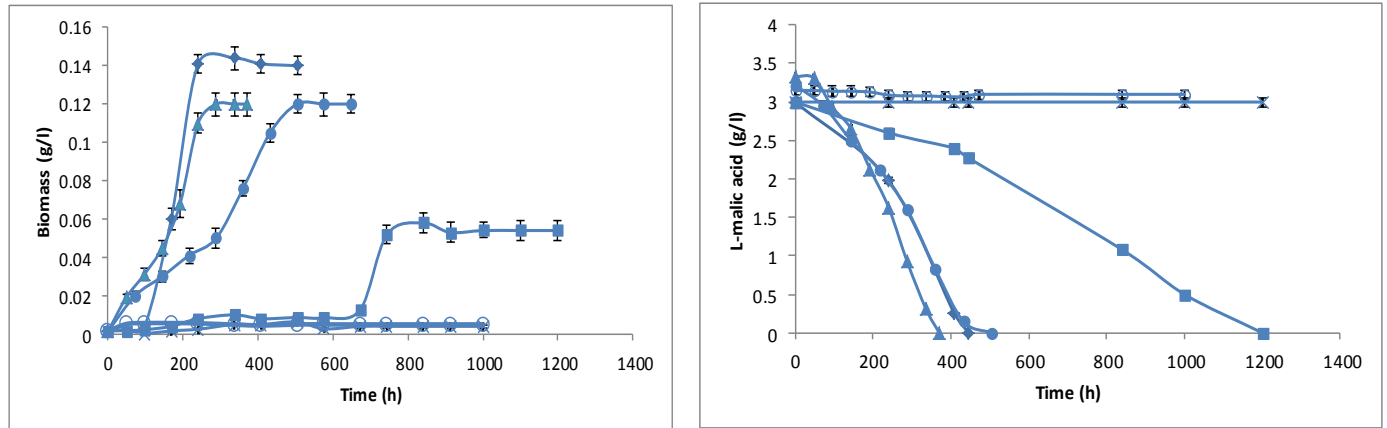


Figure III.5. Growth of *O. oeni* strain X and malate consumption in the controls and in the SGJ and NGJ media fermented by *S. cerevisiae* strains A and D. (♦) Bacterial control culture in the SGJ medium, (■) Bacterial control culture in the SW medium, (▲) NGJ fermented by *S. cerevisiae* strain A, (●) SGJ fermented by *S. cerevisiae* strain A, (○) NGJ fermented by *S. cerevisiae* strain D, (x) SGJ fermented by *S. cerevisiae* strain D. Each value is the mean of triplicate experiments \pm SD

Table III.6. Determination of the average specific growth rates and the average specific rates of malate consumption during the MLF carried out by *O. oeni* strain X in the SGJ and NGJ fermented by *S. cerevisiae* strains A and D as well as in the controls

| | SGJ control | SW control | SGJ medium fermented by <i>S. cerevisiae</i> strain D | NGJ medium fermented by <i>S. cerevisiae</i> strain D | SGJ medium fermented by <i>S. cerevisiae</i> strain A | NGJ medium fermented by <i>S. cerevisiae</i> strain A |
|---|----------------------------|----------------------------|---|---|---|---|
| X_0 : Initial biomass (g l ⁻¹) | 0.0016 (± 0.0002) | 0.0020 (± 0.0002) | 0.0016 (± 0.0002) | 0.0016 (± 0.0002) | 0.0016 (± 0.0002) | 0.0016 (± 0.0002) |
| X_f : Final biomass (g l ⁻¹) | 0.140 (± 0.007) | 0.054 (± 0.004) | 0.0044 (± 0.0004) | 0.0040 (± 0.0002) | 0.12 (± 0.005) | 0.12 (± 0.006) |
| dt_1 : Growth duration (h) | 240 | 648 | 408 | 408 | 504 | 288 |
| ^a μ^2 : average specific growth rate (h ⁻¹) | 0.0080 (± 0.0004) | 0.0060 (± 0.0002) | 0.0023 (± 0.0002) | 0.0021 (± 0.0003) | 0.004 (± 0.0002) | 0.007 |
| S_0 : Initial L-malic acid (g l ⁻¹) | 3.00 (± 0.06) | 3.00 (± 0.06) | 3.00 (± 0.07) | 3.10 (± 0.06) | 3.22 (± 0.06) | 3.33 (± 0.07) |
| S_f : Final L-malic acid (g l ⁻¹) | 0 (± 0) | 0 (± 0) | 3.00 (± 0.07) | 3.10 (± 0.06) | 0 (± 0) | 0 (± 0) |
| dt_2 : Duration of MLF (h) | 446 | 1200 | 1000 | 1000 | 504 | 370 |
| ^b Q_s^3 : average specific rate of malate consumption (g g ⁻¹ h ⁻¹) | 0.10 (± 0.01) | 0.100 (± 0.008) | 0 (± 0) | 0 (± 0) | 0.105 (± 0.01) | 0.15 (± 0.02) |

$$^a\mu \text{ (h}^{-1}\text{)} = (dX/dt_1) \cdot 1/X \text{ with } dX = (X_f - X_0) \text{ and } X = (X_f + X_0)/2$$

$$^bQ_s \text{ (g g}^{-1}\text{ h}^{-1}\text{)} = (dS/dt_2) \cdot 1/X \text{ with } dS = (S_0 - S_f) \text{ and } X = (X_f + X_0)/2$$

Figure III.5 and table III.6 show that *O. oeni* strain X was strongly inhibited in the SGJ and NGJ media fermented by *S. cerevisiae* strain D. The bacterial biomass formed in these media was 13.5

times lower than in the SW control and no malate was consumed. However, in the media fermented by *S. cerevisiae* strain A, an active bacterial growth was observed and the malate was completely consumed. The final bacterial biomass (0.12 g/l) was twice better in these media than in the SW control (0.054 g/l) and no lag phase was observed. The specific rate of malate consumption in the NGJ fermented by strain A was 1.5 times faster than in the SW control. Close values were obtained in the SGJ fermented by strain A and the SW control. Besides, the MLF duration was respectively 2.4 and 3.24 times shorter in the SGJ (504 h) and NGJ (370 h) fermented by strain A in comparison to the SW control (1200 h) because of the higher biomass produced (0.12 g/l vs 0.054 g/l).

To further comprehend the causes of growth and malate consumption inhibition observed during the sequential fermentations performed with the pair *S. cerevisiae* strain D/*O. oeni* strain X, the yeast fermented media were subjected to biochemical analyses in order to search for possible inhibitory molecules (Table III.3). Both strains A and D produced 11 % ethanol (v/v) and as already explained in paragraph III.1, ethanol can reduce the bacterial biomass produced and extend the MLF duration but do not stop it. The concentrations of SO₂ and medium chain fatty acids produced by strain D were considerably lower than the ones reported in the literature to cease MLF (Wibowo et al., 1985, Lonvaud-Funel et al., 1988, Edwards et al., 1990, Capucho and San Romão, 1994, Guzzo et al., 1998, Carreté et al., 2002). Besides, nutrient depletion was excluded from inhibitory factors as the addition of 55.3 g/l of MRS to the media fermented by *S. cerevisiae* strain D before bacterial inoculation did not remove the inhibition. The MRS was chosen because it is very favorable for the growth and metabolism of *O. oeni*. All these data encouraged us to search for other yeast antibacterial metabolites. More specifically, the production of proteinaceous metabolites by *S. cerevisiae* strain D was investigated (Part B of chapter III).

The strong inhibition of the bacterial growth and malate consumption was reproducible in both SGJ and NGJ media fermented by strain D. Moreover, the MLF inhibition was maintained when sequential fermentations with the same pair were performed in grape musts of Syrah and Cabernet-Sauvignon presenting a high phenolic content. The importance of these results regarding the production of anti-MLF peptides by *S. cerevisiae* strain D will be discussed in part B of this chapter.

Unlike *S. cerevisiae* strain D, the strain A was rather stimulatory. The MLF was faster than in the SW control (1200 h, $0.1 \text{ g g}^{-1}\text{h}^{-1}$) and as rapid as in the SGJ control (446 h, $0.1 \text{ g g}^{-1}\text{h}^{-1}$) when carried out in the SGJ media fermented by strain A (504 h, $0.105 \text{ g g}^{-1}\text{h}^{-1}$). It was even faster when performed in the NGJ media fermented by strain A (370 h, $0.15 \text{ g g}^{-1}\text{h}^{-1}$). The MLF stimulation can be attributed to the release of some activating nutrients by strain A during AF such as amino acids (Fourcassier, 1992), vitamins, nitrogen bases, long chain fatty acids (Alexandre et al., 2004) and peptides of low MW (Guilloux-Benatier and Chassagne, 2003) in addition to mannoproteins (285 mg/l produced by this strain) (Guilloux-Benatier et al., 1993, 1995). Although this strain produced higher amounts of SO_2 and medium chain fatty acids than strain D, their concentrations were still not inhibitory. Besides, no inhibitory proteinaceous compounds were produced by strain A unlike strain D (confer part B of this chapter).

Finally, it can be concluded that the bacterial growth and the malate consumption during sequential fermentations varied widely from one pair to another and strongly depended on the yeast strain chosen. A previous study carried out by Nehme et al., (2008) showed various degrees of MLF inhibition when four strains of *S. cerevisiae* were tested with *O. oeni* strain X during sequential fermentations.

In the part B of this chapter, the production of proteinaceous compounds by *S. cerevisiae* strain D responsible for the MLF inhibition with *O. oeni* strain X is investigated.

Part B- Production of anti-MLF peptides by *S. cerevisiae* strain D

I. Introduction

The ethanol, SO₂ and medium chain fatty acids produced by *S. cerevisiae* strain D as well as the nutritional behavior of this strain could not explain the strong inhibition of the bacterial growth of *O. oeni* strain X and the malate consumption during sequential fermentations. Therefore, we aimed to identify the production of proteinaceous compounds by strain D involved in the MLF inhibition. Nehme et al., (2010) have previously shown that *S. cerevisiae* strain D was able to produce anti-MLF compounds of protein nature. However, the work was interrupted for almost 6 years. Therefore, the objective of this thesis was first to demonstrate that the strain D has not lost its ability to produce the inhibitory proteinaceous compounds and second to further characterize these compounds. In this part of the study, protease and heat treatments were first performed on the SGJ media fermented by strain D in order to demonstrate the proteinaceous nature of the inhibitory compounds. Then the fermented SGJ media were fractionated by ultrafiltration and ammonium sulfate precipitation in order to identify the apparent MW of these compounds and to further purify them. The timing of the release of these compounds during AF was also assessed and the maintenance of the inhibition due to these compounds in natural winemaking conditions was investigated.

II. Protease and heat treatments of the SGJ media fermented by *S. cerevisiae* strain D

The supernatants obtained after centrifugation of the SGJ media fermented by *S. cerevisiae* strain D were subjected to different protease treatments (papain, pepsin and trypsin) and heat treatments (121 °C/20 min and 100 °C/30 min). The treated and untreated fermented media were then inoculated with *O. oeni* strain X and the MLF were followed by regular sampling until cessation of malate consumption. The kinetics of the bacterial growth and the malate consumption were compared to those of bacterial controls performed in SGJ and SW media. The results are given in figure III.6 and table III.7. The detailed values of bacterial biomass produced and malate consumed are given in appendix B-I.

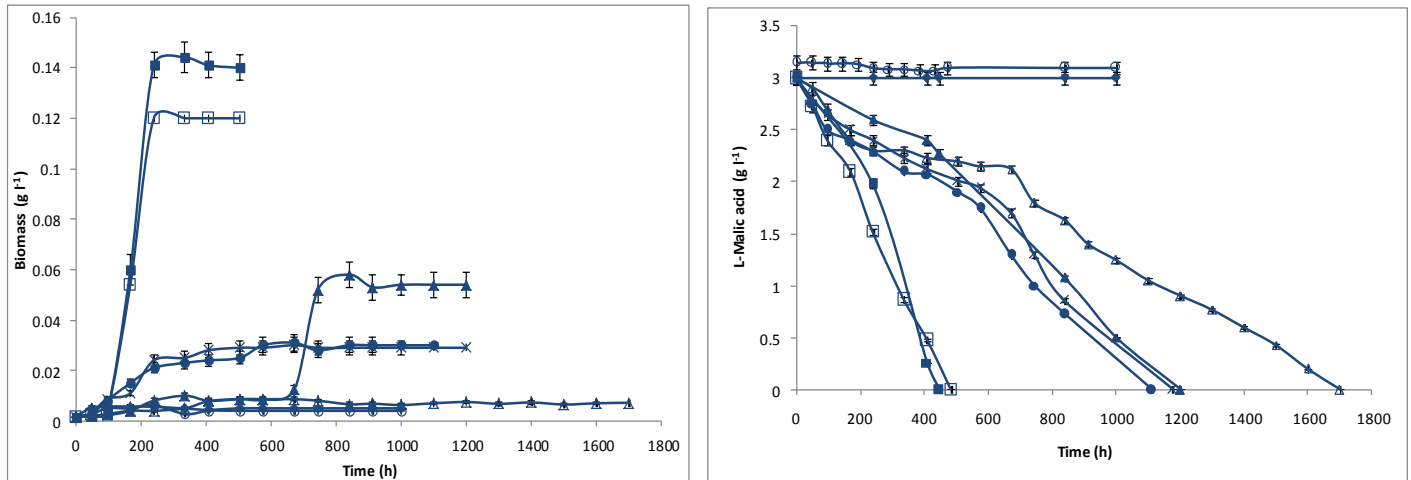


Figure III.6. Growth and malic acid consumption kinetics of *O. oeni* strain X in the untreated and treated yeast fermented media and in the bacterial control cultures. (■) SGJ control, (▲) SW control, (◆) Untreated fermented SGJ medium, (○) Untreated fermented NGJ medium, Treated fermented SGJ media: (□) Papain treatment, (●) Pepsin treatment, (Δ) Trypsin treatment, (x) Heat treatments (mean values of the treatments at 100°C/30 min and 121°C/20 min). Each value is the mean of triplicate experiments ± SD

Table III.7. Determination of the average specific growth rates and the average specific rates of malate consumption during the MLF carried out by *O. oeni* strain X in the treated and untreated fermented media as well as in the controls¹

| | SGJ control | SW control | Untreated fermented SGJ medium | Untreated fermented NGJ medium | Papain treated fermented medium | Pepsin treated fermented medium | Trypsin treated fermented medium | Heat treated fermented medium |
|--|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------------|---|---|---|
| X_0 : Initial biomass (g/l) | 0.0016 ±0.0002 | 0.0020 ±0.0002 | 0.0016 ±0.0002 | 0.0016 ±0.0002 | 0.0016 ±0.0002 | 0.0016 ±0.0002 | 0.0016 ±0.0002 | 0.0016 ±0.0002 |
| X_f : Final biomass (g/l) | 0.140 ±0.007 | 0.054 ±0.004 | 0.0044 ±0.0004 | 0.0040 ±0.0002 | 0.120 ±0.007 | 0.030 ±0.002 | 0.0090 ±0.0006 | 0.030 ±0.002 |
| dt_1 : Growth duration (h) | 240 | 648 | 408 | 408 | 240 | 576 | 576 | 576 |
| μ^2 : average specific growth rate (h ⁻¹) | 0.0080 ±0.0004 ^a | 0.0060 ±0.0002 ^b | 0.0023 ±0.0002 ^c | 0.0021 ±0.0003 | 0.0080 ±0.0004 ^{a b' c'} | 0.0030 ±0.0002 ^{a' b'' c''} | 0.0024 ±0.0003 ^{a''' b''' c'''} | 0.0030 ±0.0004 ^{a' b'' c''} |
| S_0 : Initial L-malic acid (g/l) | 3.00 ±0.06 | 3.00 ±0.06 | 3.00 ±0.07 | 3.10 ±0.06 | 3.00 ±0.05 | 3.00 ±0.06 | 3.00 ±0.05 | 3.00 ±0.05 |
| S_f : Final L-malic acid (g/l) | 0 ±0 | 0 ±0 | 3.00 ±0.07 | 3.10 ±0.06 | 0 ±0 | 0 ±0 | 0 ±0 | 0 ±0 |
| dt_2 : Duration of MLF (h) | 446 | 1200 | 1000 | 1000 | 484 | 1110 | 1700 | 1180 |
| Q_s^3 : average specific rate of malate consumption (g g ⁻¹ h ⁻¹) | 0.10 ±0.01 ^a | 0.100 ±0.008 ^b | 0 ±0 ^c | 0 ±0 | 0.100 ±0.007 ^{a b' c'} | 0.170 ±0.014 ^{a' b'' c''} | 0.33 ±0.02 ^{a''' b''' c'''} | 0.160 ±0.005 ^{a' b'' c''} |

¹ Results are mean ±SD values of three replications. In comparison to the same control (SGJ or SW) or to the untreated fermented SGJ medium, values of average specific rates (growth or malate consumption) within the same row followed by the same letter but with different number of primes are significantly different ($p < 0.05$) according to Tukey's HSD test

$$^2 \mu \text{ (h}^{-1}\text{)} = (dX/dt_1) \cdot 1/X \text{ with } dX = (X_f - X_0) \text{ and } X = (X_f + X_0)/2$$

$$^3 Q_s \text{ (g g}^{-1} \text{ h}^{-1}\text{)} = (dS/dt_2) \cdot 1/X \text{ with } dS = (S_0 - S_f) \text{ and } X = (X_f + X_0)/2$$

Figure III.6 and Table III.7 compare the results of the MLF conducted by *O. oeni* strain X in the untreated yeast fermented media (SGJ and NGJ media) and in the yeast fermented SGJ media subjected to different protease and heat treatments as well as in the controls.

In the SGJ control, the MLF was the fastest (446 h) and the final bacterial biomass reached was the highest (0.14 g/l). In the SW control, the MLF was 2.7 times longer (1200 h) and the final biomass was 2.6 times lesser (0.054 g/l) than in the SGJ control. Moreover a long lag phase of about 670 hours was observed in this case. The specific growth rate in the SW control (0.006 h^{-1}) was reduced by 25 % compared to the one obtained in the SGJ control (0.008 h^{-1}). However, the specific rate of malate consumption remained the same ($0.1 \text{ g g}^{-1} \text{ h}^{-1}$). The longer duration required for total malate consumption in the SW control was a consequence of the reduced bacterial biomass formed. Therefore, we can conclude that the 10 % ethanol (v/v) along with the reduced amount of certain nutrients in the SW medium inhibited the bacterial growth rather than the ability of each cell to consume L-malic acid.

The different heat and protease treatments applied to the yeast fermented SGJ media showed an improvement of the bacterial growth and a total consumption of the L-malic acid (Figure III.6). The most relevant result was the one obtained with the papain treatment. Papain is known to cleave peptide bonds by preferentially attacking the carbonyl carbon of basic amino acids, particularly arginine and lysine following phenylalanine or any hydrophobic residue (Amri and Mamboya, 2012). The final biomass reached in the papain treated medium (0.12 g/l) was twice higher than the one obtained in the SW control (0.054 g/l) with an average specific growth rate 1.3 times greater ($p < 0.05$). The average specific rates of malate consumption were the same ($0.1 \text{ g g}^{-1} \text{ h}^{-1}$). The bacterial growth and the malate consumption kinetics in the papain treated medium were similar to those obtained in the SGJ control ($p > 0.05$). These results suggest that the papain treatment had on one hand degraded the yeast inhibitory proteinaceous compounds and on the other hand enriched the medium with stimulatory factors of the malolactic activity and the bacterial growth (e.g., amino acids, oligopeptides) (Guilloux-Benatier et al., 2006).

The mean values of both heat treatments (100 °C/30 min and 121 °C/20 min) were presented in figure III.6 since they gave similar results ($p > 0.05$). The pepsin and heat treatments also gave similar results for bacterial growth ($p > 0.05$). The specific growth rate was 0.003 h^{-1} with a maximum biomass of 0.03 g/l (Table III.7). Compared to the SW control, the specific growth rate and the final biomass reached were twice lower. Additionally, the specific rate of malate

consumption in the SW control ($0.1 \text{ g g}^{-1} \text{ h}^{-1}$) was slower than in the pepsin and heat treated media (0.17 and $0.16 \text{ g g}^{-1} \text{ h}^{-1}$ respectively with $p < 0.05$). The improvement of the specific rate of malate consumption after pepsin treatment was probably due to the release of some activating agents of the MLF (e.g., amino acids released by proteolysis). The heat treatment altered the inhibitory proteinaceous compounds and totally evaporated ethanol.

The treatment with trypsin gave the lowest final biomass and growth rate compared to the other treatments but the highest specific rate of malate consumption (Table III.7). The growth rate was similar to those obtained in the untreated fermented media ($p > 0.05$) but with a final biomass twice higher. These values suggest that the yeast proteinaceous compounds responsible for the inhibition of the malate consumption were successfully degraded by trypsin thus activating this pathway. However, the growth ability was still weak suggesting that some other proteinaceous compounds involved in the growth inhibition were not completely degraded. The growth and malate consumption with *O. oeni* are not always correlated and the MLF can be successfully carried out without any noticeable growth (Capucho and San Romaô, 1994). The degradation of L-malic acid by non-growing or poorly growing cells of *O. oeni* can be even more rapid at low extracellular pH values due to an increase in the ΔpH ($\text{pH}_{\text{in}} - \text{pH}_{\text{ex}}$) (Bouix and Ghorbal, 2015).

The previous results were indicative of the proteinaceous nature of the yeast metabolites involved in the MLF inhibition. The increased values of the malate consumption rates obtained with the pepsin, trypsin and heat treatments indicated that the inhibitory compounds degraded, targeted mainly the malate consumption rather than the bacterial growth. The conservation of the MLF inhibition by yeast proteinaceous compounds with the same pair in comparison to the study carried out by Nehme et al., (2010) indicated that the strains did not undergo genotypic or phenotypic modifications. Neither the yeasts lost their ability to produce the inhibitory proteinaceous compounds nor did the bacteria acquire resistance.

The strong inhibition of the bacterial growth and the malate consumption was reproducible in both the SGJ and the NGJ media fermented by the yeasts and not subjected to any treatment. The bacterial biomass formed in these media was 13.5 times lower than in the SW control and no malate was consumed. The maintenance of the inhibitory effect in the fermented natural red grape juice despite the presence of phenolic compounds is an interesting finding and will be further discussed in paragraph V of this part along with the results obtained in Syrah and Cabernet Sauvignon musts.

III. Fractionation of the SGJ media fermented by *S. cerevisiae* strain D by ultrafiltration

The SGJ media fermented by *S. cerevisiae* strain D were fractionated by ultrafiltration and four different MW fractions were obtained and tested in a modified MRS medium. The fractions with a MW between 3 and 5 kDa, 5 and 10 kDa and ≥ 10 kDa were 10.5 times concentrated unlike the fraction ≤ 3 kDa which was not concentrated. The media were then inoculated with *O. oeni* strain X and the bacterial growth and malate consumption were followed by regular sampling. The results were compared to those obtained in a modified MRS control (Figure III.7). The detailed values of the biomass produced and the malate consumed are given in appendix B-II.

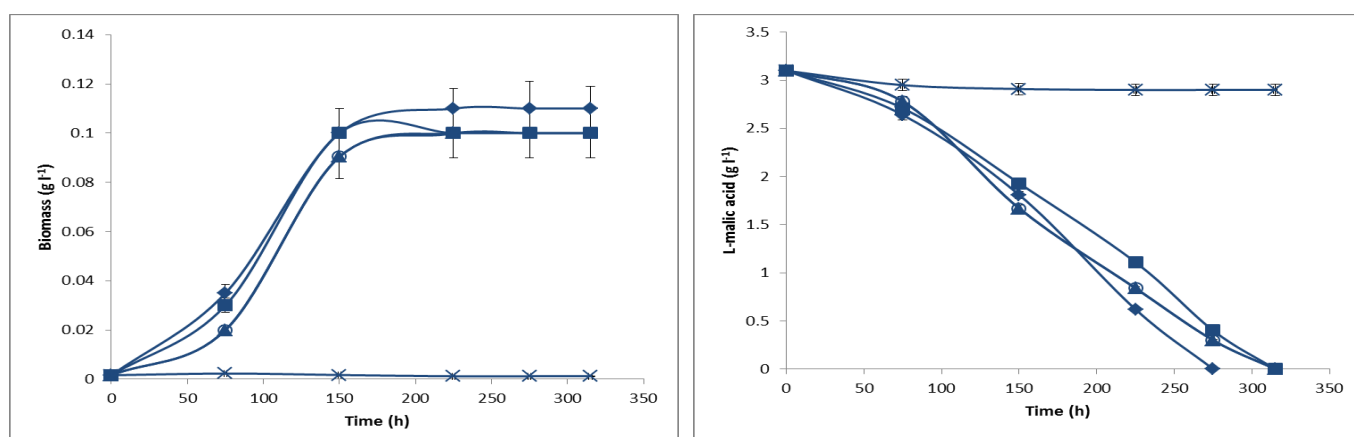


Figure III.7. Growth and malic acid consumption kinetics of *O. oeni* strain X in the control and in the presence of four different MW fractions from the SGJ media fermented by *S. cerevisiae* strain D. (♦) Control: modified MRS medium, (■) MW ≤ 3 kDa, (▲) MW between 3 and 5 kDa, (x) MW between 5 and 10 kDa, (○) MW ≥ 10 kDa. Each value is the mean of triplicate experiments \pm SD

Figure III.7 shows that the kinetic profiles of the bacterial growth and the malate consumption in the presence of the fractions presenting a MW ≤ 3 kDa, between 3 and 5 kDa and ≥ 10 kDa were very similar to those obtained in the modified MRS control, thus showing no inhibition. However, both the bacterial growth and the malate consumption were totally inhibited in the presence of the fraction presenting a MW between 5 and 10 kDa.

A 5-10 kDa fraction isolated at the end of the AF from the SGJ medium fermented by *S. cerevisiae* strain A (the non-inhibitory strain) was also tested in the modified MRS medium and showed no inhibition. The same conditions were applied and the results were identical to those of the control (data given in appendix B-III).

Therefore, we can conclude that *S. cerevisiae* strain D was able to produce an extracellular anti-MLF peptidic compound having an apparent MW between 5 and 10 kDa. This result is in accordance with the results published in Nehme et al., (2010). In the latter, the ultrafiltration units used were different and only 3 fractions with different sizes and concentrations were tested. The first one had a MW between 3.5 and 5 kDa and was not concentrated. The second and the third ones had MW ≥ 5 kDa and ≥ 10 kDa respectively and were both 3.5 times concentrated. The only fraction that showed inhibition was the one with a MW ≥ 5 kDa.

So far, few authors have demonstrated the ability of certain *S. cerevisiae* strains to produce anti-MLF compounds of protein nature. Besides, the compounds found presented different MW and were strain dependent. Dick et al., (1992) were the first to isolate two antibacterial cationic proteins produced by the yeast strain R107. One of them had the characteristics of lysozyme and the other one was a small protein with a high pI. Later on, Comitini et al., (2005) found that *S. cerevisiae* F63 was able to produce a proteinaceous compound with a MW greater than 10 kDa that strongly inhibited the growth of *O. oeni* CHR as well as its MLF. Besides, Osborne and Edwards (2007) identified a 5.9 kDa peptide produced by *S. cerevisiae* RUBY. *ferm* and inhibiting *O. oeni* Viniflora oenos. Mendoza et al., (2010) showed that *S. cerevisiae* mc2 released a proteinaceous compound presenting a MW between 3 and 10 kDa that inhibited the growth of *O. oeni* X₂L but not its ability to consume L-malic acid. Finally, Branco et al., (2014) showed that *S. cerevisiae* CCMI 885 secreted antimicrobial peptides (AMP) that were active against a wide variety of wine-related yeasts in addition to *O. oeni*. However, only the microbial growth was evaluated. These AMP corresponded to two fragments of the C-terminal amino acid sequence of the *S. cerevisiae* glyceraldehyde 3-phosphate deshydrogenase (GAPDH) enzyme. They presented a MW of 1.638 and 1.622 kDa.

All these data in addition to our findings show the wide variety of antibacterial peptides/proteins that could be released by different strains of *S. cerevisiae*. Additional tests are required to determine the exact MW of the putative antibacterial peptides of the 5-10 kDa fraction. These tests will be described in part D of this chapter.

IV. Timing of the release of the 5-10 kDa anti-MLF peptidic fraction produced by *S. cerevisiae* strain D during AF

In order to assess the timing of the release of the 5-10 kDa peptidic fraction, the AF in the SGJ medium was followed until total sugar consumption. It lasted 120 h whereas the stationary phase started after 50 h. A peptidic fraction of 5-10 kDa was collected each 24 h of the AF. Five fractions were obtained and each one was tested in a modified MRS medium where it was finally 10.5 times concentrated. The results were compared to those obtained in a modified MRS control (Figure III.8, Table III.8). The detailed values of the bacterial biomass produced and the malate consumed are given appendix B-IV.

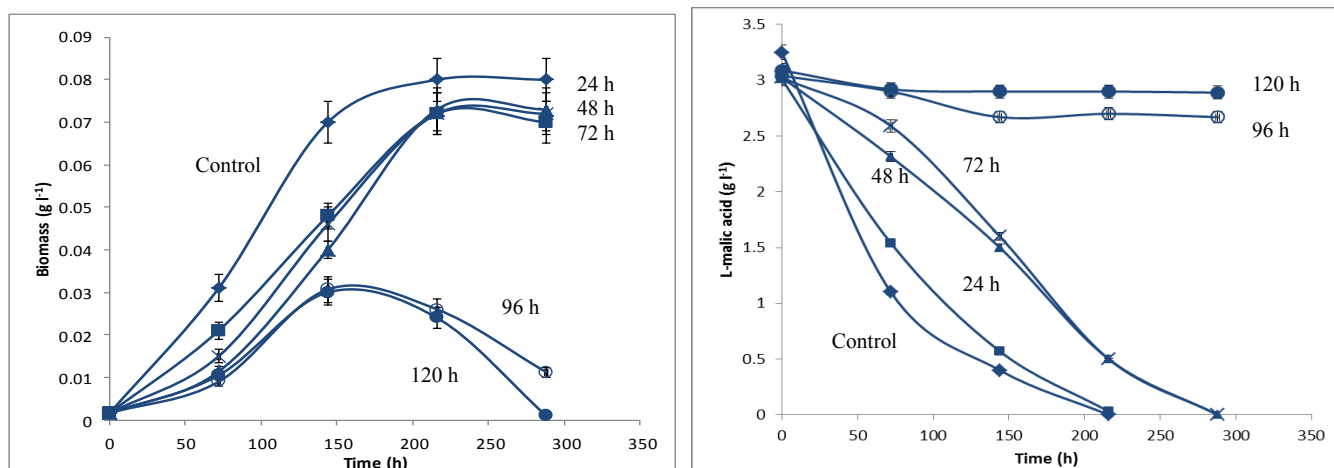


Figure III.8. Growth and malic acid consumption kinetics of *O. oeni* strain X in the control and in the presence of the 5-10 kDa inhibitory fractions collected at different intervals of the AF. (♦) Control: modified MRS medium, (■) 5-10 kDa collected after 24 h of the AF, (▲) 5-10 kDa collected after 48 h of the AF, (x) 5-10 kDa collected after 72 h of the AF, (○) 5-10 kDa collected after 96 h of the AF, (●) 5-10 kDa collected after 120 h of the AF. Each value is the mean of triplicate experiments \pm SD

Table III.8. Determination of the average specific growth rates and the average specific rates of malate consumption during the MLF carried out by *O. oeni* strain X in the modified MRS media containing the 5-10 kDa fractions collected each 24 h of the AF as well as in the control¹

| | Control: modified MRS medium | modified MRS medium + D1 ⁴ fraction | modified MRS medium + D2 ⁵ fraction | modified MRS medium + D3 ⁶ fraction | modified MRS medium + D4 ⁷ fraction | modified MRS medium + D5 ⁸ fraction |
|--|---------------------------------------|--|--|--|--|--|
| X ₀ : Initial biomass (g/l) | 0.0016 ±0.0002 | 0.0016 ±0.0002 | 0.0016 ±0.0002 | 0.0016 ±0.0002 | 0.0016 ±0.0002 | 0.0016 ±0.0002 |
| X _f : Final biomass (g/l) | 0.080 ±0.006 | 0.072 ±0.006 | 0.073 ±0.005 | 0.072 ±0.006 | 0.031 ±0.002 | 0.030 ±0.002 |
| dt ₁ : Growth duration (h) | 216 | 216 | 216 | 216 | 144 | 144 |
| μ ² : average specific growth rate (h ⁻¹) | 0.009 ±0.001 ^a | 0.009 ±0.001 ^a | 0.009 ±0.001 ^a | 0.009 ±0.001 ^a | 0.013 ±0.002 ^b | 0.013 ±0.001 ^b |
| S ₀ : Initial L-malic acid (g/l) | 3.25 ±0.16 | 3.01 ±0.16 | 3.03 ±0.20 | 3.03 ±0.15 | 3.04 ±0.15 | 3.09 ±0.12 |
| S _f : Final L-malic acid (g/l) | 0 ±0 | 0.03 ±0 | 0 ±0 | 0 ±0 | 2.67 ±0.12 | 2.90 ±0.16 |
| dt ₂ : Duration of MLF (h) | 216 | 216 | 288 | 288 | 144 | 144 |
| Q _s ³ : average specific rate of malate consumption (g g ⁻¹ h ⁻¹) | 0.37 ±0.02 ^a | 0.375 ±0.020 ^a | 0.28 ±0.02 ^b | 0.290 ±0.015 ^b | 0.16 ±0.01 ^c | 0.084 ±0.006 ^d |

¹ Results are mean ± SD values of three replications. In comparison to the control, values of average specific rates (growth or malate consumption) within the same row followed by different letters are significantly different ($p < 0.05$) according to Tukey's HSD test

² μ (h⁻¹) = (dX/dt₁).1/X with dX = (X_f - X₀) and X = (X_f + X₀)/2

³ Q_s (g g⁻¹ h⁻¹) = (dS/dt₂).1/X with dS = (S₀ - S_f) and X = (X_f + X₀)/2

⁴D1 (day 1): fraction collected 24 h after the beginning of the AF

⁵D2 (day 2): fraction collected 48 h after the beginning of the AF

⁶D3 (day 3): fraction collected 72 h after the beginning of the AF

⁷D4 (day 4): fraction collected 96 h after the beginning of the AF

⁸D5 (day 5): fraction collected 120 h after the beginning of the AF

Figure III.8 shows that the growth kinetic profiles of *O. oeni* strain X in the presence of the fractions collected after 24, 48 and 72 h were very similar. Although the average specific growth rates were identical to that of the control (0.009 h⁻¹), the final biomass reached (0.072 g/l) was 10 % lesser than the one reached in the control (0.08 g/l) (Table III.8). When the fractions collected after 96 and 120 h were tested, the specific growth rates were slightly higher (0.013 h⁻¹, $p < 0.05$), but the maximum biomass reached (0.03 g/l) was reduced by 62.5 % compared to the

control (Table III.8). Moreover, a decline phase was detected with these two fractions leading to the death of the whole population with the last fraction (120 h).

The malate was totally consumed in the presence of the fractions collected after 24 h (D1), 48 h (D2) and 72 h (D3) but with a gradual decrease of the consumption kinetics (Figure III.8 and Table III.8). The average specific rate of malate consumption in the presence of the fraction D1 was similar to that of the control ($0.37 \text{ g g}^{-1} \text{ h}^{-1}$, $p > 0.05$). In the presence of the fractions D2 and D3, the specific rates were 23 % lower than in the control ($p < 0.05$). The fractions taken after 96 h (D4) and 120 h (D5) of AF were the most inhibiting ones as only 0.37 and 0.19 g/l of L-malic acid were consumed reducing the control specific rate of 57 and 77 % respectively ($p < 0.05$).

Therefore, we can conclude that the 5-10 kDa peptidic fraction was gradually released during the AF and reached its maximum inhibitory concentration at late stages of the stationary phase (96 and 120 h). Its effect started to be detectable with the fraction D2 (48 h) reducing mainly the rate of malate consumption. The same was noticed with the fraction D3 (72 h). The strong inhibition of malate consumption with the fractions D4 (96 h) and D5 (120 h) can somewhat explain the weak final biomasses formed. It is known that the MLF produces ATP through a chemiosmotic mechanism thus enhancing the bacterial growth (Cox and Henick-Kling, 1995; Bouix and Ghorbal, 2015). The MLF is one of the main sources of energy for bacteria in winemaking conditions. Therefore, an inhibition of malate consumption can also affect the bacterial growth.

Information about the timing of the release of antibacterial peptides/proteins is scarce and to our best knowledge no previous work has described simultaneously the kinetics of their production and the kinetics of their anti-MLF activity. Albergaria et al., (2010) showed that *S. cerevisiae* CCMI 885 started to secrete three antifungal peptides presenting an apparent MW of about 6, 4.5 and 4 kDa at the end of the AF exponential growth phase (day 2) with a gradual increase of their concentration during the stationary growth phase (days 4 and 7). Later on, Branco et al., (2014) demonstrated that these peptides were also active against the growth of *O. oeni* and corresponded to GAPDH-derived peptides of 1.6 kDa. They suggested that the peptides were released by apoptotic yeast cells during the stationary phase. In fact, Silva et al., (2011) showed that GAPDH is presumably a substrate of metacaspases during apoptosis. Our findings present some similarities with these conclusions since the most important antibacterial effect was detected with the fractions obtained from late stationary phase. However, further investigation must be carried out to see if any of the 5-10 kDa peptides is possibly a GAPDH fragment.

Osborne and Edwards (2007) did not specify the timing of the release of the 5.9 kDa peptide secreted by *S. cerevisiae* RUBY.ferm. They only showed that samples taken at different intervals of the AF (days 9, 16 and 23) were able to inhibit *O. oeni* Viniflora oenos.

The timing results are also in accordance with the co-culture results published by Nehme et al., (2010) who demonstrated that the co-culture strategy improved the MLF output with the same pair (*S. cerevisiae* strain D/*O. oeni* strain X). During co-cultures, AF and MLF were conducted simultaneously by inoculating yeasts and bacteria at the same time from the beginning in a membrane bioreactor using the same experimental conditions. The bacterial growth was twice better and 74 % of the initial malate were successfully consumed in three weeks without any risk of increased volatile acidity.

The present study shows that the 5-10 kDa peptides gradually appeared during the AF, thus giving the bacteria enough time to grow better and especially to better consume L-malic acid.

More recently, co-inoculation was suggested as a worthwhile alternative for winemaking both for better malate consumption and improved sensorial characteristics when compared to the traditional sequential inoculation (Cañas et al., 2015; Versari et al., 2015; Tristezza et al., 2016). Information about the timing of the release of the inhibitory peptides is very useful for the determination of the best moment for bacterial inoculation and for an optimum extraction of the targeted molecules.

V. Maintenance of the anti-MLF effect of the 5-10 kDa peptidic fraction produced by *S. cerevisiae* strain D in natural winemaking conditions

In part A of this chapter, we demonstrated that the MLF inhibition during sequential fermentations with the pair *S. cerevisiae* strain D/*O. oeni* strain X was reproducible in both synthetic and natural red grape juices without giving any further explanation. *S. cerevisiae* strain A which was rather stimulatory towards *O. oeni* strain X gave also similar results in both synthetic and natural grape juices. In the current part (part B), it was demonstrated that *S. cerevisiae* strain D was able to release a peptidic fraction presenting an apparent MW between 5 and 10 kDa responsible for the MLF inhibition. It was gradually released during the AF and reached its highest concentration at late stages of the stationary phase. This fraction may have

worked synergistically with the 11 % ethanol (v/v) produced by *S. cerevisiae* strain D to strongly inhibit the bacterial growth and the malate consumption.

Sequential fermentations with the same pairs were conducted in Syrah and Cabernet Sauvignon grape musts in order to evaluate the reproducibility of the previous results in natural winemaking conditions.

Table III.9 gives the phenolic content of the Cabernet Sauvignon and the Syrah wines at the end of the AF carried out by *S. cerevisiae* strains A and D while table III.10 describes the growth of *O. oeni* strain X and the malate consumption during the MLF performed in these wines.

Table III.9. Total phenolic content (mg/l equivalent gallic acid) in Cabernet Sauvignon and Syrah wines at the end of the AF carried out by *S. cerevisiae* strains A and D

| | Cabernet Sauvignon wine | Syrah Wine | Cabernet Sauvignon wine | Syrah wine |
|---|---|--------------|---|--------------|
| *Pre-fermentation maceration temperature (°C) | Phenolic content at the end of the AF carried out by <i>S. cerevisiae</i> strain A (mg/l) | | Phenolic content at the end of the AF carried out by <i>S. cerevisiae</i> strain D (mg/l) | |
| 10 | 513 (± 18) | 365 (± 7) | 868 (± 18) | 378 (± 32) |
| 60 | 2635 (± 21) | 2425 (± 106) | 2925 (± 7) | 2718 (± 117) |
| 70 | 3725 (± 21) | 3043 (± 88) | 3978 (± 11) | 3645 (± 170) |
| 80 | 2985 (± 21) | 2295 (± 35) | 3078 (± 11) | 2585 (± 42) |

*Pre-fermentation maceration temperature: Cabernet Sauvignon and Syrah musts were macerated at 10, 60, 70 or 80 °C for 48 h before AF

Table III.10. Growth of *O. oeni* strain X and malate consumption during MLF performed in the Syrah and Cabernet Sauvignon wines obtained after AF by *S. cerevisiae* strains A or D

| | AF carried out by <i>S. cerevisiae</i> strain A | | | | AF carried out by <i>S. cerevisiae</i> strain D | | | |
|--|--|----------------------|----------------------|----------------------|--|----------------------|----------------------|----------------------|
| | Cabernet Sauvignon wine | | Syrah wine | | Cabernet Sauvignon wine | | Syrah wine | |
| Pre-fermentation maceration temperature (°C) | 10 | 60, 70 and 80 | 10 | 60, 70 and 80 | 10 | 60, 70 and 80 | 10 | 60, 70 and 80 |
| Initial bacterial biomass (g/l) | 0.0016 (± 0.0002) | 0.0016 (± 0.0002) | 0.0016 (± 0.0002) | 0.0016 (± 0.0002) | 0.0016 (± 0.0002) | 0.0016 (± 0.0002) | 0.0016 (± 0.0002) | 0.0016 (± 0.0002) |
| Final bacterial biomass (g/l) | 0.120 (± 0.005) | 0.080 (± 0.004) | 0.120 (± 0.006) | 0.080 (± 0.004) | 0.0040 (± 0.0004) | 0.0020 (± 0.0002) | 0.0060 (± 0.001) | 0.0020 (± 0.0002) |
| Initial L-malic acid (g/l) | 3.00 (± 0.06) | 3.00 (± 0.06) | 3.00 (± 0.06) | 3.00 (± 0.06) | 3.00 (± 0.06) | 3.00 (± 0.06) | 3.00 (± 0.06) | 3.00 (± 0.06) |
| Final L-malic acid (g/l) | 0 (± 0) | 0 (± 0) | 0 (± 0) | 0 (± 0) | 3 (± 0.06) | 3 (± 0.06) | 3 (± 0.06) | 3 (± 0.06) |
| Malolactic fermentation duration (days) | 30 | 45 | 30 | 45 | 90 | 90 | 90 | 90 |

Table III.9 gives the total phenolic content at the end of the AF carried out by *S. cerevisiae* strains A and D in Cabernet Sauvignon and Syrah wines. The corresponding grape musts were previously macerated at 10, 60, 70 and 80 °C for 48 h before AF. High phenolic contents were reached at maceration temperatures of 60, 70 and 80 °C because of a better phenolic extraction from the grape skin and seeds. Sequential fermentations were carried out in these wines by inoculating *O. oeni* strain X at the end of the AF. MLF were followed for three months and were totally inhibited in wines fermented by *S. cerevisiae* strain D. Both the bacterial growth and the malate consumption were repressed regardless of the phenolic content (Tables III.9 and III.10). Despite high phenolic contents, MLF conducted by *O. oeni* strain X in wines produced by *S. cerevisiae* strain A were totally completed. In comparison to the pre-fermentation maceration at 10 °C, the higher phenolic content obtained at 60, 70 and 80 °C reduced the final bacterial biomass reached and slowed down the MLF but malate was totally consumed (Table III.10).

Therefore, MLF results were reproducible in both synthetic and natural grape juice media with both yeasts/bacteria pairs tested.

The maintenance of the inhibitory effect due to the 5-10 kDa yeast peptidic fraction in natural winemaking conditions despite the presence of phenolic compounds is an interesting finding. It is usually known that phenolic compounds are able to interact with proteins leading either to

their precipitation or to changes of their properties (Sims et al., 1995; Yokotsuka and Singleton 1995). Wine proteins derive mostly from grapes but also from yeasts during AF. Both reversible (van der Waals forces, hydrogen bonding and hydrophobic binding) and irreversible (covalent bonds) interactions are involved. Proline rich proteins like collagen, gelatin and casein, which are commonly used as protein fining agents in wine, and some salivary proteins reported to be involved in astringency perception, are particularly prone to interact with phenolic compounds. The precipitation depends on the phenols and proteins type and concentration. Low MW phenolic compounds display moderate affinity for proteins (phenolic acids, flavonols and non-galloylated flavan-3-ol monomers). Interaction and precipitation increase with the number of phenolic rings and thus with the degree of polymerization and the presence of galloyl substituents (Dangles, 2005; McRae and Kennedy, 2011).

Few studies investigated the possible interactions between peptides of low MW and phenolic compounds in wine. Besides, no previous studies dealt with peptides from wine origin. Yokotsuka and Singleton (1995) demonstrated that gelatin peptides (2, 5 and 10 kDa) and synthetic peptides of low MW (between 1.3 and 2.5 kDa) were very effective for fining wines, at least at the same levels as whole gelatin (70 kDa) with high affinity for phenols. The affinity depended on the pH and temperature. Osborne and Edwards (2007) found that *S. cerevisiae* RUBY.ferm was able to produce a peptide of 5.9 kDa that inhibited *O. oeni* Viniflora oenos during sequential fermentations in synthetic grape juice media lacking phenols. However, Larsen et al., (2003) who previously used the same strains combination were able to successfully achieve MLF in Chardonnay wines. Therefore, it was suggested that the Chardonnay wine contained phenolic compounds that may have interacted with the 5.9 kDa peptide.

The 5-10 kDa peptides of this study may have not presented binding sites for phenolic compounds and thus were not removed. Regardless of the different types and concentrations of phenolic compounds, the inhibition caused by the 5-10 kDa peptidic fraction released by *S. cerevisiae* strain D was preserved in natural winemaking conditions.

VI. Fractionation of the SGJ media fermented by *S. cerevisiae* strain D by ammonium sulfate precipitation

The SGJ media fermented by *S. cerevisiae* strain D were centrifuged to recuperate the supernatant. Different amounts of $(\text{NH}_4)_2\text{SO}_4$ were added to reach the following saturation degrees: A: 0-20 %, B: 20-40 %, C: 40-60 %, D: 60-80 % and E: 80-100 %. For each saturation degree, the medium was centrifuged and the protein pellet obtained was suspended in a phosphate buffer. The samples were then desalted using ultrafiltration units of 5 kDa cut-off, thus retaining compounds with a $\text{MW} \geq 5$ kDa. This choice was made because the fractionation by ultrafiltration has previously shown that compounds with a $\text{MW} \leq 5$ kDa were not inhibitory (paragraph III of part B). The inhibitory effect of the five proteinaceous precipitated fractions (A, B, C, D and E) was evaluated separately in modified MRS media inoculated with *O. oeni* strain X. The MLF was followed by regular sampling until cessation of malate consumption and compared to that of a modified MRS control (Figure III.9). The detailed values of the biomass produced and the malate consumed in the presence of each precipitate are given in appendix B-V.

This experiment was a further step towards purification and enabled us to select the protein fraction that will undergo additional purification and characterization (part D of this chapter).

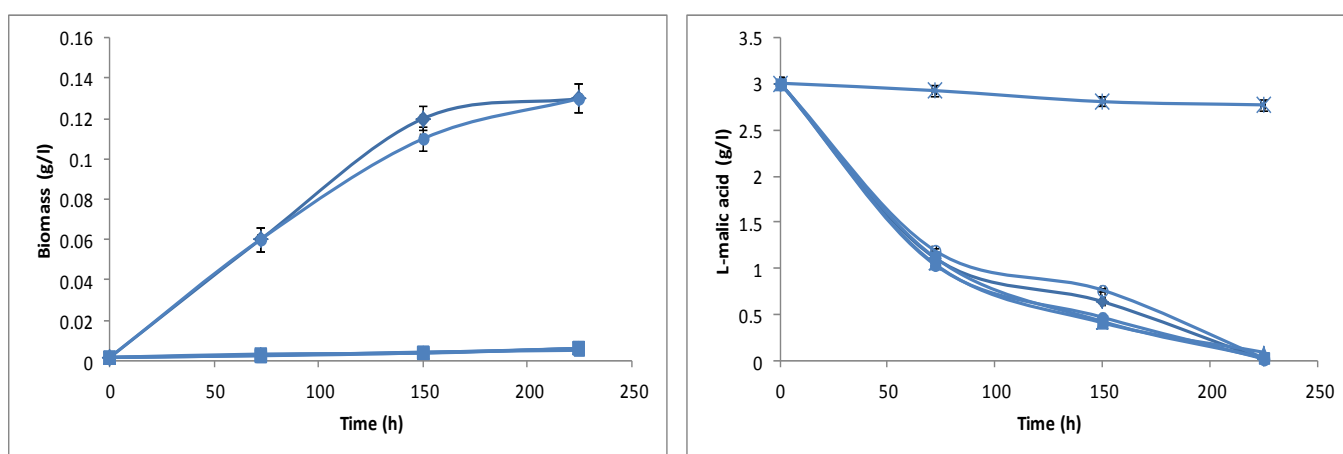


Figure III.9. Growth and malic acid consumption kinetics of *O. oeni* strain X in the control and in the presence of the proteinaceous fractions obtained by ammonium sulfate precipitation in the SGJ media fermented by *S. cerevisiae* strain D. (♦) Control: modified MRS medium; precipitated fractions at different saturation degrees: (■) 0-20 %, (▲) 20-40 %, (○) 40-60 %, (x) 60-80 %, (●) 80-100 %. Each value is the mean of triplicate experiments \pm SD

The protein concentration of the different precipitates was measured before addition to the MRS media and is given in table III.11.

Table III.11. Protein concentration (mg/l) of the different precipitates before addition to the MRS media*

| Saturation degree (%) | Protein concentration (mg/l) |
|-----------------------|---|
| 0-20 | 256.36 (\pm 0.0013) |
| 20-40 | 264.09 (\pm 0.0023) |
| 40-60 | 232.3 (\pm 0.0017) |
| 60-80 | 650.45 (\pm 0.0019) |
| 80-100 | 273 (\pm 0.006) |
| Residual supernatant | 332 (\pm 0.003) |

*The protein concentration was determined by the Lowry method. The standard curve is given in the appendix of part B

Figure III.9 shows that the growth of *O. oeni* strain X in the presence of the precipitate 80-100 % was similar to that obtained in the control. However, with all the other precipitates, the growth was strongly inhibited. These results suggest the presence of different proteinaceous compounds responsible for the bacterial growth inhibition. These compounds presented different solubility levels since they were precipitated by increased amounts of ammonium sulfate. The results also show that the malate consumption kinetics were the same and similar to the control in the presence of all the precipitates to the exception of the 60-80 % one. The latter strongly inhibited the malate consumption.

The 80-100 % precipitate affected neither the growth nor the malate consumption and was similar to the control. However, the precipitates of 0-20 %, 20-40 % and 40-60 % strongly inhibited the bacterial growth but not the malate consumption. The 60-80 % precipitate was the only one that strongly inhibited both the bacterial growth and the malate consumption. Table III.11 indicates that this precipitate presented the highest protein concentration.

These results suggest the presence of 2 categories of inhibitory proteinaceous compounds. The first one only inhibited the bacterial growth while the second one only inhibited the malate consumption. The latter was solely recuperated in the 60-80 % precipitate. With this precipitate,

the inhibition of the bacterial growth could be a consequence of the malate consumption inhibition or it could be due to the presence of both categories of protein compounds. In winemaking conditions, the MLF is one of the main sources of energy for bacterial survival and growth (Cox and Henick-Kling, 1995; Bouix and Ghorbal, 2015). Thus, an inhibition of the malate consumption can also cause an inhibition of the bacterial growth.

The same work was carried out on the SGJ media fermented by *S. cerevisiae* strain A used as a reference strain in this study. The results with the different precipitates obtained were similar to the ones obtained in the modified MRS control, thus showing no inhibition, neither on the bacterial growth nor on the malate consumption.

Finally, the combined results of the fractionation by ultrafiltration and ammonium sulfate precipitation enabled us to conclude that the most inhibiting proteinaceous fraction of both the bacterial growth and the malate consumption presented an apparent MW of 5-10 kDa and was recuperated with a saturation degree of 60-80 %. Therefore, the 5-10 kDa fraction of the 60-80 % ammonium sulfate precipitate was retained for further purification steps (part D of this chapter).

Well plate tests were performed in order to qualitatively evaluate the inhibitory effect of the proteinaceous compounds isolated by ultrafiltration and ammonium sulfate precipitation of the SGJ media fermented by *S. cerevisiae* strain D on the bacterial growth of *O. oeni* strain X. The results are given in appendix B-VI.

The following part (part C) of this chapter, will examine the mechanism of action of the 5-10 kDa peptidic fractions and the 60-80 % precipitate. Their direct effect on the malolactic enzyme activity will be evaluated in order to better elucidate their involvement in MLF inhibition.

Part C- *In vitro* evaluation of the malolactic enzyme inhibition by the 5-10 kDa peptidic fractions and the 60-80 % precipitate

I. Introduction

In an attempt to determine the mechanism of action of the 5-10 kDa fractions, an *in vitro* enzymatic reaction was designed and the fractions of 5-10 kDa collected each 24 h of the AF were tested as described in paragraph II.2.4 of chapter II. The proteinaceous fractions obtained by ammonium sulfate precipitation were also tested. The objective was to evaluate the direct impact of these fractions on the malolactic enzyme activity in order to evaluate their involvement in the MLF inhibition.

The growth of *O. oeni* strain X was followed in modified MRS media and the bacterial pellets were recuperated and suspended in specific buffers (citrate buffer pH 3.5 and 5.5, phosphate buffer pH 6 and 6.7). Different buffers were chosen in order to conduct the reaction at different pH. Bacterial cell lysis was then performed and the cell free cytosolic content comprising the malolactic enzyme was recovered and divided into 2 aliquots. One was used as a control and the other as a test medium to which the proteinaceous fractions were added. The reaction mixture is given in table II.17 of chapter II. The enzymatic reaction was performed at 30°C for 30 min at pH 5.5 and 6, 40 min at pH 6.7 and 70 min at pH 3.5. At the end of each assay, the concentrations of the L-malic acid consumed and the L-lactic acid produced were measured in both aliquots (control and test) and compared (Tables III.12 and III.13). The experiment performed at pH 5.5 gave results similar to those obtained at pH 6 and thus the results corresponding to pH 5.5 are given in the appendix of part C.

II. *In vitro* evaluation of the malolactic enzyme inhibition by the 5-10 kDa peptidic fractions

Table III.12. Amount of L-malic acid consumed (g/l) and L-lactic acid produced (g/l) during the enzymatic reaction *in vitro* performed at different pH in the presence of the 5-10 kDa fractions (ABP) collected each 24 h of the AF¹

| Sampling time of the 5-10 kDa fractions during the AF | pH=6.7 t=40 min | | | pH=6 t=30 min | | | pH=3.5 t= 70min | | |
|---|-------------------------------|-------------------------------|---|-------------------------------|-------------------------------|------------------------------------|------------------------------|-------------------------------|------------------------------------|
| | Consumed L-malic acid (g/l) | Produced L-lactic acid (g/l) | ² Inhibition % of malate consumption | Consumed L-malic acid (g/l) | Produced L-lactic acid (g/l) | Inhibition % of malate consumption | Consumed L-malic acid (g/l) | Produced L-lactic acid (g/l) | Inhibition % of malate consumption |
| Control ³ (absence of ABP) | 1.00 ± 0.02 ^a | 0.670 ± 0.013 ^a | 0 ± 0 | 1 ± 0 ^a | 0.670 ± 0.013 ^a | 0 ± 0 | 0.96 ± 0.02 ^a | 0.65 ± 0.01 ^a | 0 ± 0 |
| After 24 h of the AF (D1) | 0.720 ± 0.015 ^b | 0.48 ± 0.01 ^b | 28.0 ± 0.6 | 0.92 ± 0.02 ^b | 0.620 ± 0.012 ^b | 8.00 ± 0.16 | 0.89 ± 0.02 ^b | 0.6 ± 0.01 ^b | 7.30 ± 0.15 |
| After 48 h of the AF (D2) | 0.630 ± 0.013 ^c | 0.420 ± 0.009 ^c | 37.0 ± 0.8 | 0.85 ± 0.02 ^c | 0.570 ± 0.011 ^c | 15.0 ± 0.3 | 0.82 ± 0.02 ^c | 0.55 ± 0.01 ^c | 14.6 ± 0.3 |
| After 72 h of the AF (D3) | 0.560 ± 0.011 ^d | 0.380 ± 0.008 ^d | 44 ± 1 | 0.70 ± 0.01 ^d | 0.47 ± 0.01 ^d | 30.0 ± 0.6 | 0.65 ± 0.01 ^d | 0.44 ± 0.01 ^d | 32.30 ± 0.65 |
| After 96 h of the AF (D4) | 0.100 ± 0.002 ^e | 0.070 ± 0.002 ^e | 90 ± 2 | 0.096 ± 0.002 ^e | 0.065 ± 0.001 ^e | 90.4 ± 2.0 | 0.06 ± 0.001 ^e | 0.040 ± 0.001 ^e | 94 ± 2 |
| After 120 h of the AF (D5) | 0.090 ± 0.002 ^f | 0.060 ± 0.002 ^f | 91 ± 2 | 0.060 ± 0.001 ^f | 0.040 ± 0.001 ^f | 94 ± 2 | 0.001 ± 0 ^f | 0.0007 ± 0 ^f | 100 ± 2 |

¹ Results are mean ±SD values of three replications. In comparison to the control, values of malate consumed and lactate produced followed by different letters within the same column are significantly different ($p < 0.05$) according to Tukey's HSD test

²Inhibition % of malate consumption = (consumed L-malic acid in the control - consumed L-malic acid in the presence of ABP) x 100/ (consumed L-malic acid in the control)

³Absence of ABP: absence of antibacterial peptides

In this experiment, the malolactic enzyme of the cell-free bacterial enzymatic extract was directly exposed to the inhibitory peptides. The five fractions of 5-10 kDa collected each 24 h of the AF (D1 to D5) were tested as described in table II.17 of chapter II. Table III.12 shows the amounts of L-malic acid consumed and the corresponding L-lactic acid produced for each 5-10 kDa fraction tested.

The first set of experiments was conducted at pH 6.7 which is around the cytoplasmic pH of the majority of lactic acid bacteria. The same experiment was repeated by changing the pH. pH 6 was chosen because it is the optimum pH for activity of the malolactic enzyme of *O. oeni* (Schumann et al., 2013). pH 3.5 was also tested because it represents the pH of wine at the

beginning of the MLF in this study. The results obtained in the controls show that the malolactic enzyme was active in the pH range tested since the L-malic acid was completely decarboxylated into L-lactic acid and CO₂ (Table III.12). The reaction was the fastest at pH 6 since it is the optimum pH and took only 30 min whereas it was the slowest at pH 3.5 and took 70 min. 40 min were required at pH 6.7 to completely decarboxylate the same amount of L-malic acid (1 g/l). Interestingly, Bouix and Ghorbal (2015) demonstrated that at low extracellular pH, *O. oeni* cells were able to drop their intracellular pH to values as low as 3.5 (equal to the extracellular pH). The MLF was then initiated indicating that the malolactic enzyme was active at this low pH. During MLF, the intracellular pH increased again due to the proton extrusion that accompanied the release of lactate and CO₂. It reached 6 at the end of the MLF and dropped again to 3.5 when the malate was totally consumed. The results of the *in vitro* experiment performed at 3.5 showed that the enzyme was indeed active at this low pH value although the enzymatic reaction was 2.3 times slower than at pH 6 which is the optimum pH (Table III.12).

The results of the experiment performed at pH 6.7 clearly show that the 5-10 kDa fractions were able to reduce the amount of L-malic acid consumed of 28 % (D1) to 91 % (D5) when compared to the control, thus exhibiting a direct inhibitory effect on the malolactic enzyme activity ($p < 0.05$). The ethanol concentration in the five fractions was evaluated and a residual amount of 0.02 g/l was found in the 5-10 kDa fraction corresponding to D5. Therefore, all the fractions were ethanol free and the inhibition observed was only due to the presence of the peptides. Besides, the kinetic of the inhibition was in agreement with the results of the timing experiment (paragraph IV of Part B) proving once again that the 5-10 kDa peptidic fraction was gradually released during the AF with the highest concentration reached at the end of the stationary phase. Moreover, it is known that during the malolactic fermentation, each mole of L-malic acid decarboxylated by the malolactic enzyme releases one mole of L-lactic acid and one mole of CO₂. Therefore, the measurement of the lactate produced was an additional indicator of the malolactic enzyme activity. The amounts of L-lactic acid produced were measured and found to be stoichiometrically equivalent to the amounts of L-malic acid consumed. As an example, in the presence of the fraction D1, 0.72 g/l of L-malic acid equivalent to 5.4 mmol/l were consumed and gave 0.48 g/l of L-lactic acid which corresponded to 5.3 mmol/l. Consequently, the gradual decrease in the amount of L-lactic acid produced proved that the 5-10 kDa peptidic fractions

targeted specifically the malolactic enzyme and not any other enzyme of the cytosolic extract. The same observations can be made with the experiments performed at pH 6 and pH 3.5.

Additionally, 5-10 kDa fractions isolated from the SGJ media fermented by *S. cerevisiae* strain A (reference strain or non-inhibitory strain) were also tested and gave results identical to the control. Therefore, the inhibition of the malolactic enzyme by the 5-10 kDa peptides of *S. cerevisiae* strain D is highly specific.

From the previous results, it can be concluded that the peptides were able to exert their inhibitory effect in a pH range between 3.5 and 6.7. We also suggest that *in vivo*, the peptides of the 5-10 kDa fraction released by *S. cerevisiae* strain D would enter the bacterial cells by mechanisms yet to be identified and directly inhibit the malolactic enzyme.

No previous works have shown the involvement of yeast proteinaceous metabolites in the direct inhibition of the malolactic enzyme activity. Few have attempted to explain the mechanism of action of the yeast antibacterial peptides/proteins and evaluated mainly their impact on the bacterial growth. The proteinaceous compound ≥ 10 kDa found by Comitini et al., (2005), was dose dependent and was able to reduce the bacterial growth with a typical saturation kinetic thus suggesting the presence of a receptor on the bacterial cell. Therefore its bacteriostatic or bactericidal effect depended on its concentration and the incomplete MLF was correlated to its bactericidal effect. Mendoza et al., (2010) found that their peptidic fraction having a MW between 3 and 10 kDa inhibited the bacterial growth but not the ability to consume L-malic acid with a typical saturation kinetic similar to that suggested by Comitini et al., (2005). Osborne and Edwards (2007) suggested that their antibacterial peptide presenting a MW of 5.9 kDa inhibited the bacteria along with SO₂. The mechanism proposed was that of bacteriocins forming membrane pores and facilitating the entry of SO₂ inside the cells thus leading to the bacterial death and arrest of MLF. The mechanism of action of the GAPDH-derived peptides identified by Branco et al., (2014) and that inhibited the growth of *O. oeni* was not elucidated. In addition no data concerning the malate consumption was shown.

III. *In vitro* evaluation of the malolactic enzyme inhibition by the proteinaceous fractions obtained by ammonium sulfate precipitation

It was shown in part B of this chapter that the proteinaceous fraction obtained by ammonium sulfate precipitation at a saturation degree of 60-80 % strongly inhibited the MLF while all the other fractions did not (0-20 %; 20-40 %; 40-60 % and 80-100 %). The precipitates were desalted with ultrafiltration membranes of 5 kDa thus retaining compounds ≥ 5 kDa.

In paragraph II of this part, we demonstrated that the 5-10 kDa peptidic fractions directly inhibited the malolactic enzyme activity. Therefore, we decided to recuperate the 5-10 kDa fraction of the 60-80 % precipitate for further studies (mechanism of action and purification).

In the following, we will evaluate the effect of the 60-80 % precipitate on the malolactic enzyme activity. First the 60-80 % precipitate containing compounds ≥ 5 kDa will be tested. Then, the 5-10 kDa fraction from this precipitate will be also tested. The 0-20 % precipitate was chosen amongst the precipitates that did not inhibit MLF to study its direct impact on the malolactic enzyme activity.

Table III.13. Amount of L-malic acid consumed (g/l) and L-lactic acid produced (g/l) during the enzymatic reaction *in vitro* performed at different pH in the presence of different proteinaceous fractions obtained by ammonium sulfate precipitation at the end of the AF (120 h)

| | pH=6.7 t=40 min | | pH=6 t=30 min | | pH=3.5 t=70 min | |
|---|-----------------------------------|------------------------------------|-----------------------------------|------------------------------------|-----------------------------------|------------------------------------|
| | Consumed L-malic acid (g/l) | Produced L-lactic acid (g/l) | Consumed L-malic acid (g/l) | Produced L-lactic acid (g/l) | Consumed L-malic acid (g/l) | Produced L-lactic acid (g/l) |
| Control (absence of ABP) | 1.00 (± 0.02) | 0.670 (± 0.013) | 0.933 (± 0.020) | 0.062 (± 0.001) | 0.974 (± 0.002) | 0.65 (± 0.01) |
| 60-80 % precipitate containing compounds ≥ 5 kDa | 0.120 (± 0.002) | 0.080 (± 0.002) | 0.120 (± 0.002) | 0.080 (± 0.002) | 0.120 (± 0.003) | 0.080 (± 0.002) |
| 5-10 kDa fraction of the 60-80 % precipitate | 0.0080 (± 0.0002) | 0.0053 (± 0.0001) | 0.0012 (± 0) | 0.0080 (± 0.0002) | 0.0090 (± 0.0002) | 0.0060 (± 0.0001) |
| 0-20 % precipitate containing compounds ≥ 5 kDa | 0.90 (± 0.02) | 0.60 (± 0.01) | 0.90 (± 0.02) | 0.60 (± 0.01) | 1.00 (± 0.02) | 0.67 (± 0.01) |

Table III.13 shows that the 60-80 % precipitate containing compounds ≥ 5 kDa was able to inhibit the malate consumption by directly targeting the malolactic enzyme activity. The results were similar with all the pH tested and were in accordance with those obtained with the 5-10 kDa fractions previously tested (paragraph II of part C). For example, at pH 6.7, the malate consumption was reduced by 88 % in comparison to the control. The inhibition was even greater when the 5-10 kDa fraction was isolated from the 60-80 % precipitate. In this case, it was reduced by 99 %. The 0-20 % precipitate showed no inhibition of the malolactic enzyme activity. This result was also in accordance with that previously obtained in the modified MRS broth (paragraph VI of part B).

In the following part (part D), the 5-10 kDa fraction of the 60-80 % precipitate will be further analyzed by SDS-PAGE and ion exchange chromatography followed by SDS-PAGE and sequencing in order to purify the putative antimicrobial peptides responsible for the MLF inhibition.

**Part D- Purification of the anti-MLF peptides of
the 5-10 kDa peptidic fraction isolated from the
60-80 % ammonium sulfate precipitate**

I. Introduction

The results of parts B and C encouraged us to retain the 5-10 kDa peptidic fraction of the ammonium sulfate precipitate obtained at a saturation degree of 60-80 % for further purification. In fact, this proteinaceous fraction isolated from the SGJ media fermented by *S. cerevisiae* strain D strongly inhibited the malate consumption in the *in vitro* experiment; thus directly inhibiting the malolactic enzyme activity. Since *S. cerevisiae* strain A was adopted as a reference strain in this study, the 5-10 kDa peptidic fraction of the 60-80 % precipitate isolated from the SGJ media fermented by this strain was used as a control in the SDS-PAGE and ion exchange chromatography (IEXC) experiments performed in this part.

II. Comparison of the protein profiles of *S. cerevisiae* strains A and D by Tricine SDS-PAGE

The 5-10 kDa peptidic fractions isolated from the 60-80 % precipitates obtained from the SGJ media fermented by *S. cerevisiae* strains A and D were analyzed and compared by Tricine SDS-PAGE. The results are given in figure III.10.

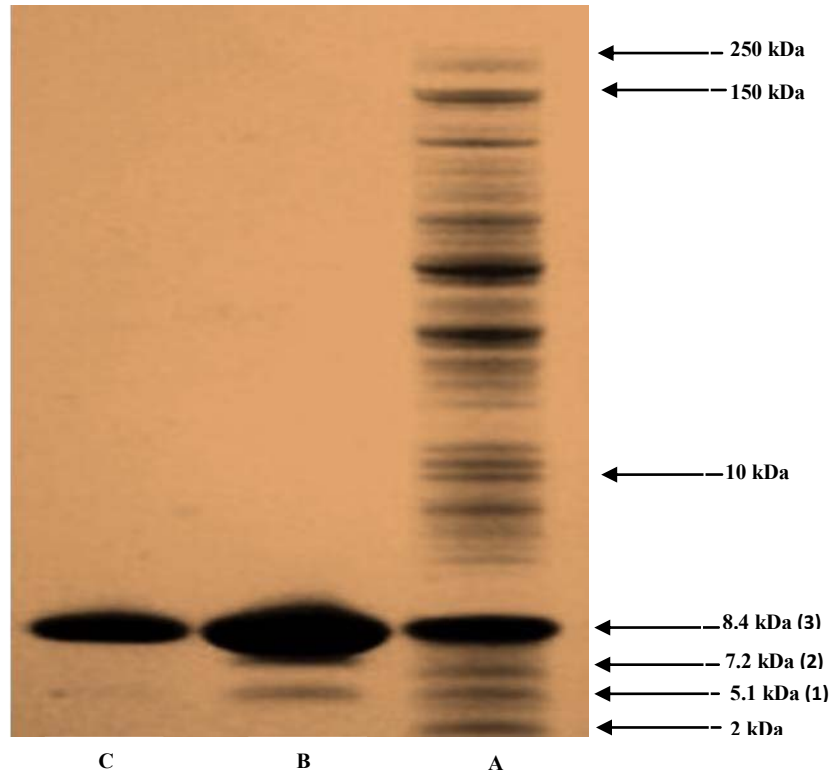


Figure III.10 Silver stained Tricine SDS-PAGE gel. Lane A corresponds to the dual-xtra MW standard 2-250 kDa (Bio-Rad). Lane B corresponds to the 5-10 kDa fraction of the 60-80 % precipitate from *S. cerevisiae* strain D. Lane C corresponds to the 5-10 kDa fraction of the 60-80 % precipitate from *S. cerevisiae* strain A (reference strain)

Tricine SDS-PAGE analysis of the inhibitory fraction released by *S. cerevisiae* strain D revealed three protein bands with an apparent MW of approximately 5.1 kDa (1), 7.2 kDa (2), and 8.4 kDa (3) as shown in lane B. Two of these protein bands, (1) and (2), were not present in the SGJ media fermented by strain A (lane C). Besides, the band of approximately 8.4 kDa in size (3) was released by both strains, A and D, but was more concentrated in the SGJ media fermented by strain D. Therefore, it is likely that the peptides of these bands were involved in the inhibition of the MLF. However, further purification steps are required to answer the following questions:

- a- How many peptides are really involved in the MLF inhibition?
- b- Do they work synergistically?
- c- What is the exact MW and type of these peptides?

The use of strain A as a reference or control was very helpful since the comparison of the protein profiles proved that strain D was able to produce additional peptides. This strategy was also adopted by Osborne and Edwards, (2007) who compared the protein profiles of *S. cerevisiae*

RUBY.ferm (inhibitory strain) and Saint-Georges S101 (non-inhibitory strain) by SDS-PAGE analysis of the peptidic fractions of 3-5 kDa isolated from the SGJ media fermented by both strains. The analysis revealed three protein bands of approximately 5.9, 5.1 and 3.4 kDa in size. The bands of 3.4 and 5.1 kDa were common for both strains, whereas the band of 5.9 kDa was present only in the lane corresponding to RUBY.ferm. Therefore, they concluded that the peptide responsible for the inhibition of the bacterial growth of *O. oeni* Viniflora oenos and its MLF had an apparent MW of 5.9 kDa.

Besides, the ultrafiltration technique used in this study to isolate the peptidic fraction of 5-10 kDa and the SDS-PAGE technique used to migrate and separate the different peptides of this fraction gave us an idea about the apparent MW of the putative antibacterial peptides. The values of the apparent MW were most probably higher than the real ones because interactions and aggregation of different peptides may have occurred during the fractionation by ultrafiltration thus retaining peptides with a MW lower than the membrane cut-off. Besides, interactions may have also occurred during electrophoresis.

As an example, Albergaria et al., (2010), showed that *S. cerevisiae* CCMI 885 produced a peptidic fraction with an apparent MW between 2 and 10 kDa that inhibited the growth of several wine-related yeast species. The peptidic fraction was obtained by ultrafiltration of the SGJ media fermented by strain CCMI 885. The Tricine SDS-PAGE analysis of this fraction gave three protein bands with an apparent MW of 6, 4.5 and 4 kDa corresponding to one or more antimicrobial peptides. Later on, Branco et al., (2014) demonstrated that these peptides were also active against the growth of *O. oeni* and corresponded to GAPDH-derived peptides of 1.6 kDa.

For further characterization and purification of the anti-MLF peptides of the 5-10 kDa fraction obtained from the 60-80 % precipitate, the latter was submitted to ion exchange chromatography. The inhibitory eluates were then submitted to SDS-PAGE in order to select the protein bands that must undergo sequencing.

III. Purification of the anti-MLF peptides of the 5-10 kDa fraction from the 60-80 % precipitate by Ion Exchange Chromatography (IEXC) followed by SDS-PAGE and sequencing

The Biological Low Pressure Chromatography (Bio-Rad) was firstly used for protein purification by IEXC. The only result obtained is given in the appendix of part D. Due to technical problems, purification by IEXC was then pursued using the Aurum mini kit (Bio-Rad). Aurum AEX (anionic exchange) and CEX (cationic exchange) columns allow rapid fractionation of complex protein mixtures in only a few steps using the common technique of IEXC (refer to paragraph II.2.3.6 of chapter II). Each Aurum AEX or CEX column contains 0.2 ml of UNOsphere™ Q [-N⁺(CH₃)₃] or S [-SO₃⁻] support respectively. The buffers used were:

- Binding buffers: 20 mM phosphate pH 6.7 for CEX and 20 mM Tris pH 8.3 for AEX
- Elution buffers: binding buffer + 0.5 M NaCl and binding buffer + 1 M NaCl (gradient elution)

The 5-10 kDa peptidic fraction of the 60-80 % ammonium sulfate precipitate was used in this experiment for further purification. 500 ml of the SGJ media fermented by *S. cerevisiae* strain D were used to recuperate the 60-80 % precipitate. The precipitate was then suspended in 10 ml of the binding buffer (either phosphate or Tris buffer depending on the type of IEXC) and submitted to ultrafiltration in the 10 kDa unit. The filtrate lower than 10 kDa was ultrafiltered again using the 5 kDa unit. The desalted retentate between 5 and 10 kDa was recuperated (0.2 ml). 1 ml of this retentate was collected for each analysis.

The same procedure was performed with the strain A used as a reference strain.

The eluted peptidic fractions from both AEXC and CEXC were tested *in vitro* in the presence of the malolactic enzyme of the cell-free enzymatic extract and the consumption of L-malic acid as well as the production of L-lactic acid were both monitored (refer to paragraph II.2.4 of chapter II). The results given in table III.14 correspond to those of the control aliquots and the test aliquots containing the different eluates. The reaction was run at 30 °C, pH 6.7 for 40 min.

Table III.14. Consumed L-malic acid (g/l) and produced L-lactic acid (g/l) in the controls and in the presence of the eluates obtained by AEXC and CEXC

| | Consumed L-malic acid (g/l) | Produced L-lactic acid (g/l) |
|------------------------------------|--------------------------------|---------------------------------|
| ^a Control 1 | 0.98 (\pm 0.02) | 0.66 (\pm 0.01) |
| ^b Control 2 | 1.00 (\pm 0.02) | 0.68 (\pm 0.01) |
| ^c Control 3 | 1.00 (\pm 0.02) | 0.68 (\pm 0.01) |
| Eluate 1 from AEXC (0.5 M NaCl) | 0.002 (\pm 0) | 0.0013 (\pm 0) |
| Eluate 1 from CEXC (0.5 M NaCl) | 0.002 (\pm 0) | 0.0013 (\pm 0) |
| Eluate 2 from AEXC (1 M NaCl) | 0.96 (\pm 0.02) | 0.65 (\pm 0.01) |
| Eluate 2 from CEXC (1 M NaCl) | 0.97 (\pm 0.02) | 0.67 (\pm 0.01) |

^aControl 1: 0.14 ml of phosphate buffer 0.1 M pH 6.7 were added instead of the peptidic eluate

^bControl 2: 0.14 ml of NaCl 0.5 M were added instead of the peptidic eluate

^cControl 3: 0.14 ml of NaCl 1 M were added instead of the peptidic eluate

Table III.15. Protein concentration of the eluates obtained by AEXC and CEXC

| Protein concentration (mg/l) | AEXC | CEXC |
|------------------------------|----------------|----------------|
| Eluate 1 with 0.5 M NaCl | 251 (\pm 5) | 303 (\pm 6) |
| Eluate 2 with 1 M NaCl | 246 (\pm 5) | 269 (\pm 5) |

Control 1 in Table III.14 is the usual control used in all the *in vitro* experiments (refer to part C of this chapter and to paragraph II.2.4 of chapter II). It enables us to monitor the malolactic enzyme activity *in vitro*. Controls 2 and 3 were added to this experiment to evaluate the effect of NaCl on the malolactic enzyme activity. In fact, the elution of peptides in both AEXC and CEXC was performed using increasing concentrations of NaCl (0.5 M then 1 M of NaCl) in the elution buffers. We wanted to make sure that any inhibition observed in the presence of the protein eluates was only due to the peptides and not to NaCl. The results show that NaCl did not affect the malolactic enzyme activity since the 1 g/l of L-malic acid initially present in the control aliquots (controls 2 and 3) were totally consumed.

Eluates obtained after elution with 0.5 M NaCl from both AEXC and CEXC (eluates 1 of table III.14) strongly inhibited the malolactic enzyme activity since only 0.2 % of the malate present were consumed. However, those eluted at 1 M NaCl did not affect the enzyme activity and the

malate was entirely consumed. Therefore the peptides of interest were recuperated in eluates 1 of both AEXC and CEXC. The same procedure was performed with strain A and none of the eluates showed inhibition (data not shown). The inhibitory eluates of strain D (eluates 1 of table III.14) were then migrated on a Tricine SDS-PAGE gel and the bands of interest were recuperated for sequencing.

Table III.15 shows the protein concentration of the eluates. The inhibitory eluates (eluates 1 from both AEXC and CEXC) were initially present in a volume of 0.6 ml. Before analyzing them by Tricine SDS-PAGE, they were first precipitated with a TCA solution then suspended in 0.1 ml of a Triton X-100 buffer. Therefore, they were finally 6 times concentrated ($0.6/0.1 = 6$). The final concentration of the proteins loaded on the gel was:

Eluate 1 from AEXC: $251 \times 6 = 1506$ mg/l or 1.506 g/l

Eluate 1 from CEXC: $303 \times 6 = 1818$ mg/l or 1.818 g/l

This information was important for sequencing purposes.

Figure III.11 shows the results of the migration of the inhibitory eluates of strain D (eluates 1 from both AEXC and CEXC) on the Tricine SDS-PAGE.

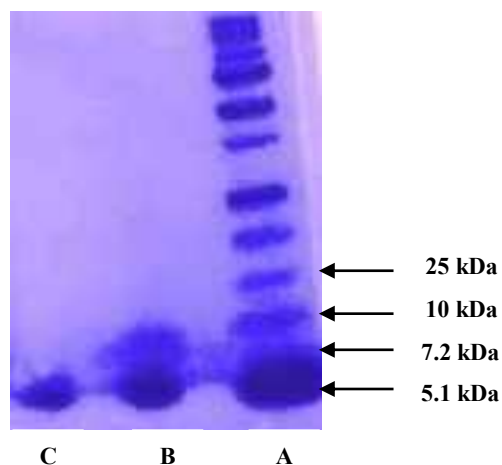


Figure III.11 Coomassie G-250 stained Tricine SDS-PAGE gel. Lane A contains dual-xtra MW standard 2-250 kDa (Bio-Rad). Lane B corresponds to eluate 1 from CEXC. Lane C corresponds to eluate 1 from AEXC

The cationic eluate presented two protein bands of approximately 5.1 and 7.2 kDa whereas the anionic eluate presented one protein band of 5.1 kDa. We finally decided to cut the three protein bands from both eluates in order to sequence them and identify the nature of the putative

antimicrobial peptides responsible for the MLF inhibition with *O. oeni* strain X. Tables III.16 and III.17 show the results of the peptides analyzed by LC1D-nanoESI-LTQ-Orbitrap. The identified peptides, their amino acid sequence, molecular weight and isoelectric point (pI) are presented.

Table III.16. Sequence analysis by LC1D-nanoESI-LTQ-Orbitrap of the peptides corresponding to the 5.1 and 7.2 kDa bands of the cationic eluate

| Cationic peptides/ proteins | | | | | | | |
|---|-------------------|--|----------------------------|------------------|-------|------|--------|
| Identified Proteins | Accession Number | Sequence of amino acids | Identification Probability | Actual Mass (Da) | Start | Stop | pI |
| Wtm2p [<i>S. cerevisiae</i> Lalvin QA23] | gi 323346419 | (D)DDDDNDDDDEEGN _x KTKSAAT(P) | 95% | 2,420.89 | 89 | 110 | 3.352 |
| Putative short-chain deshydrogenase/reductase [<i>S. cerevisiae</i> S288c] | gi 6322742 (+4) | (K)DmAVSYLSR(Y) | 100% | 1,056.49 | 118 | 126 | 6.499 |
| Actin [<i>S. cerevisiae</i> S288c] | gi 14318479 (+2) | (K)AGFAGDDAPR(A) | 100% | 975.4408 | 19 | 28 | 3.94 |
| Conserved protein [<i>S. cerevisiae</i> YJM789] | gi 151944993 (+1) | (S)SAASAGVSR(I) | 96% | 804.4088 | 15 | 23 | 10.467 |
| Glyceraldehyde-3-phosphate deshydrogenase [<i>S. cerevisiae</i> YJM789] | gi 151943468 (+4) | (K)KVVITAPSS(T) | 99% | 900.5276 | 116 | 124 | 9.074 |
| Glyceraldehyde-3-phosphate deshydrogenase [<i>S. cerevisiae</i> YJM789] | gi 151943468 (+4) | (R)TASGNIIPSSTGAAK(A) | 100% | 1,373.71 | 199 | 213 | 9.074 |
| YNL092W-like protein [<i>S. cerevisiae</i> FostersB] | gi 323303237 | (D)LD _x SKTcSLLT(Q) | 95% | 1,237.62 | 97 | 107 | 5.722 |
| K7_Ykr096wp [<i>S. cerevisiae</i> Kyokai no.7] | gi 349579655 | (N)DNNNNNDNNNNNNNSNSRD(N) | 97% | 2,404.88 | 227 | 247 | 3.498 |
| YDL025C-like protein [<i>S. cerevisiae</i> AWRI796] | gi 323334240 (+1) | (-)mEVVTNHTQR(Q) | 97% | 1,271.60 | 1 | 10 | 7.378 |
| Ltv1p [<i>S. cerevisiae</i> JAY291] | gi 256271082 (+2) | (K)NVEDLFIEPK(Y) | 97% | 1,202.61 | 114 | 123 | 3.962 |
| Petite colonies protein [<i>S. cerevisiae</i> YJM789] | gi 151944772 (+1) | (K)TNGAASLDPTKER(K) | 95% | 1,358.68 | 53 | 65 | 6.651 |

Table III.17. Sequence analysis by LC1D-nanoESI-LTQ-Orbitrap of the peptides corresponding to the 5.1 kDa band of the anionic eluate

| Anionic peptides/proteins | | | | | | | |
|---|-------------------|--|----------------------------|------------------|-------|------|-------|
| Identified Proteins | Accession Number | Sequence of amino acids | Identification Probability | Actual Mass (Da) | Start | Stop | pI |
| Wtm2p [<i>S. cerevisiae</i> Lalvin QA23] | gi 323346419 | (D)DDDDNDDDDEEGN _x KTKSAAT(P) | 98% | 2,420.89 | 89 | 110 | 3.352 |
| Utr2p [<i>S. cerevisiae</i> EC1118] | gi 259145860 (+3) | (F)CNATQACPEDKPCCSQYGEcGTG(Q) | 97% | 2,420.89 | 27 | 49 | 3.96 |

The cationic eluate revealed 11 different protein fragments corresponding to 10 different proteins of *S. cerevisiae* (Table III.16) whereas the anionic eluate revealed 2 protein fragments corresponding to 2 different yeast proteins (Table III.17). The identified proteins play diverse vital roles in *S. cerevisiae* cells. The short-chain deshydrogenase/reductase protein binds and stabilizes pre-tRNAs and RNA polymerase III transcripts (Schenk et al., 2012). Yeast actin is a structural protein involved in cell polarization, endocytosis, cytoskeleton functions and histone acetyltransferase activity (Greer and Schekman, 1982; Moseley and Good, 2006). YNL092W-like protein is a methyltransferase (Borneman et al., 2011; Szczepinska et al., 2014), K7_Ykr096wp is an RNase (Akao et al., 2011) and YDL025C-like protein is a major protein kinase involved in protein phosphorylation (Ptacek et al., 2005). Ltv1p is a protein complex that contains GTPases located in the late endosomal membrane that functions in nuclear export of the ribosomal small subunits (Gao et al., 2006; Seiser et al., 2006), petite colonies protein or Pet127p is a membrane-associated protein involved in stability and processing of *S. cerevisiae* mitochondrial RNAs (Wiesenberger and Fox, 1997) and Utr2p has a hydrolase activity, hydrolyzing O-glycosyl compounds (Novo et al., 2009).

No previous studies have described antimicrobial activities related to the previous proteins and/or their corresponding fragments, neither against bacteria nor against fungi.

Interestingly, the Wtm2p fragment was found in both eluates and was mainly recuperated in the anionic eluate due to its negative charge (*pI* 3.352 lower than the pH of both eluting buffers; phosphate buffer pH 6.7 and Tris buffer pH 8.3).

Wtm1p (WD repeat-containing transcriptional modulator) is a protein present in a large nuclear complex and presenting two homologs Wtm2p and Wtm3p, which probably arose by gene duplications. These proteins are transcriptional modulators with roles in meiotic regulation and silencing, RNR genes expression and are also involved in response to replication stress. They are relocalized in the cytosol in response to hypoxia (Pemberton and Blobel, 1997). The fragment of Wtm2p seems to play a main role in the inhibition since the corresponding anionic eluate strongly inhibited the malolactic enzyme activity *in vitro* as previously shown in Table III.14. There is no previous study on the antimicrobial role of the Wtm2p proteins. Giving its *pI* (3.352), the Wtm2p peptidic fragment is anionic at pH 6.7 which is the pH of the *in vitro* experiment. Therefore, it may deprive the malolactic enzyme from its Mn^{2+} cofactors by chelating them, thus inhibiting the malolactic enzyme activity and the malate consumption.

The sequencing also revealed two peptidic fractions of GAPDH in the cationic eluate, one having a MW of 0.9 kDa and the other of 1.373 kDa. They both presented the same *pI* (9.074) (Table III.16). Branco et al., (2014) previously demonstrated that GAPDH fragments were involved in the growth inhibition of *O. oeni* and some non-*Saccharomyces* yeast. However, the two GAPDH fragments identified by these authors were different and had a MW of 1.622 and 1.638 kDa and a *pI* of 4.37. They corresponded to two fragments of the C-terminal amino acid sequence of the GAPDH enzyme. Their antibacterial activity was only tested against the growth and not against the malate consumption.

In the present study, the cationic eluate strongly inhibited the malate consumption *in vitro* (Table III.14). Therefore, we suggest that the GAPDH fragments which are positively charged at pH 6.7 (pH of the enzymatic reaction), may have inhibited the malolactic enzyme activity by competing for the binding site of the NAD⁺ coenzyme. In fact, NAD⁺ is also positively charged and has a MW of 0.662 kDa which is close to the MW of the GAPDH peptides. By depriving the malolactic enzyme from NAD⁺, it will become dysfunctional.

Since there is no additional information about the possible antimicrobial role of the identified peptidic fractions, it is likely that one or more of them have worked synergistically to inhibit the malolactic enzyme activity. In real winemaking conditions, they are supposed to enter the bacterial cytoplasm by mechanisms yet to be identified in order to reach the malolactic enzyme. Therefore, the bacterial growth inhibition could be a consequence of the MLF inhibition which is one of the main energy sources during winemaking. Besides, it would be interesting to see if any of the peptides can directly inhibit the bacterial growth by targeting the membrane and/or the cell wall. Future work should also investigate their antimicrobial range of action, their biochemical and inhibitory properties and the possibility of using them as natural alternative biopreservatives in food products.

Chapter IV. Conclusions and Perspectives

The completion of malolactic fermentation in winemaking is not always successful. The major causes of this failure are the high ethanol content at the end of AF in addition to other yeast metabolites such as endogenous SO₂, medium chain fatty acids, and peptides/proteins. Besides, wine sulphitizing, the high acidity at the end of AF and the deficiency in some essential nutrients such as amino acids and vitamins contribute to the MLF inhibition. The failure of the MLF when it is desired leads to a depreciation of the quality of the end product and thus to a huge economic loss.

For a better understanding of the possible reasons of failure or success of MLF, it is crucial to study the microbial interactions that may occur between specific strains of *S. cerevisiae* and *O. oeni*. These interactions are strain dependent and are either mediated by extracellular yeast metabolites or by cell-to-cell contact mechanisms. Therefore, it is important to identify and quantify the yeast metabolites responsible for the inhibition or stimulation of *O. oeni* growth and its ability to consume L-malic acid.

The current study dealt with the MLF inhibition and the identification of the yeast inhibitory metabolites. More specifically, yeast metabolites of protein nature were investigated. The latter were studied by few authors who demonstrated that the peptides/proteins responsible for the inhibition of *O. oeni* were strain specific. They presented different apparent MW and their exact nature and MW were identified in only one study (Branco et al., 2014). Besides, the inhibition of the bacterial growth was usually the main parameter evaluated. Thus, the MLF inhibition was always considered as a consequence of the bacterial growth inhibition.

In the present work, two strains of *S. cerevisiae* (A and D) were tested with one strain of *O. oeni* (X) in sequential fermentations. One was found inhibitory and the second stimulatory of the MLF. The inhibitory yeast strain (strain D) was subjected to different experiments in order to prove the protein nature of the metabolites responsible for the MLF inhibition. Their apparent MW, the timing of their release during AF and the reproducibility of the results in synthetic and natural grape juices were evaluated. An attempt to further purify these metabolites was also undertaken.

The first part of the work was devoted to the study of the pure cultures of both yeast strains and the bacterial strain. Kinetic and biochemical parameters were evaluated. Then, sequential

fermentations were carried out by inoculating the bacteria in the SGJ media fermented by the yeasts. *S. cerevisiae* strain D strongly inhibited the growth of *O. oeni* strain X as well as its ability to consume L-malic acid whereas the yeast strain A stimulated them. The differences in the biochemical profiles of both yeast strains could not explain the opposite effects observed with the bacterial strain tested (ethanol, SO₂, medium chain fatty acids, nutrients). This result encouraged us to search for metabolites of protein nature. Strain A was considered as a reference strain all over the study.

Protease and heat treatments of the SGJ media fermented by strain D enabled us to demonstrate the protein nature of the yeast inhibitory metabolites. Fractionation by ultrafiltration of the same media showed that an extracellular peptidic fraction of 5-10 kDa was responsible for the inhibition. It was gradually released during AF and reached its highest concentration at late stages of the stationary phase. The MLF inhibition was maintained in natural grape juices presenting low and high phenolic contents. Therefore, the peptides were not inactivated by phenolic compounds, which is an interesting finding.

Fractionation by ammonium sulfate precipitation of the SGJ media fermented by strain D and desalting with membranes of 5 kDa, showed that the fraction recuperated at a saturation degree of 60- 80 % containing compounds ≥ 5 kDa was the only fraction that inhibited both the bacterial growth and the malate consumption. The fractions between 0 and 60 % inhibited the bacterial growth only and the 80-100 % fraction was not inhibitory. Therefore, we decided to retain the 5-10 kDa fraction of the 60-80 % precipitate for further purification steps.

The five fractions of 5-10 kDa recuperated each 24 h of the AF (120 h) as well as the 60-80 % precipitates were able to directly inhibit the malolactic enzyme activity in a well-designed *in vitro* experiment. The malolactic enzyme of the cell-free cytosolic extract of *O. oeni* strain X was put in direct contact with the inhibitory proteinaceous compounds. The kinetic of the inhibition was in agreement with the timing result. The results also showed that the malolactic enzyme was active at pH 3.5, 6 (optimum pH) and 6.7. The inhibitory peptides also exerted their inhibitory effect in the same range of pH.

Finally, the 5-10 kDa fraction of the 60-80 % precipitate was first analyzed by SDS-PAGE, then by ion exchange chromatography (IEXC) followed by SDS-PAGE and sequencing. The SDS-

PAGE showed 2 protein bands of 5.1 and 7.2 kDa only produced by the yeast strain D in comparison to the strain A. A common band of 8.4 kDa but more concentrated with strain D was also detected. Eluates of both anionic and cationic IEXC obtained after elution with 0.5 M NaCl were the only eluates that strongly inhibited the malolactic enzyme activity *in vitro*. The migration of both eluates by SDS-PAGE showed a common band of 5.1 kDa and a second band of 7.2 kDa specific to the cationic eluate. The 3 bands were excised and sequenced by LC1D-nanoESI-LTQ-Orbitrap. The results showed 12 different peptides originating from 12 different proteins of *S. cerevisiae*. The peptides may have worked synergistically. 2 GAPDH fragments of 0.9 and 1.373 kDa having a *pI* of 9.074 and a Wtm2p fragment of 2.42 kDa having a *pI* of 3.35 were involved in the MLF inhibition.

Ultimately, this work has resulted in the following advances:

- Demonstration of two types of yeast-bacteria interactions (inhibition/stimulation) by testing two strains of yeasts with one bacterial strain in sequential fermentations
- Quantification of the MLF inhibition and stimulation
- Demonstration of the involvement of a yeast peptidic fraction with an apparent MW between 5 and 10 kDa in the MLF inhibition. It was gradually released during AF and reached its highest concentration at late stages of the stationary phase. In a previous study, it was shown that the co-inoculation of the yeast strain D with the bacterial strain X improved the MLF output. Therefore, knowing the timing of the release helps decide the best moment for bacterial inoculation
- Maintenance of the MLF inhibition in natural winemaking conditions, thus suggesting that the peptides of the 5-10 kDa fraction did not interact with the phenolic compounds
- Determination of the mechanism of action of the 5-10 kDa inhibitory peptides. The latter were able to directly inhibit the malolactic enzyme activity *in vitro*. The inhibition increased with their concentration and was exerted in a pH range between 3.5 and 6.7
- *In vitro* inhibition of the malolactic enzyme activity by the 5-10 kDa peptidic fraction from the 60-80 % ammonium sulfate precipitate. Its migration by SDS-PAGE revealed 2 bands of 5.1 and 7.2 kDa produced only by *S. cerevisiae* strain D and not by strain A and a common band of 8.4

kDa more concentrated with strain D. At least, one of the three bands contained the inhibitory peptides

- The IEXC of the 5-10 kDa fraction from the 60-80 % precipitate revealed that anionic and cationic eluates obtained with 0.5 M NaCl were the only ones containing the inhibitory peptides. The cationic eluate revealed 2 bands of 5.1 and 7.2 kDa by SDS-PAGE whereas the anionic revealed one band of 5.1 kDa. The 3 bands were excised and sequenced by LC1D-nanoESI-LTQ-Orbitrap. The results showed 12 different peptides originating from 12 different proteins of *S. cerevisiae*. The peptides may have worked synergistically. 2 GAPDH fragments of 0.9 and 1.373 kDa having a pI of 9.074 and a Wtm2p fragment of 2.42 kDa having a pI of 3.35 were involved in the MLF inhibition.

It would be interesting for future studies to evaluate the antimicrobial spectrum of activity of the identified peptides against other *O. oeni* strains and against wine spoilage microorganisms such as *Dekkera/Brettanomyces* and other non-*Saccharomyces* as well as some lactic acid bacteria such as *Pediococcus* and *Lactobacillus*. It would be also interesting to see if they can inhibit or kill pathogenic bacteria such as *Salmonella* and *Listeria*. In that way, they could be used as biopreservatives in food products, thus reducing or removing the use of conventional chemical preservatives. In such case, it will be mandatory to perform toxicity testing in order to determine the doses allowed and to avoid harming the consumer's health. Also, it provides a screening tool that helps to determine the best choice for the use of the oenological yeast/LAB pairs in winemaking.

It would be interesting for future studies to evaluate the antimicrobial spectrum of activity of the identified peptides against other *O. oeni* strains and against wine spoilage microorganisms such as *Dekkera/Brettanomyces* and other non-*Saccharomyces* as well as some lactic acid bacteria such as *Pediococcus* and *Lactobacillus*. Testing different *O. oeni* strains provides a screening tool that will help determine the best choice of oenological *S. cerevisiae/O. oeni* pairs for winemaking. It would be also interesting to see if the peptides can inhibit or kill pathogenic bacteria such as *Salmonella* and *Listeria*. In that way, they could be used as biopreservatives in

food products, thus reducing or removing the use of conventional chemical preservatives. In such case, it will be mandatory to perform toxicity testing in order to determine the doses allowed and to avoid harming the consumer's health.

These findings will encourage future research to move to molecular arena, from proteomics to genomics to study the genes that encoded these peptides/proteins. This will permit to observe singularities (SNPs), in other terms, to search for modifications of bases (deletion, insertion) and also to search for the conservative domains with putative functions that may explain their effect. Moreover, it would be curious to completely purify and separate the peptides of the inhibitory protein mixture and to discover the mechanisms by which they enter inside the bacterial cytoplasm (use of receptors, translocation, endocytosis, etc...) and inhibit the malolactic enzyme activity. Besides, the determination of their spatial conformation and their binding sites will clarify their mechanism of action regarding the malolactic enzyme and eventually other cell components. It is possible that some of them may target the cell membrane or disrupt the DNA and/or protein synthesis.

It would be also interesting to study the mechanisms of resistance against these peptides used by *O. oeni* strains that were not affected by *S. cerevisiae* strain D.

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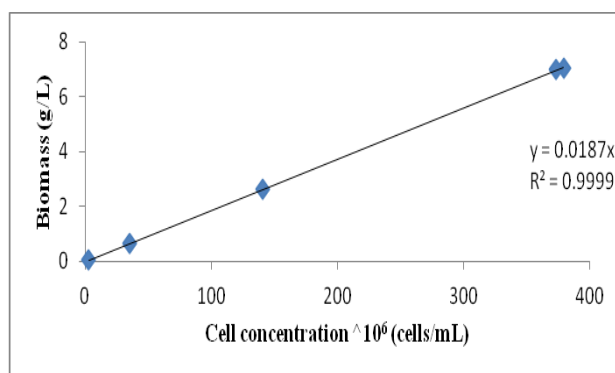
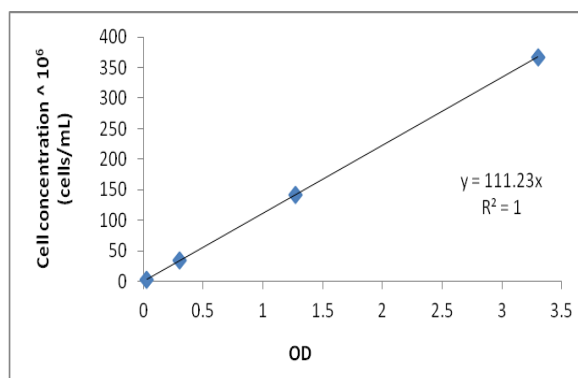
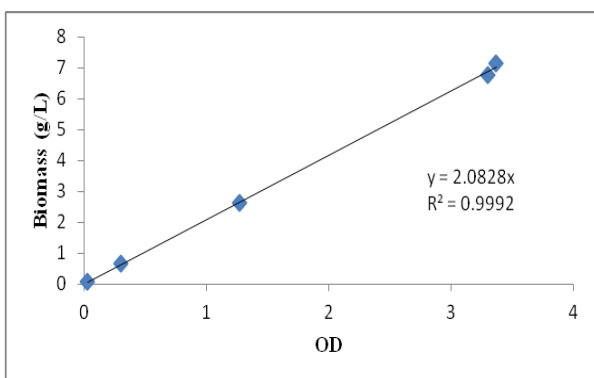
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Appendices

Appendix of part A: Alcoholic and malolactic fermentation Appendix

A-I. Correlation curves between the biomass and the OD, the cell concentration and the OD and the biomass and the cell concentration of *S. cerevisiae* strain D



Appendix A-II. Pure culture of *S. cerevisiae* strain D in SGJ media containing yeast nitrogen base without amino acids and peptides

Table 1. Kinetic parameters of *S. cerevisiae* strain D during AF in SGJ media containing yeast nitrogen base without amino acids and peptides

| Time (h) | Cell concentration $\times 10^6$ (cells/ml) | Viability (%) | OD | Biomass (g/l) | Sugar (g/l) |
|----------|--|------------------|-------|----------------------|-----------------------|
| 0 | 2.95 (± 0.06) | - | 0.068 | 0.05 (± 0) | 202 (± 1.41) |
| 8 | 18.25 (± 0.91) | 94.3 | 0.073 | 0.31 (± 0.016) | 190 (± 0) |
| 24 | 38.45 (± 0.68) | 91.7 | 0.079 | 1.88 (± 0.016) | 175.6 (± 0.56) |
| 32 | 52.27 (± 0.89) | 92 | 0.086 | 2.2 (± 0) | 161 (± 2.05) |
| 48 | 100 (± 1.62) | 88.23 | 0.184 | 2.88 (± 0.037) | 153 (± 0.47) |
| 56 | 120 (± 3.26) | 85.11 | 0.23 | 3.18 (± 0.032) | 133 (± 0.47) |
| 72 | 65 (± 1.47) | 80 | 0.095 | 2.4 (± 0.014) | 123.4 (± 2.43) |
| 80 | 62.7 (± 0.47) | 76 | 0.093 | 2.2 (± 0.094) | 109.09 (± 1.74) |
| 96 | 60.54 (± 1.42) | 75.12 | 0.093 | 2.08 (± 0.012) | 101.73 (± 0.82) |
| 104 | 57.15 (± 0.78) | 72 | 0.087 | 2.01 (± 0.057) | 81.5 (± 0.7) |

Table 2. Biochemical parameters of *S. cerevisiae* strain D during AF in SGJ media containing yeast nitrogen base without amino acids and peptides

| Biochemical parameters | Values |
|-----------------------------|-----------------------|
| Initial sugar (g/l) | 202 (± 1.4) |
| Final sugar (g/l) | 81.5 (± 0.7) |
| Initial L-malic acid (g/l) | 3.2 (± 0.26) |
| Final L-malic acid (g/l) | 2 (± 0.13) |
| Consumed L-malic acid (g/l) | 1.2 |
| Initial pH/Final pH | 3.5/3.35 |
| Ethanol (g/l) | 33.65 (± 0.007) |
| Acetate (g/l) | 0.097 (± 0.009) |
| Consumed citrate (g/l) | 0.0023 |

Appendix A-III. Pure culture of *S. cerevisiae* strain D in SGJ media containing yeast nitrogen base with amino acids but without peptides

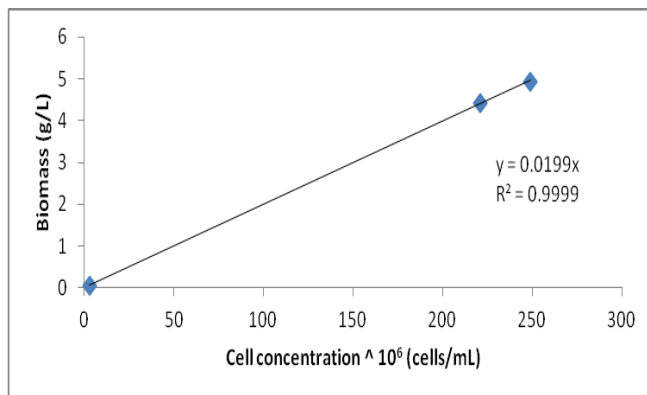
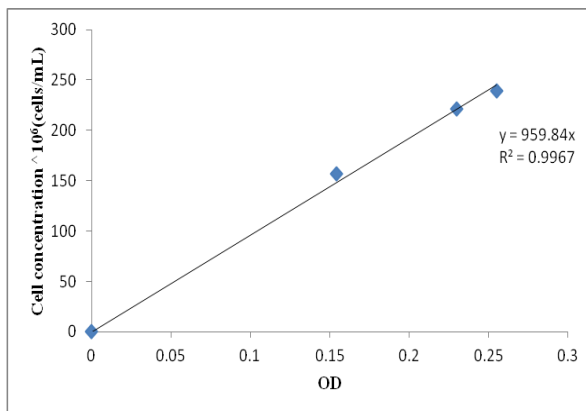
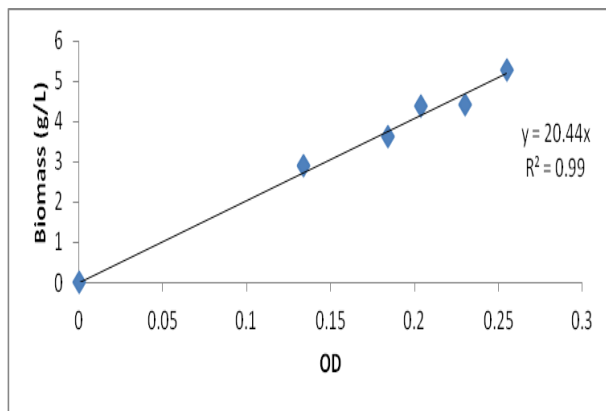
Table 1. Kinetic parameters of *S. cerevisiae* strain D during AF in SGJ media containing yeast nitrogen base with amino acids but without peptides

| Time (h) | Cell concentration $\times 10^6$ (cells/ml) | Viability (%) | OD | Biomass (g/l) | Sugar (g/l) |
|----------|--|------------------|-------|---------------|-------------|
| 0 | 3.01 (± 0.008) | - | 0.027 | 0.012 | 200 |
| 24 | 33.35 (± 0.91) | 95.3 | 0.3 | 0.4 | 167 |
| 48 | 123.69 (± 1.68) | 93.7 | 0.6 | 2.4 | 134 |
| 72 | 133 (± 2.16) | 90 | 0.67 | 3.6 | 108 |
| 96 | 153.7 (± 1.19) | 86.3 | 0.74 | 4.8 | 89 |
| 120 | 220 (± 1.41) | 80.6 | 0.98 | 5.4 | 69 |
| 144 | 153.4 (± 21.51) | 78 | 0.74 | 4.8 | 62 |
| 168 | 93.4 (± 1.65) | 76 | 0.61 | 2.2 | 60 |
| 192 | 86 (± 1.4) | 75.12 | 0.58 | 2.1 | 58.75 |
| 216 | 78.4 (± 2.8) | 72 | 0.53 | 2 | 57.9 |

Table 2. Biochemical parameters of *S. cerevisiae* strain D during AF in SGJ media containing yeast nitrogen base with amino acids but without peptides

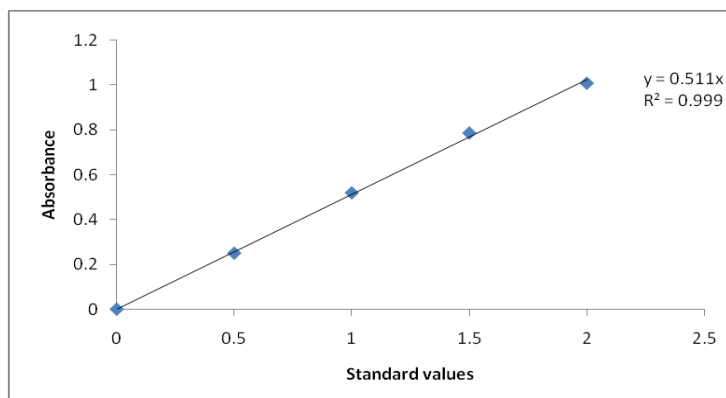
| Biochemical parameters | Values |
|-----------------------------|-----------------------|
| Initial sugar (g/l) | 200 |
| Final sugar (g/l) | 57.9 |
| Initial L-malic acid (g/l) | 3.04 |
| Final L-malic acid (g/l) | 1.88 |
| Consumed L-malic acid (g/l) | 1.16 |
| Initial pH/Final pH | 3.5/3.32 |
| Ethanol (g/l) | 36.35 (± 0.007) |
| Acetate (g/l) | 0.091 (± 0.009) |
| Consumed citrate (g/l) | 0.0027 |

Appendix A-IV. Correlation curves between the biomass and the OD, the cell concentration and the OD and the biomass and the cell concentration of *S. cerevisiae* strain A

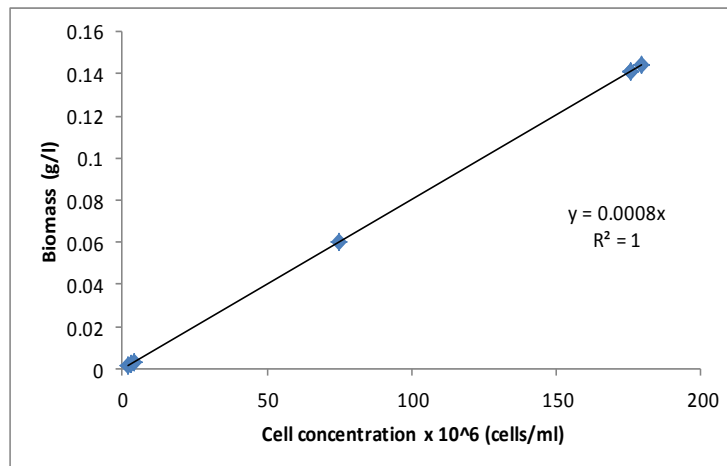
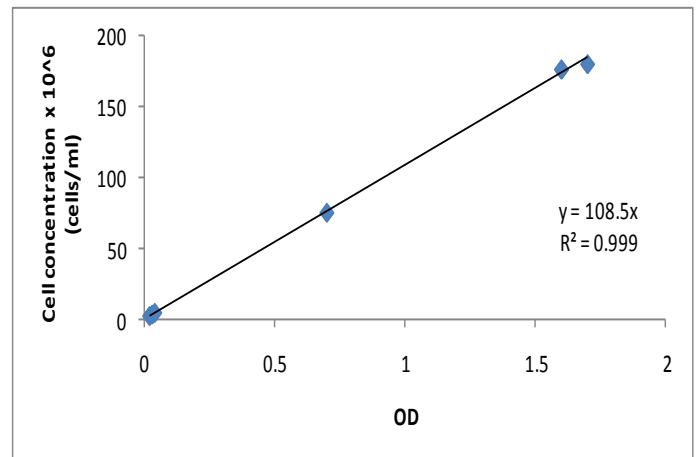
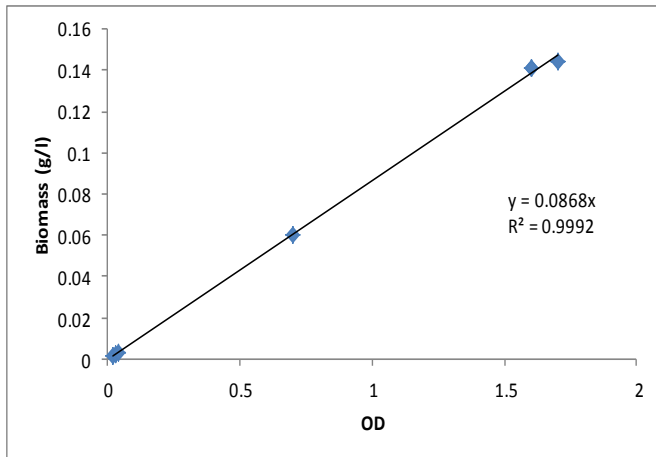


Appendix A-V. Standard curve for the sugar analysis by DNS**Table 1. Standard values (sugar concentrations g/l) and corresponding absorbance values**

| Standard values (g/l) | Absorbance (560 nm) |
|-----------------------|---------------------|
| 0 | 0 |
| 0.5 | 0.25 |
| 1 | 0.519 |
| 1.5 | 0.786 |
| 2 | 1 |

**Figure 1. Standard curve for the sugar analysis by DNS**

Appendix A-VI. Correlation curves between the biomass and the OD, the cell concentration and the OD and the biomass and the cell concentration of *O. oeni* strain X



Appendix A-VII. Sequential fermentation with the pair *S. cerevisiae* strain A/*O. oeni* strain X

Table 1. Kinetic parameters of *O. oeni* strain X during the MLF in the SGJ media fermented by *S. cerevisiae* strain A

| Time ±3 (h) | Cell concentration ^10⁶ (cells/ml) | Biomass (g/l) | L-malic acid (g/l) |
|------------------------|--|--------------------------|-------------------------------|
| 0 | 1.96 (±0.25) | 0.0016 (±0.0002) | 3.22 (±0.06) |
| 72 | 24 (±2.5) | 0.02 (±0.002) | 2.95 (±0.06) |
| 144 | 36.26 (±3.75) | 0.03 (±0.003) | 2.5 (±0.05) |
| 216 | 51(±5) | 0.041 (±0.004) | 2.12 (±0.04) |
| 288 | 63 (±6.25) | 0.05 (±0.005) | 1.6 (±0.03) |
| 360 | 95 (±5) | 0.076 (±0.004) | 0.83 (±0.02) |
| 432 | 131 (±6.25) | 0.105 (±0.005) | 0.15 (±0.003) |
| 504 | 150 (±6) | 0.12 (±0.005) | 0 (±0) |
| 576 | 196 (±7.5) | 0.12 (±0.006) | - |
| 648 | 170 (±6) | 0.12 (±0.005) | - |

Table 2. Kinetic parameters of *O. oeni* strain X during the MLF in the NGJ media fermented by *S. cerevisiae* strain A

| Time ±3 (h) | Cell concentration ^10⁶ (cells/ml) | Biomass (g/l) | L-malic acid (g/l) |
|------------------------|--|--------------------------|-------------------------------|
| 0 | 1.99 (±0.25) | 0.0016 (±0.0002) | 3.33 (±0.07) |
| 48 | 24 (±2.5) | 0.019 (±0.002) | 3.31 (±0.07) |
| 96 | 38.58 (±4) | 0.031 (±0.003) | 2.93 (±0.06) |
| 144 | 55.62 (±5) | 0.045 (±0.004) | 2.65 (±0.05) |
| 192 | 85.5 (±9) | 0.068 (±0.007) | 2.13 (±0.043) |
| 240 | 132.5 (±6) | 0.11 (±0.005) | 1.63 (±0.033) |
| 288 | 152 (±7.5) | 0.12 (±0.006) | 0.92 (±0.02) |
| 336 | 119.5 (±8) | 0.12 (±0.006) | 0.31 (±0.006) |
| 370 | 120 (±8) | 0.12 (±0.006) | 0 (±0) |

Appendix A-VIII. Sequential fermentation with the pair *S. cerevisiae* strain D/*O. oeni* strain X

Table 1. Kinetic parameters of *O. oeni* strain X during the MLF in the SGJ media fermented by *S. cerevisiae* strain D

| Time ±3 (h) | Cell concentration x 10 ⁶ (cells/ml) | Biomass (g/l) | L-malic acid (g/l) |
|----------------|--|------------------|-----------------------|
| 0 | 2 (±0.25) | 0.0016 (±0.0002) | 3 (±0.06) |
| 48 | 6.5 (±0.75) | 0.0052 (±0.0006) | 3 (±0.06) |
| 96 | 7.13 (±1.1) | 0.0057 (±0.0009) | 3 (±0.06) |
| 168 | 7 (±0.8) | 0.0057 (±0.0006) | 3 (±0.06) |
| 240 | 7 (±0.8) | 0.0056 (±0.0006) | 3 (±0.06) |
| 336 | 6 (±0.6) | 0.0049 (±0.0005) | 3 (±0.06) |
| 408 | 5.5 (±0.5) | 0.0044 (±0.0004) | 3 (±0.06) |
| 504 | 6.25 (±0.8) | 0.005 (±0.0006) | 3 (±0.06) |
| 576 | 6 (±0.8) | 0.005 (±0.0006) | 3 (±0.06) |
| 672 | 6 (±0.8) | 0.005 (±0.0006) | 3 (±0.06) |
| 744 | 6 (±0.8) | 0.005 (±0.0006) | 3 (±0.06) |
| 840 | 6 (±0.8) | 0.005 (±0.0006) | 3 (±0.06) |
| 912 | 6 (±0.8) | 0.005 (±0.0006) | 3 (±0.06) |
| 1000 | 6 (±0.8) | 0.005 (±0.0006) | 3 (±0.06) |

Table 2. Kinetic parameters of *O. oeni* strain X during the MLF in the NGJ media fermented by *S. cerevisiae* strain D

| Time (h) | Cell concentration x 10 ⁶ (cells/ml) | Biomass (g/l) | L-malic acid (g/l) |
|-------------|--|------------------|-----------------------|
| 0 | 2.04 (±0.25) | 0.0016 (±0.0002) | 3.15 (±0.063) |
| 48 | 3.16 (±0.4) | 0.0025 (±0.0003) | 3.15 (±0.063) |
| 96 | 6.23 (±0.6) | 0.005 (±0.0005) | 3.14 (±0.063) |
| 144 | 6.36 (±0.6) | 0.005 (±0.0005) | 3.14 (±0.063) |
| 192 | 7.29 (±0.8) | 0.0063 (±0.0006) | 3.13 (±0.063) |
| 240 | 7.91 (±0.8) | 0.0063 (±0.0006) | 3.09 (±0.062) |
| 288 | 4.24 (±0.4) | 0.0034 (±0.0003) | 3.08 (±0.062) |
| 336 | 3.37 (±0.4) | 0.003 (±0.0003) | 3.08 (±0.062) |
| 384 | 5.06 (±0.5) | 0.004 (±0.0004) | 3.07 (±0.062) |
| 432 | 5 (±0.5) | 0.004 (±0.0004) | 3.07 (±0.062) |

Appendix A-IX. Sequential fermentations in the natural red grape juices

Table 1. Kinetic parameters of the AF performed by *S. cerevisiae* strains A, D and R in the natural red grape juices

| Time ± 3 (h) | <i>S. cerevisiae</i> strain D | | <i>S. cerevisiae</i> strain A | | <i>S. cerevisiae</i> strain R | |
|------------------|-------------------------------|---|-------------------------------|---|-------------------------------|---|
| | Sugar (g/l) | Cell concentration $\times 10^6$ (cells/ml) | Sugar (g/l) | Cell concentration $\times 10^6$ (cells/ml) | Sugar (g/l) | Cell concentration $\times 10^6$ (cells/ml) |
| 0 | 219 (± 4.32) | 3.09 (± 0.05) | 212 (± 1.7) | 2.98 (± 0.02) | 212.8 (± 1.76) | 2.98 (± 0.005) |
| 24 | 182.77 (± 4.83) | 46.87 (± 1.33) | 188 (± 1.88) | 59.91 (± 5.02) | 184.3 (± 0.78) | 41.50 (± 1.87) |
| 48 | 141.67 (± 2.49) | 162.74 (± 3.29) | 158 (± 2.87) | 228.2 (± 3.53) | 144.6 (± 2.05) | 157.47 (± 1.2) |
| 72 | 94.43 (± 2.06) | 286.33 (± 2.49) | 105 (± 6.15) | 328 (± 12.83) | 90.9 (± 3.58) | 216.5 (± 3.51) |
| 96 | 43.35 (± 1.39) | 341.04 (± 0.82) | 67 (± 0.83) | 256.67 (± 3.77) | 60.4 (± 1.64) | 286.37 (± 2.44) |
| 120 | 9.27 (± 0.68) | 324.57 (± 4.16) | 21 (± 1.55) | 210 (± 6.16) | 25.1 (± 3.24) | 312.38 (± 0.54) |
| 144 | 1.43 (± 0.06) | 305 (± 2.94) | 5 (± 0.67) | 176.76 (± 6.6) | 6.2 (± 0.87) | 293.37 (± 2.1) |
| 168 | 0.01 (± 0.001) | 302 (± 0.001) | 1 (± 0.28) | 152.67 (± 2.36) | 1.3 (± 0.53) | 288.11 (± 2.3) |

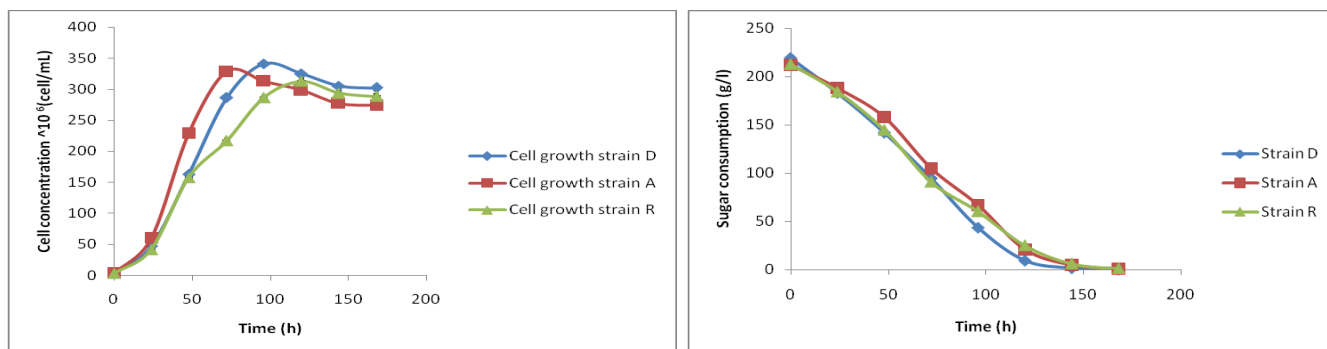


Figure 1. Growth and sugar consumption during AF with the yeast strains A, D and R in the natural red grape juices

Table 2. Kinetic parameters of the MLF performed by *O. oeni* strain X in the natural red grape juices fermented by *S. cerevisiae* strains A, D and R

| Time ± 3 (h) | NGJ fermented by the strain D | | NGJ fermented by the strain A | | NGJ fermented by the strain R | |
|------------------|-------------------------------|---|-------------------------------|---|-------------------------------|---|
| | Malate (g/l) | Bacterial cell concentration $\times 10^6$ (cells/ml) | Malate (g/l) | Bacterial cell concentration $\times 10^6$ (cells/ml) | Malate (g/l) | Bacterial cell concentration $\times 10^6$ (cells/ml) |
| 0 | 3.15 (± 0.063) | 2.04 (± 0.25) | 3.33 (± 0.07) | 1.99 (± 0.25) | 3.28 (± 0.04) | 2.01 (± 0.02) |
| 48 | 3.15 (± 0.063) | 3.16 (± 0.4) | 3.31 (± 0.07) | 24 (± 2.5) | 3.2 (± 0.0012) | 36 (± 0.22) |
| 96 | 3.14 (± 0.063) | 6.23 (± 0.6) | 2.93 (± 0.06) | 38.58 (± 4) | 2.4 (± 0.091) | 53.21 (± 0.11) |
| 144 | 3.14 (± 0.063) | 6.36 (± 0.6) | 2.65 (± 0.05) | 55.62 (± 5) | 1.42 (± 0.046) | 87.14 (± 0.12) |
| 192 | 3.13 (± 0.063) | 7.29 (± 0.8) | 2.13 (± 0.043) | 85.5 (± 9) | 1.05 (± 0.041) | 126.33 (± 0.06) |
| 240 | 3.09 (± 0.062) | 7.91 (± 0.8) | 1.63 (± 0.033) | 132.5 (± 6) | 0.76 (± 0.042) | 141 (± 0.02) |
| 288 | 3.08 (± 0.062) | 4.24 (± 0.4) | 0.92 (± 0.02) | 152 (± 7.5) | 0.09 (± 0.017) | 134 (± 0.05) |
| 336 | 3.08 (± 0.062) | 3.37 (± 0.4) | 0.31 (± 0.006) | 119.5 (± 8) | - | - |
| 370 | 3.07 (± 0.062) | 5.06 (± 0.5) | 0 (± 0) | 120 (± 8) | - | - |
| 432 | 3.07 (± 0.062) | 5 (± 0.5) | - | - | - | - |
| 472 | 3.1 (± 0.062) | | | | | |
| 840 | 3.1 (± 0.062) | | | | | |
| 1000 | 3.1 (± 0.062) | | | | | |

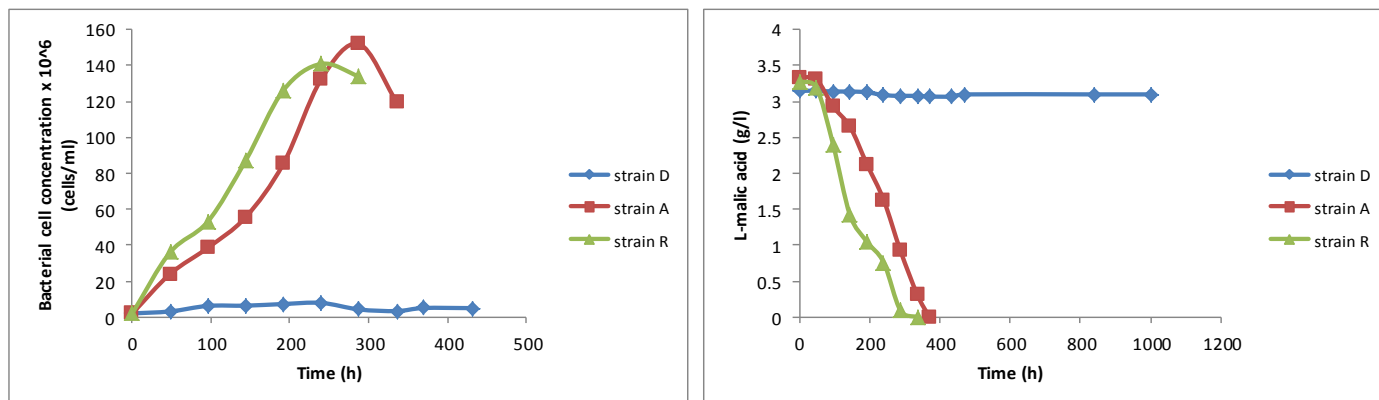


Figure 2. Bacterial growth and malate consumption during MLF carried out by *O. oeni* strain X in the natural red grape juices fermented by the yeast strains A, D and R

Appendix of part B: Production of anti-MLF peptides by *S. cerevisiae* strain D

Appendix B-I. Protease and heat treatments of the SGJ media fermented by *S. cerevisiae* strain D

Table 1. Evolution of the bacterial biomass of *O. oeni* strain X in the SGJ media fermented by strain D and subjected to different heat and protease treatments

| Time (h) | Heat treated media | Papain treated media | Pepsin treated media | Trypsin treated media |
|-------------|------------------------|------------------------|------------------------|------------------------|
| 0 | 0.0016 (\pm 0.0002) | 0.0016 (\pm 0.0002) | 0.0016 (\pm 0.0002) | 0.0016 (\pm 0.0002) |
| 48 | 0.003 (\pm 0.0003) | 0.0024 (\pm 0.0002) | 0.0029 (\pm 0.0003) | 0.002 (\pm 0.0002) |
| 96 | 0.0087 (\pm 0.0009) | 0.0034 (\pm 0.0003) | 0.008 (\pm 0.0008) | 0.0027 (\pm 0.0003) |
| 168 | 0.011 (\pm 0.001) | 0.054 (\pm 0.005) | 0.015 (\pm 0.002) | 0.004 (\pm 0.0004) |
| 240 | 0.024 (\pm 0.002) | 0.12 (\pm 0.01) | 0.021 (\pm 0.002) | 0.004 (\pm 0.0004) |
| 336 | 0.025 (\pm 0.003) | 0.12 (\pm 0.01) | 0.023 (\pm 0.002) | 0.005 (\pm 0.0005) |
| 408 | 0.028 (\pm 0.003) | 0.12 (\pm 0.01) | 0.024 (\pm 0.002) | 0.0078 (\pm 0.0008) |
| 504 | 0.029 (\pm 0.003) | 0.12 (\pm 0.01) | 0.025 (\pm 0.003) | 0.0087 (\pm 0.0009) |
| 576 | 0.029 (\pm 0.003) | | 0.03 (\pm 0.003) | 0.0086 (\pm 0.0009) |
| 672 | 0.03 (\pm 0.003) | | 0.031 (\pm 0.003) | 0.0086 (\pm 0.0009) |
| 744 | 0.029 (\pm 0.003) | | 0.028 (\pm 0.003) | 0.008 (\pm 0.0008) |
| 840 | 0.029 (\pm 0.003) | | 0.03 (\pm 0.003) | 0.0066 (\pm 0.0007) |
| 912 | 0.029 (\pm 0.003) | | 0.03 (\pm 0.003) | 0.0068 (\pm 0.0007) |
| 1000 | 0.029 (\pm 0.003) | | 0.03 (\pm 0.003) | 0.0063 (\pm 0.0006) |
| 1100 | | | | 0.007 (\pm 0.0007) |
| 1200 | | | | 0.0075 (\pm 0.0007) |
| 1300 | | | | 0.0068 (\pm 0.0007) |
| 1400 | | | | 0.0073 (\pm 0.0007) |
| 1500 | | | | 0.0065 (\pm 0.0007) |
| 1600 | | | | 0.007 (\pm 0.0007) |
| 1700 | | | | 0.0072 (\pm 0.0007) |

Table 2. Evolution of the malate consumption by *O. oeni* strain X in the SGJ media fermented by strain D and subjected to different heat and protease treatments

| Time (h) | Heat treated media | Papain treated media | Pepsin treated media | Trypsin treated media |
|----------------------|---------------------|----------------------|----------------------|-----------------------|
| 0 | 3 (\pm 0.06) | 3 (\pm 0.06) | 3 (\pm 0.06) | 3 (\pm 0.06) |
| 48 | 2.8 (\pm 0.06) | 2.73 (\pm 0.06) | 2.75 (\pm 0.06) | 2.9 (\pm 0.06) |
| 96 | 2.65 (\pm 0.05) | 2.4 (\pm 0.05) | 2.5 (\pm 0.05) | 2.7 (\pm 0.05) |
| 168 | 2.5 (\pm 0.05) | 2.1 (\pm 0.04) | 2.4 (\pm 0.05) | 2.4 (\pm 0.05) |
| 240 | 2.4 (\pm 0.05) | 1.52 (\pm 0.03) | 2.29 (\pm 0.05) | 2.3 (\pm 0.045) |
| 336 | 2.23 (\pm 0.045) | 0.87 (\pm 0.02) | 2.1 (\pm 0.04) | 2.3 (\pm 0.045) |
| 408 | 2.126 (\pm 0.04) | 0.48 (\pm 0.01) | 2.07 (\pm 0.04) | 2.23 (\pm 0.045) |
| 484 | 2.01 (\pm 0.04) | 0 (\pm 0) | 1.9 (\pm 0.04) | 2.2 (\pm 0.045) |
| 576 | 1.94 (\pm 0.04) | | 1.75 (\pm 0.035) | 2.15 (\pm 0.04) |
| 672 | 1.7 (\pm 0.04) | | 1.3 (\pm 0.03) | 2.12 (\pm 0.04) |
| 744 | 1.3 (\pm 0.03) | | 1 (\pm 0.02) | 1.8 (\pm 0.035) |
| 840 | 0.86 (\pm 0.02) | | 0.73 (\pm 0.02) | 1.63 (\pm 0.03) |
| 1180 (heat) | 0 (\pm 0) | | 0 (\pm 0) | 1.4 (\pm 0.025) |
| 1110 (pepsin) | | | | |
| 1000 | | | | 1.25 (\pm 0.025) |
| 1100 | | | | 1.05 (\pm 0.02) |
| 1200 | | | | 0.9 (\pm 0.02) |
| 1300 | | | | 0.77 (\pm 0.015) |
| 1400 | | | | 0.6 (\pm 0.01) |
| 1500 | | | | 0.43 (\pm 0.09) |
| 1600 | | | | 0.2 (\pm 0.004) |
| 1700 | | | | 0 (\pm 0) |

Appendix B-II. Fractionation of the SGJ media fermented by *S. cerevisiae* strain D by ultrafiltration

Table 1. Evolution of the biomass of *O. oeni* strain X and the malate consumption during MLF performed in the modified MRS media in the presence of the different MW fractions obtained by ultrafiltration as well as in the control

| Time (h) | Modified MRS control | | Fraction ≤ 3 kDa | | Fraction between 3 and 5 kDa | | Fraction between 5 and 10 kDa | | Fraction ≥ 10 kDa | |
|----------|----------------------------|------------------------|----------------------------|------------------------|------------------------------|------------------------|-------------------------------|------------------------|----------------------------|------------------------|
| | Biomass (g/l) | Malate (g/l) | Biomass (g/l) | Malate (g/l) | Biomass (g/l) | Malate (g/l) | Biomass (g/l) | Malate (g/l) | Biomass (g/l) | Malate (g/l) |
| 0 | 0.0016 (± 0.0002) | 3.1 (± 0.06) | 0.0016 (± 0.0002) | 3.1 (± 0.06) | 0.0016 (± 0.0002) | 3.1 (± 0.06) | 0.0016 (± 0.0002) | 3.1 (± 0.06) | 0.0016 (± 0.0002) | 3.1 (± 0.06) |
| 75 | 0.035 (± 0.004) | 2.64 (± 0.05) | 0.03 (± 0.003) | 2.71 (± 0.06) | 0.02 (± 0.002) | 2.78 (± 0.06) | 0.0023 (± 0.0002) | 2.95 (± 0.06) | 0.02 (± 0.002) | 2.71 (± 0.05) |
| 150 | 0.1 (± 0.01) | 1.81 (± 0.04) | 0.1 (± 0.01) | 1.93 (± 0.04) | 0.0904 (± 0.01) | 1.67 (± 0.02) | 0.0017 (± 0.0002) | 2.91 (± 0.06) | 0.1 (± 0.01) | 1.8 (± 0.04) |
| 225 | 0.11 (± 0.01) | 0.62 (± 0.01) | 0.1 (± 0.01) | 1.11 (± 0.02) | 0.1 (± 0.01) | 0.84 (± 0.02) | 0.0012 (± 0.0001) | 2.9 (± 0.06) | 0.1 (± 0.01) | 0.86 (± 0.01) |
| 275 | 0.11 (± 0.01) | 0 (± 0) | 0.1 (± 0.01) | 0.4 (± 0.01) | 0.1 (± 0.01) | 0.3 (± 0.01) | 0.0012 (± 0.0001) | 2.9 (± 0.06) | 0.1 (± 0.01) | 0.3 (± 0.01) |
| 315 | 0.11 (± 0.01) | | 0.1 (± 0.01) | 0 (± 0) | 0.1 (± 0.01) | 0 (± 0) | 0.0012 (± 0.0001) | 2.9 (± 0.06) | 0.1 (± 0.01) | 0 (± 0) |

Appendix B-III. Fractionation of the SGJ media fermented by *S. cerevisiae* strain A by ultrafiltration

Table 1. Evolution of the biomass of *O. oeni* strain X and the malate consumption during MLF performed in the modified MRS media in the presence of the 5-10 kDa fraction obtained by ultrafiltration as well as in the control

| Time (h) | Modified MRS control | | Fraction between 5 and 10 kDa | |
|----------|----------------------|------------------|-------------------------------|-----------------|
| | Biomass (g/l) | Malate (g/l) | Biomass (g/l) | Malate (g/l) |
| 0 | 0.0016 (± 0.0002) | 3 (± 0.06) | 0.0016 (± 0.0002) | 3 (± 0.06) |
| 75 | 0.035 (± 0.004) | 2.64 (± 0.05) | 0.03 (± 0.004) | 2.5 (± 0.06) |
| 150 | 0.1 (± 0.01) | 1.81 (± 0.04) | 0.009 (± 0.01) | 1.6 (± 0.04) |
| 225 | 0.11 (± 0.01) | 0.62 (± 0.01) | 0.12 (± 0.01) | 0.4 (± 0.01) |
| 275 | 0.11 (± 0.01) | 0 (± 0) | 0.12 (± 0.01) | 0 (± 0) |

Appendix B-IV. Timing of the release of the 5-10 kDa anti-MLF peptidic fraction produced by *S. cerevisiae* strain D during AF

Table 1. Evolution of the biomass of *O. oeni* strain X and the malate consumption in the modified MRS media containing the 5-10 kDa fractions collected each 24 h of the AF as well as in the control

| Time (h) | Modified MRS control | | 5-10 kDa fraction collected after 24h | | 5-10 kDa fraction collected after 48h | | 5-10 kDa fraction collected after 72h | | 5-10 kDa fraction collected after 96h | | 5-10 kDa fraction collected after 120h | |
|----------|----------------------|-----------------|---------------------------------------|------------------|---------------------------------------|-----------------|---------------------------------------|-----------------|---------------------------------------|-----------------|--|-----------------|
| | Biomass (g/l) | Malate (g/l) | Biomass (g/l) | Malate (g/l) | Biomass (g/l) | Malate (g/l) | Biomass (g/l) | Malate (g/l) | Biomass (g/l) | Malate (g/l) | Biomass (g/l) | Malate (g/l) |
| 0 | 0.0016 (±0.0002) | 3.25 (±0.07) | 0.0016 (±0.0002) | 3.01 (±0.06) | 0.0016 (±0.0002) | 3.03 (±0.06) | 0.0016 (±0.0002) | 3.02 (±0.06) | 0.0016 (±0.0002) | 3.04 (±0.06) | 0.0016 (±0.0002) | 3.1 (±0.06) |
| 72 | 0.031 (±0.002) | 1.1 (±0.02) | 0.021 (±0.001) | 1.54 (±0.03) | 0.011 (±0.002) | 2.32 (±0.05) | 0.015 (±0.001) | 2.6 (±0.05) | 0.009 (±0.001) | 2.9 (±0.06) | 0.011 (±0.003) | 2.92 (±0.06) |
| 144 | 0.07 (±0.003) | 0.4 (±0.01) | 0.048 (±0.002) | 0.57 (±0.01) | 0.04 (±0.004) | 1.5 (±0.03) | 0.046 (±0.003) | 1.6 (±0.03) | 0.031 (±0.003) | 2.7 (±0.05) | 0.03 (±0.005) | 2.9 (±0.06) |
| 216 | 0.08 (±0.005) | 0 (±0) | 0.072 (±0.005) | 0.03 (±0.001) | 0.073 (±0.005) | 0.5 (±0.01) | 0.072 (±0.003) | 0.5 (±0.01) | 0.026 (±0.002) | 2.7 (±0.05) | 0.024 (±0.005) | 2.9 (±0.06) |
| 288 | 0.08 (±0.005) | | 0.07 (±0.005) | | 0.073 (±0.005) | 0 (±0) | 0.072 (±0.001) | 0 (±0) | 0.011 (±0.0001) | 2.7 (±0.05) | 0.001 (±0.005) | 2.9 (±0.06) |

Appendix B-V. Fractionation of the SGJ media fermented by *S. cerevisiae* strain D by ammonium sulfate precipitation

Table 1. Growth of *O. oeni* strain X and malate consumption in the modified MRS control and in the presence of the proteinaceous fractions obtained by ammonium sulfate precipitation at different saturation degrees

| Time ± 3 (h) | Modified MRS control | | Saturation degree: 0-20 % | | Saturation degree: 20-40 % | | Saturation degree: 40-60 % | | Saturation degree: 60-80 % | | Saturation degree: 80-100 % | |
|--------------|----------------------|-------------------|---------------------------|-----------------|----------------------------|-----------------|----------------------------|------------------|----------------------------|-----------------|-----------------------------|-----------------|
| | Biomass (g/l) | Malate (g/l) | Biomass (g/l) | Malate (g/l) | Biomass (g/l) | Malate (g/l) | Biomass (g/l) | Malate (g/l) | Biomass (g/l) | Malate (g/l) | Biomass (g/l) | Malate (g/l) |
| 0 | 0.0017 (±0.0002) | 3.01 (±0.06) | 0.0015 (±0.0002) | 3.01 (±0.06) | 0.0015 (±0.0002) | 3.01 (±0.06) | 0.0016 (±0.0002) | 3.01 (±0.06) | 0.0016 (±0.0002) | 3.01 (±0.06) | 0.0017 (±0.0002) | 3.01 (±0.06) |
| 72 | 0.06 (±0.006) | 1.12 (±0.02) | 0.0025 (±0.0003) | 1.12 (±0.04) | 0.0028 (±0.0003) | 1.05 (±0.02) | 0.0032 (±0.0003) | 1.2 (±0.02) | 0.0024 (±0.0002) | 2.93 (±0.06) | 0.06 (±0.006) | 1.04 (±0.02) |
| 150 | 0.12 (±0.006) | 0.64 (±0.01) | 0.0042 (±0.0004) | 0.43 (±0.09) | 0.004 (±0.0004) | 0.41 (±0.08) | 0.0037 (±0.0004) | 0.77 (±0.02) | 0.0038 (±0.0004) | 2.81 (±0.06) | 0.11 (±0.006) | 0.47 (±0.01) |
| 225 | 0.13 (±0.007) | 0.013 (±0.003) | 0.0051 (±0.0005) | 0.03 (±0.01) | 0.006 (±0.0006) | 0.09 (±0.02) | 0.006 (±0.0006) | 0.029 (±0.01) | 0.006 (±0.0006) | 2.78 (±0.06) | 0.13 (±0.007) | 0.019 (±0) |

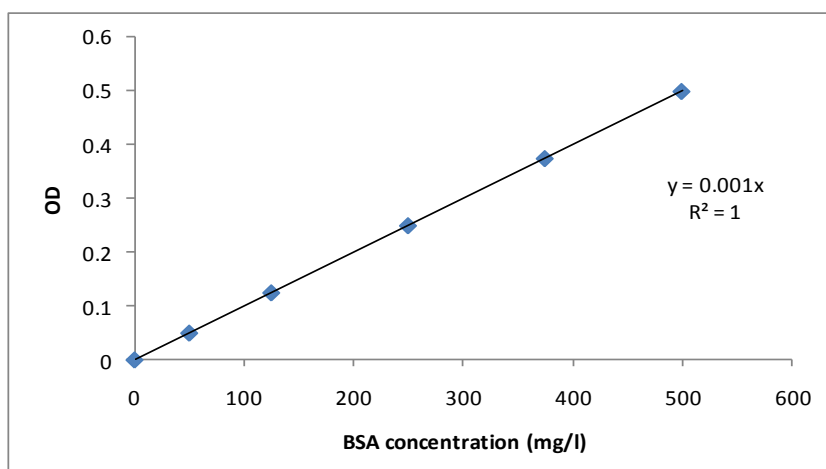


Figure 1. Standard curve used for the determination of protein concentrations by the Lowry method

Appendix B-VI. Screening of the antimicrobial activity of *S. cerevisiae* strain D during well plate tests

The antimicrobial activity of the four peptidic fractions obtained by ultrafiltration of the SGJ media fermented by *S. cerevisiae* strain D (≤ 3 kDa, 3-5 kDa, 5-10 kDa and ≥ 10 kDa) as well as the five fractions obtained by ammonium sulfate precipitation (A: 0-20 %; B: 20-40 %; C: 40-60 %; D: 60-80 % and E: 80-100 %) were tested against *O. oeni* strain X. All the fractions obtained by ultrafiltration were 75 times concentrated to the exception of the fraction ≤ 3 kDa. The fractions precipitated by ammonium sulfate were 2500 times concentrated. Different volumes of each peptidic fraction (10, 25, 50, 75, 100, 125, 150, 175 and 200 μ l) were introduced in the wells and tested. The MRS agar plates inoculated with *O. oeni* strain X were incubated at 30°C for 72 h before measuring the diameter of the clear zones around the wells. The following figure shows one example of results obtained. In the corresponding MRS agar plate, the fractions tested were: 5-10 kDa and ≥ 10 kDa from the SGJ media fermented by the strain D. The third well was used as a control and contained the 5-10 kDa fraction from the SGJ media fermented by the strain A (reference strain).

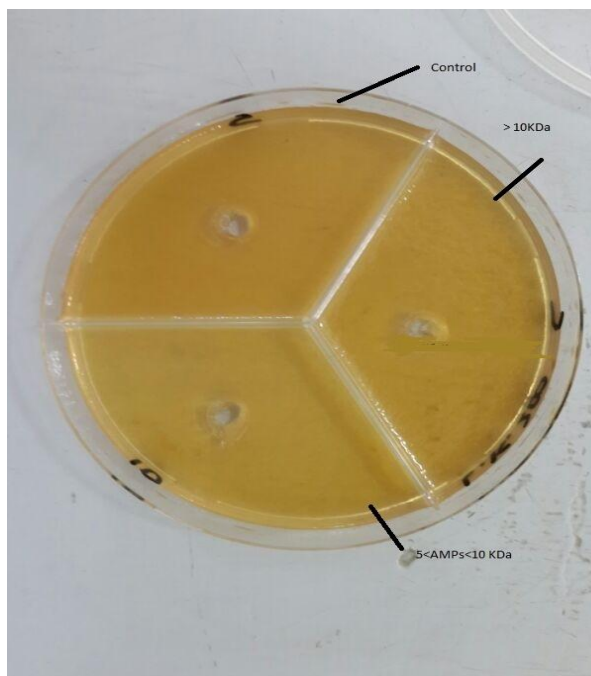


Figure 1. Screening of the antimicrobial activity of 2 peptidic fractions (5-10 kDa and ≥ 10 kDa) harvested from the SGJ media fermented by the yeast strain D against *O. oeni* strain X. Control: 5-10 kDa fraction from the SGJ media fermented by the strain A; AMP: antimicrobial peptides

The results show that the growth of *O. oeni* strain X was strongly inhibited only in the presence of the 5-10 kDa peptidic fraction isolated from the SGJ media fermented by *S. cerevisiae* strain D. 200 μ l of this fraction were required to show inhibition. A clear zone around the well was detected in this case. No death or loss in culturability was detected with the other fractions obtained by ultrafiltration. We can conclude that the inhibitory effect exerted on the bacterial growth was limited to the 5-10 kDa fraction. The fraction obtained by ammonium sulfate precipitation at a saturation degree of 60-80 % showed exactly the same clear zone as the one observed with the 5-10 kDa peptidic fraction. The same was observed with the precipitates 0-20 %, 20-40 % and 40-60 % to the exception of the 80-100 % precipitate (data not shown).

The same test was performed with proteinaceous fractions extracted from the SGJ media fermented by the yeast strain A. No inhibition was detected with any of the fractions tested and the bacteria continued to grow for 96 h before final opacity.

From the results above, we can conclude that the 5-10 kDa peptidic fraction and the precipitates obtained at a saturation degree between 0 and 80 % from the SGJ media fermented by *S. cerevisiae* strain D affected the bacterial growth and culturability of *O. oeni* strain X.

Appendix of part C: In vitro evaluation of the malolactic enzyme inhibition by the 5-10 kDa peptidic fractions and the 60-80 % precipitate

Table 1. Amount of L-malic acid consumed (g/l) and L-lactic acid produced (g/l) during the enzymatic reaction *in vitro* performed at pH 5.5 in the presence of the 5-10 kDa fractions (ABP) collected each 24 h of the AF

| Sampling time of the 5-10 kDa fractions during the AF | pH=5.5 t=30 min | |
|---|-----------------------------|------------------------------|
| | Consumed L-malic acid (g/l) | Produced L-lactic acid (g/l) |
| Control (absence of ABP) | 1.00 (±0.02) | 0.670 (±0.001) |
| After 24 h of AF | 0.87 (±0.02) | 0.40 (±0.01) |
| After 48 h of AF | 0.82 (±0.02) | 0.55 (±0.01) |
| After 72 h of AF | 0.60 (±0.01) | 0.40 (±0.01) |
| After 96 h of AF | 0.072 (±0.001) | 0.050 (±0.001) |
| After 120 h of AF | 0.080 (±0.002) | 0.050 (±0.001) |

Table 2. Amount of L-malic acid consumed (g/l) and L-lactic acid produced (g/l) during the enzymatic reaction *in vitro* performed at pH 5.5 in the presence of the proteinaceous fractions obtained by ammonium sulfate precipitation at the end of the AF (120 h)

| | pH = 5.5 t = 30 min | |
|---|-----------------------------|------------------------------|
| | Consumed L-malic acid (g/l) | Produced L-lactic acid (g/l) |
| Control (absence of ABP) | 1.00 (±0.02) | 0.670 (±0.001) |
| 60-80 % precipitate containing compounds \geq 5 kDa | 0.170 (±0.003) | 0.110 (±0.002) |
| 5-10 kDa fraction of the 60-80 % precipitate | 0.0090 (±0.0002) | 0.0060 (±0.0001) |
| 0-20 % precipitate containing compounds \geq 5 kDa | 1.00 (±0.02) | 0.670 (±0.001) |

Appendix of part D: Purification of the anti-MLF peptides of the 5-10 kDa peptidic fraction isolated from the 60-80 % ammonium sulfate precipitate**Purification of the anti-MLF peptides of the 5-10 kDa fraction from the 60-80 % precipitate by Ion Exchange Chromatography (IEXC)**

The Biological Low Pressure Chromatography (Bio-Rad) was firstly used for protein purification. Only one result was obtained before technical problems occurred and obliged us to use the Aurum mini kit (Bio-Rad). The columns used were Bio-Scale™ Mini cartridges of 5 ml; UNOsphere Q [-N⁺(CH₃)₃] as strong anion exchanger and UNOsphere S [-SO₃⁻] as strong cation exchanger (Bio-Rad). The flow rate was set to 1ml/min for the purification protocol.

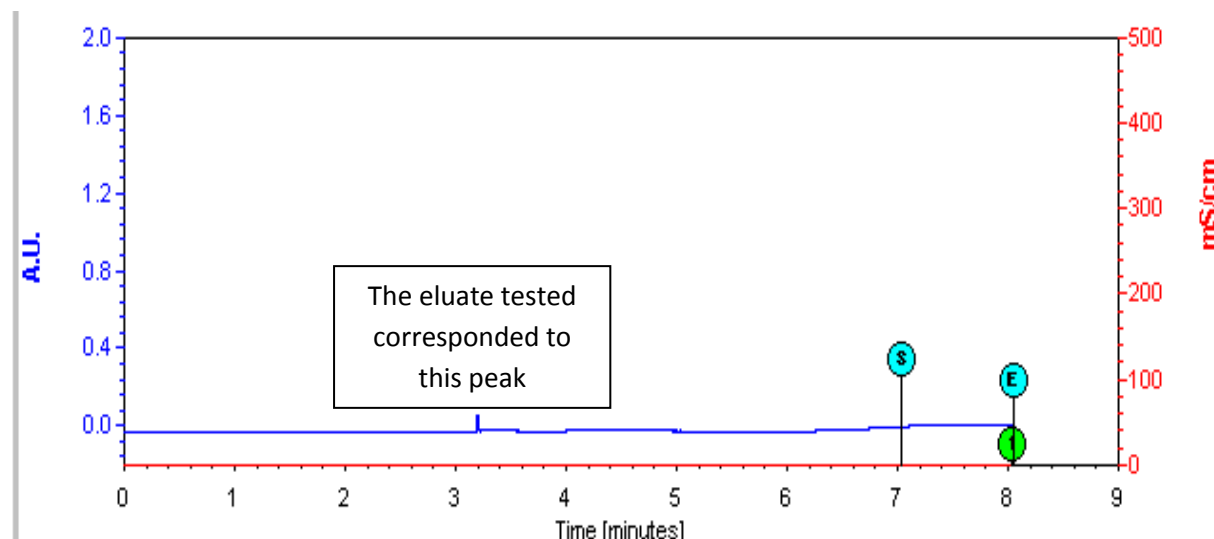
1 ml of the 5-10 kDa fraction from the 60-80 % ammonium sulfate precipitate was used for purification. First, 500 ml of the SGJ media fermented by *S. cerevisiae* strain D were used to recuperate the 60-80 % precipitate. The precipitate was then suspended in 10 ml of the corresponding binding buffer (50 X) and submitted to ultrafiltration in the 10 kDa unit. The filtrate lower than 10 kDa was ultrafiltered again using the 5 kDa unit. The desalted retentate between 5 and 10 kDa was recuperated (0.2 ml, 2500 X). 1 ml of this retentate was collected for each analysis.

Binding buffers: 1 M Tris buffer pH 8.3 was used for the anionic exchanger (AEXC). 10 mM phosphate buffer pH 6.7 was used for the cationic exchanger (CEXC).

Elution buffers: For both anionic and cationic exchange chromatography, increasing salt gradients were adopted for elution. The elution buffers were therefore composed of binding buffers coupled to increasing concentrations of NaCl (0.1, 0.5 and 1 M). The eluted samples were concentrated by the speed-vacuum-concentrator (each 1 ml of eluate gave a final volume of 0.5 ml) and used to test their inhibitory effect *in vitro* on the malolactic enzyme activity of the enzymatic extract (refer to paragraph II.2.4 of chapter II).

Results

(a)



(b)

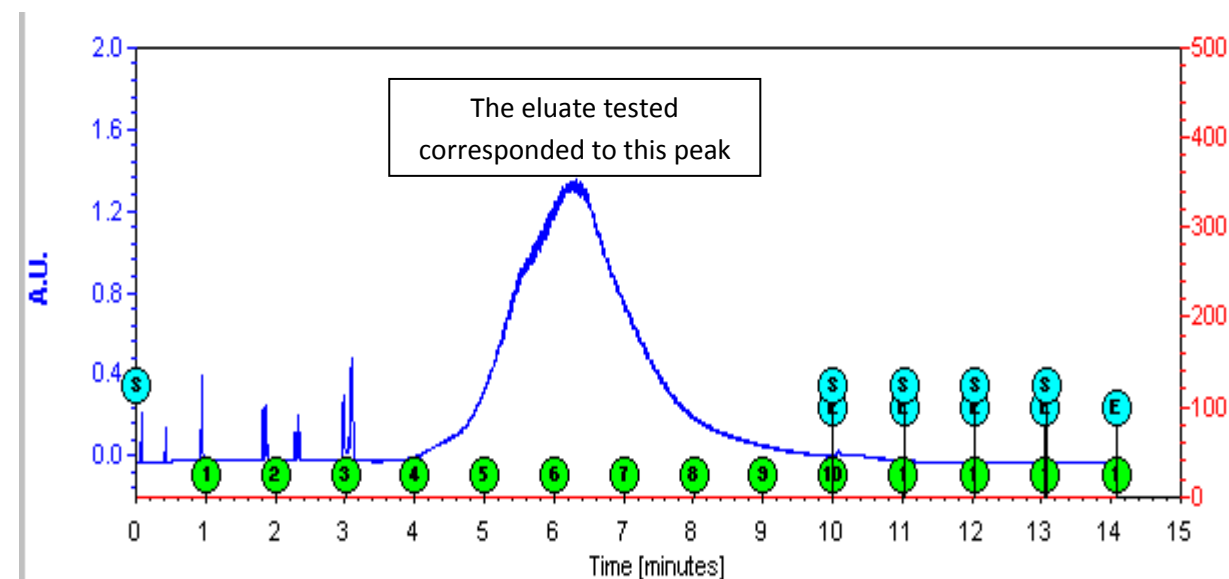


Figure 1. Chromatograms obtained during elution with 0.5 M NaCl of the 5-10 kDa peptidic fraction from the 60-80 % precipitate. (a) AEXC (b) CEXC

The eluates that correspond to the peaks shown on figure a.1 were tested *in vitro* on the enzymatic extract containing the malolactic enzyme as explained in paragraph II.2.4 of chapter

II. The enzymatic reaction was performed at 30 °C, at pH 6.7 for 40 min. The results are given in table a.1

Table 1. Consumed L-malic acid (g/l) and produced L-lactic acid (g/l) in the control and in the presence of the eluates obtained by AEXC and CEXC

| | Protein concentration of the eluates (mg/l) | Malate consumed (g/l) | Lactate produced (g/l) |
|----------------------|---|-----------------------|------------------------|
| ^a Control | - | 0.98 (± 0.02) | 0.66 (± 0.01) |
| Eluate from AEXC | 612 (± 10) | 0.33 (± 0.01) | 0.220 (± 0.005) |
| Eluate from CEXC | 511 (± 10) | 0.32 (± 0.01) | 0.21 (± 0.005) |

^a Control: 0.14 ml of phosphate buffer 0.1 M pH 6.7 were added instead of the peptidic eluate

Discussion

The eluates that correspond to the peaks shown on figure a.1 and obtained after elution with 0.5 M NaCl were tested *in vitro* on the enzymatic extract comprising the malolactic enzyme. Results of table a.1 show that with both eluates obtained from AEXC and CEXC, the malate consumption was reduced by 66 % in comparison to the control. Thus, both eluates contained inhibitory peptides that directly inhibited the malolactic enzyme activity. The *pI* of these peptides is most probably between 6.7 and 8.3. The results were very encouraging but technical problems obliged us to further characterize and purify the inhibitory peptides by using the Aurum mini kit for IEXC (Bio-Rad).

Publications and Scientific Contributions

List of international conferences and publications

1-Macrowine: Macromolecules and secondary metabolites of grapevine and wine. Stellenbosch, South Africa, 7-10 September 2014. Oral presentation

Nancy Nehme, **Ziad Rizk**, Youssef El Rayess, Florence Mathieu, Patricia Taillandier

Timing of the release of yeast-derived peptides involved in malolactic fermentation inhibition and impact of the inoculation strategy on the output of malolactic fermentation

2- Oeno 2015, 10th International Symposium of Oenology, June 29- 1st of July, 2015. Bordeaux-France. Oral presentation with proceedings

Ziad Rizk, Youssef El Rayess, Chantal Ghanem, Florence Mathieu, Patricia Taillandier, Nancy Nehme
Involvement of yeast-derived peptides in the inhibition of malolactic fermentation and impact of these peptides on the malolactic enzyme activity

3- Oeno 2015, 10th International Symposium of Oenology, June 29- 1st of July, 2015. Bordeaux-France. Poster with proceedings

Chantal Ghanem, Patricia Taillandier, Lara Hanna-Wakim, **Ziad Rizk**, Nancy Nehme, Jean-Pierre Souchard, Youssef El Rayess

Impact of maceration time and temperature on the extraction and evolution of polyphenols in Lebanese musts

4- **Ziad Rizk**, Youssef El Rayess, Chantal Ghanem, Florence Mathieu, Patricia Taillandier, Nancy Nehme (2016) Role of peptides released by *Saccharomyces cerevisiae* BDX in the inhibition of the malolactic enzyme extracted from *Oenococcus oeni* Vitilactic F. Submitted to International Journal of Food Microbiology (*In process*).

5- Youssef El Rayess, **Ziad Rizk**, Chantal Ghanem, Nancy Nehme (2016) Clarification and stabilization of wines using membrane processes. In: Jordao Antonio, Cosme Fernanda (Eds.) Recent advances in wine stabilization and conservation technologies. Nova Science Publishers Inc., New-York. Expected date of publication : first trimester of 2016.

