STUDY OF THE MICROBIAL BIODIVERSITY OF IRPINIAN GRAPEVINES FOR OPTIMIZING THE PRODUCTION OF TYPICAL WINES (AGLIANICO AND FIANO)

Fabio Santomauro

Dottorato in Scienze Biotecnologiche – XXII ciclo
Indirizzo Biotecnologie Industriali
Università di Napoli Federico II

Dottorato in Scienze Biotecnologiche – XXII ciclo
Indirizzo Biotecnologie Industriali
Università di Napoli Federico II

STUDY OF THE MICROBIAL BIODIVERSITY OF IRPINIAN GRAPEVINES FOR OPTIMIZING THE PRODUCTION OF TYPICAL WINES (AGLIANICO AND FIANO)

Fabio Santomauro

Dottorando: Fabio Santomauro

Relatore: Prof. Mosè Rossi

Tutor: Dott.ssa Maria Grazia Volpe

Cotutor: Dott. Francesco La Cara

Coordinatore: Prof. Ettore Benedetti

A Giulia.

L'essenziale è invisibile agli occhi.

INDEX

SUMMARY.....pag.10
RIASSUNTO.....pag.13

INTRODUCTION.....pag.20

1-What wine is:chemical composition and production.....pag.21
2- The vine and wine: yeasterday and today.....pag.23
2.1- Hystory.....pag.23
2.2- Legislation.....pag.25
2.3- Wine in the moder market.....pag.26
3- Biotechnology in the wine industry.....pag.27
3.1- Improvement of the vine.....pag.27
3.2- Improvement in yeast.....pag.29
3.3- Limits to the application of Biotechnology.....pag.32
4- Indigenous yeasts: the egg of Columbus?.....pag.32

MATERIALS AND METHODS.....pag.36

1- Grape and must sampling from Aglianico and Fiano.....pag.37
2- Isolation of yeasts.....pag.37
3- Identification of yeasts.....pag.38
3.1- Biochemical and morphological identification.....pag.38
3.2- Molecular identification of yeasts.....pag.39
4- Selection of yeasts.....pag.40
4.1- Selection for resistance to sulphur dioxide.....pag.40
4.2- Selection for the secretion of a β-glucosidase.....pag.40
5- Technological cycle of wine production.....pag.41
5.1- Aglianico.....pag.41
5.2- Fiano.....pag.43
6- Analysis of wine samples.....pag.45
6.1- Analysis of sugars.....pag.45
6.2- Determination of alcohol content.....pag.45
6.3- Analysis of total acidity.....pag.45
6.4- Analysis of acetic acid.....pag.45
6.5- Analysis of total polyphenol content.....pag.46
6.6- Total flavonoid assay.....pag.46
6.7- Determination of total and coloured anthocyanins.....pag.46
6.8- Measurement of the antiradical activity.....pag.47
6.9- Measurement of the reducing power.....pag.47
6.10- Analysis of colour intensity and hue.....pag.47
6.11- Analysis of polyphenols by HPLC.....pag.48
6.12- Determination of wine aroma.....pag.48
6.12.1- Sample preparation for HS-SPME-GC analysis.....pag.48

6.12.2- GC-MS analysis.....pag.48
6.13- Sensory analysis…….....pag.49
6.13.1- Sensory analysis of 2007 wines….....pag.49
6.13.2- Sensory analysis of 2008 wines…….....….pag.49
6.14- Statistical analysis.....pag.49
7- Purification of a β-glucosidase from *Rhodotorula mucilaginosa* AGWLR12…….....pag.50
7.1- Growth in liquid medium…….....pag.50
7.2- Determination of the enzyme activity.....pag.50
7.3- Determination of protein concentration.....pag.51
7.4- Concentration of the β-glucosidase.....pag.51
7.5- Anion-exchange chromatography on DEAE Fast Flow.....pag.51
7.6- Chromatography on Superdex 200 XK 26/60.....pag.51
8- Characterization of the β-glucosidase from *R.mucilaginosa* AGWLR12.....pag.52
8.1- Determination of the optimum pH.....pag.52
8.2- Effect of temperature upon enzyme activity: thermostability…….....…….....…...…...pag.52
8.3- Effect of temperature upon enzyme activity: thermophily.....pag.52
8.4- Purity of the β-glucosidase and determination of its molecular weight ….....pag.52
8.5- Determination of kinetic constants.....pag.53
8.6- Specificity of β-glucosidase for synthetic substrates and disaccharides.....pag.54
8.7- Effect of ethanol and sugar on enzyme activity.....pag.54
8.8- Effect of β-glucosidase activity upon hydrolysis of piceid.....pag.55

RESULTS AND DISCUSSION pag.56

1-Isolation of yeast strains….....pag.57
2-Sulphur dioxide resi stance…….....pag.59
3-Presence of β-glycosidase activity…….....pag.60
4-Fermentation with indigenous yeasts.....pag.61
4.1- 2007 vintage.....pag.61
4.2- 2008 Aglianico vintage.....pag.65
4.3- 2008 Fiano vintage.....pag.70
5- HPLC analysis of polyphenols.....pag.73
5.1- 2007 vintage.....pag.73
5.2- 2008 vintage.....pag.73
6- Analysis of the aroma profile by GC-MS.....pag.76
7- Sensory analysis…….....……...pag.82
7.1- Sensory analysis of 2007 wines……..…….…….....…….....…...pag.82
7.2- Sensory analysis of 2008 Aglianico wines…….……..…….…….....……pag.83
7.3- Sensory analysis of 2008 Fiano wines…….….....….....pag.84
8- Purification and characterization of a β-glucosidase from *Rhodotorula*

mucilaginosa **AGWLR12…….…….……..….....….....pag.85**
8.1- Study of the β-glucosidase output
in relation to the yeast growth…….....….…pag.85
8.2- Purification of the β-glucosidase.....pag.85
8.3- Dependance of enzyme activity upon pH.....pag.88
8.4- Temperature dependance of enzyme activity.....pag.88
8.5- Study of kinetic constants.....pag.89
8.6- Specificity of the β-glucosidase toward synthetic substrates
and disaccharides…….....pag.90
8.7- Dependence of the β-glucosidase activity
upon ethanol and sugar concentration.....pag.91
8.8- Effect of the β-glucosidase activity upon hydrolysis of piceid.....…pag.92

CONCLUTIONS.....pag.93
BIBLIOGRAPHY.....pag.96

APPENDIX.....pag.104
RINGRAZIAMENTI…….....…….…….....….pag.110

Summary

At the industrial level, the study of microbial biodiversity of different vine cultivars of the wine industry could provide an effective way to meet the demands for greater health, quality and typicality of the wine by a consumer, without colliding with the statutory prohibition or prejudice associated with the use of genetically modified vine and yeast. This research involves the study of microbial biodiversity of two cultivars of vine which originate from Irpinia, a region possessing an established vine industry: Aglianico and Fiano. As a first step, we created a collection of yeasts isolated from grapes and grape vines and these were later identified. Analysis of their morphology and biochemical characteristics identified them as belonging to four main genera: *Saccharomyces spp., Kloeckera spp., Candida spp.* and *Metschinikowia spp.* With regards to the Aglianico, *Saccharomyces* yeasts were found only in the musts, whereas in Fiano they were also present on grapes. In both cases the non-*Saccharomyces* genera were the predominant ones on grapes. Most of the yeasts selected were resistant to high concentrations of SO_2 (100 ppm and 250 ppm), including non-*Saccharomyces* generally considered very sensitive to this compound. Also, only a small fraction of them were able to secrete β-glucosidase, an enzyme that, in enology, catalyzes the release in the must of odorous molecules in the form of volatile terpenes. The analysis of data obtained from the selections highlighted some yeasts, and *Saccharomyces spp.* and non-*Saccharomyces spp.*, which have been used in various combinations for wine production on an industrial scale: these belong to the species *S. cerevisiae, Rhodotorula mucilaginosa, Metschnokowia pulcherrima* and *Hanseniaspora guilliermondii*. In the case of *R. mucilaginosa*, it was used alongside commercial yeast in the fermentation of both types of must for the 2007 vintage, while the others have been used in various combinations with each other, and the commercial yeast in the harvest of 2008, only with musts from the vineyards of origin. *S. cerevisiae* strains AGYP37 and *M. pulcherrima* AGYP28 were used in the fermentation of Aglianico while *S. cerevisiae* strains FYP69 and *H. guilliermondii* FWL66 in that of Fiano. As for the fermentation of 2007, the strain *R. mucilaginosa* AGWLR12 had better results in Aglianico than in Fiano, reaching an alcohol content and total acidity levels higher than the control, without producing too much acetic acid and completing fermentation one week in advance. Aromatic analysis of wines made with this yeast shows that it has influence on the content of methanol, and yields more higher alcohols and especially terpenes. Compared to the control, the latter increased by three times in Aglianico and by almost nine times in Fiano. This increase is due to the action of a β-glucosidase of AGWLR12 which is quite active under the conditions of the must and also responsible for increasing the content of resveratrol in the Aglianico wine, through its capacity for hydrolysis of the precursor trans-piceid. Sensory analysis confirmed the positive contribution of *Rhodotorula* AGWLR12 to both Fiano and Aglianico, resulting in a greater intensity of almost all parameters. Furthermore, on 2008 wines, it has shown some positive differences among samples as well, with regard the colour, the aroma and taste-tactile sensation. The analysis of wine and biochemical parameters of the wines of 2008 vintage shows that selected strains of native yeasts are efficient in completing fermentation and can affect wine quality. The best results were obtained in the fermentation of Aglianico grape where, in comparison to the control, native yeasts increased the total acidity without producing excessive amounts of acetic acid, reached more or less constant values of pH and alcohol content, increased colour, increased the content of polyphenols, flavonoids and coloured anthocyanins, and increased the reducing power but not the anti-radical activity of wine, except in one case. HPLC analysis showed a significant increase of gallic acid, catechin and once

of resveratrol in experimental fermentation of Aglianico. The magnitude of these changes seems to be influenced by the interaction of yeasts. During the fermentation of Fiano musts, native yeasts achieved the same alcohol content, pH, polyphenol and flavonoid content, antiradical activity and reducing power of the control, not producing excessive amounts of acetic acid. A positive feature was an increase of catechin levels. These results indicate that indigenous yeasts, at least those isolated from Aglianico, can help to confer quality and wholesomeness to wine.

Riassunto

Così come ogni altro settore, anche quello vitivinicolo è soggetto a cambiamenti, e in questo primo decennio del nuovo millennio esso sta attraversando una fase di transizione. Sul mercato mondiale si sono affacciati nuovi paesi (Argentina, Australia, California, Cile, Nuova Zelanda e Sud Africa) a fare concorrenza ai tradizionali produttori di vino del Vecchio Continente, tra cui vi è ovviamente l'Italia. Il progressivo aumento della produzione (si parla di circa 27 miliardi di ettolitri prodotti ogni anno) ha saturato il mercato, con un'eccedenza produttiva pari al 15-20% sul totale. Questo surplus è costituito da vino di non particolare qualità e questo è indice che le preferenze del consumatore si sono spostate su vini di alta e altissima qualità, ma non solo.
Il cliente è sempre più consapevole del valore di una sana alimentazione ed è quindi più attento al contenuto del vino e a parametri quali la quantità e la composizione della frazione polifenolica (in primo luogo il contenuto di resveratrolo, composto largamente pubblicizzato come apportatore di effetti salutari), la concentrazione di solfiti, il grado alcolico, la presenza di agenti tossici (i.e. l'etilcarbammato, un noto cancerogeno, e le ammine biogeniche, agenti neurotossici), la scelta delle uve utilizzate, i metodi di coltivazione del vigneto, senza però ignorare fattori classici di influenza come regione di provenienza, gusto, aroma, colore, intensità, tatto, originalità e tipicità. Nasce da qui l'esigenza di migliorare la qualità finale del vino e nel contempo di differenziare il prodotto in modo da renderlo unico, con un sguardo sempre vigile sul rapporto tra qualità e prezzo.
All'evoluzione del settore vinicolo verso una più alta qualità e una più grande varietà di offerta concorre in grande misura anche la legislazione che, regolamentando questo mercato, ha inteso proteggere e valorizzare lo straordinario patrimonio italiano di biodiversità e tradizioni. La considerazione di cui gode il settore vitivinicolo è espressa chiaramente all'articolo 1 della proposta di legge n.475 del 2006 volta a disciplinarlo:

" *La vite e il vino sono elementi inseparabili dalla storia, dalla cultura, dal paesaggio, dalla vita sociale e dall'economia della Nazione Italiana che, riconoscendo tale ruolo, regolamenta e tutela nell'ambito della normativa dell'Unione Europea, i vigneti, il territorio in cui sono presenti ed i prodotti da essi ottenuti".*

Ecco allora che i vini migliori vengono classificati con sigle (IGT, DOC, DOCG, VQPRD) che attestano il rispetto di precise caratteristiche produttive a garanzia del livello qualitativo.
In tutto questo la biotecnologia ha tentato di apportare il proprio contributo attraverso la sperimentazione sulla vite e sul lievito. Per quanto riguarda la vite, il recente completamento della sua sequenza nucleotidica rappresenta solo l'ultimo dei progressi ottenuti per il miglioramento di questa pianta. Tecniche come la trasformazione mediata da *Agrobacterium* e il bombardamento biolistico permettono la manipolazione del genoma vegetale e di perseguire obiettivi quali il miglioramento della salute della vite tramite resistenza ai patogeni, il miglioramento della sua coltivazione tramite la resistenza agli stess ambientali e quello della sua qualità tramite modifica dei pattern metabolici.
Per quanto riguarda il lievito, principalmente *S. cerevisiae*, la biotecnologia si è focalizzata sul miglioramento della sua efficienza di fermentazione, il controllo che esso poteva avere sulla contaminazione microbiologica e la sua influenza sulla salubrità e qualità del vino. L'introduzione in filiera di viti e ceppi di lievito geneticamente modificati risulta però problematico in quanto il loro utilizzo spesso si

scontra con i pregiudizi del consumatore, i divieti legislativi e la diffidenza dei produttori.
Come allora affrontare le nuove tendenze del mercato? La risposta potrebbe trovarsi nella serie di studi che nell'ultimo decennio hanno messo in evidenza le potenzialità della grande varietà di lieviti autoctoni dei vigneti che con i loro enzimi e i loro metaboliti possono influenzare profondamente la qualità finale del vino. Alcuni autori hanno riportato che essi sono responsabili delle caratteristiche uniche che lega un vino alla sua regione di produzione. Questi lieviti sono quelli che normalmente si ritrovano durante una fermentazione spontanea del mosto. Grandi vini quali il Barolo e il Chianti in Italia, o i vini francesi detti *Premier Cru*, devono la loro complessità proprio al fatto che vengono prodotti tramite fermentazione spontanea, e quindi ai molteplici generi di microrganismi già presenti sulle uve. Tuttavia, data l'imprevedibilità di una fermentazione spontanea che può portare a fenomeni quali fermentazioni incomplete o produzione di aromi sgadevoli (off-flavours), di solito durante il processo di vinificazione si preferisce eliminare questi microrganismi spontanei a favore di colture starter di lieviti *Saccharomyces spp.* selezionate in modo da rispondere ad esigenze industriali quali completare efficientemente la fermentazione degli zuccheri, produrre poco acido acetico, resistere alle concentrazioni di SO_2 normalmente utilizzate in cantina e ad alte concentrazioni di etanolo.
Un più attento studio dei microrganismi autoctoni dei vigneti potrebbe però essere d'aiuto nella ricerca di una maggiore qualità del vino in risposta alle nuove esigenze del mercato, senza scortrarsi contro pregiudizi o divieti ma anzi reinterpretando la tradizione e permettendo ad ogni territorio di produzione di stabilire in maniera più forte la propria unicità. L'obiettivo del mio dottorato è stato quello di studiare la biodiversità microbica dei microrganismi rinvenuti in uve e mosti di due vigneti della regione irpina, uno nero e uno bianco, rispettivamente l' "Aglianico" e il "Fiano", da cui si producono il rosso Taurasi e il bianco Fiano di Avellino, entrambi DOCG, per poter isolare ceppi di lieviti sia *Saccharomyces* che non-*Saccharomyces* che possano influire positivamente su qualità e tipicità di questi vini. Per fare questo mi sono avvalso dei vigneti e degli impianti di fermentazione dell'Istituto Tecnico Agrario "F. De Sanctis" di Avellino.
Il primo passo è stato quello ovviamente quello di creare una ceppoteca adeguatamente rappresentativa della biodiversità microbica delle uve dei vigneti in questione. Il prelievo dei campioni di uve delle cultivar "Aglianico" e "Fiano" è stato effettuato a intervalli di tempo tali da avere un campione rappresentativo nei diversi stadi di maturazione, dall'allegagione all'invaiatura dell'uva, mentre i campioni di mosto sono stati prelevati durante le fasi di pigiatura e fermentazione presso la cantina dell'Istituto Tecnico Agrario "F. De Sanctis". Per l'isolamento della microflora blastomicetica, sono stati utilizzati tre diversi terreni di coltura: YPD, WL Nutrient (Oxoid) substrato in grado di differenziare le colonie attraverso il colore, Lysine Medium (Oxoid) terreno selettivo, in grado di permettere la crescita di lieviti non-*Saccharomyces*. La crescita microbica è stata effettuata incubando le piastre a 28°C per 5 giorni. Il riconoscimento dei lieviti è stata effettuata tramite analisi morfologica al microscopio e utilizzando il kit di identificazione API 20C AUX (BioMérieux Italy) che determina l'assimilazione o meno di substrati selettivi. Per alcuni lieviti è stata effettuata l'analisi di sequenza della regione D1/D2 del gene per l'rRna 26S. Alla fine si è ottenuta una ceppoteca di circa 400 ceppi di lievito composta sia da *Saccharomyces* che non-*Saccharomyces*. Per quanto riguarda il vitigno Aglianico sulle uve i lieviti isolati appartengono ai generi *Candida spp* e *Kloeckera spp*, con

una prevalenza del primo genere. Nel mosto a inizio fermentazione invece *Candida* non è più riscontrabile, al contrario di *Kloechera* che invece è secondo solo ai *Saccharomyces*. In questa fase è riscontrabile anche una sensibile percentuale di *Metschnikowia spp*. Col proseguire della fermentazione e il conseguente aumento del grado alcolico solo il genere *Saccharomyces spp*. sopravvive. Per quanto riguarda invece il vitigno Fiano sulle uve sono riscontrabili lieviti dei generi *Candida spp*, *Metshnikowia spp*, *Kloeckera spp* e *Saccharomyces spp*. Nel mosto a inizio fermentazione i *Saccharomyces* prendono il sopravvento ma sono riscontrabili ancora i generi *Metshnikowia* e *Kloeckera*, e quest'ultimo anzi si ritrova anche a fine fermentazione. Questi dati indicano che per entrambe le cultivar i non-*Saccharomyces* rappresentano la maggioranza della popolazione di lieviti sulle uve. *Candida*, in entrambi i vigneti, non si adatta alle condizioni del mosto ma è isolabile solo da uve. Inoltre sull'uva Fiano è riscontrabile una buona percentuale di lieviti *Saccharomyces*, al contrario di quanto riportato in letteratura. Inoltre il genere *Kloeckera* può essere ritrovato fino a fine fermentazione. Ciò può essere dovuto alla più bassa temperatura con cui è condotta la fermentazione del Fiano che rende questo genere di lieviti più resistenti ad alte concentrazioni di etanolo.
Sulla ceppoteca sono stati effettuati dei saggi per saggiare la resistenza alla SO_2, agente chimico comunemente usato in cantina, e la presenza di attività β-glicosidasica, utile per la liberazione dei terpeni dalla loro forma glicosilata inodore. La determinazione della resistenza alla SO_2 è stata monitorata facendo crescere i ceppi isolati in mosto pastorizzato in presenza di 100 e 250 ppm di metabisolfito (MBK). Si tenga presente che in cantina normalmente viene usata una concentrazione di MBP di 100 ppm.
Per quanto riguarda l'attività β-glicosidasica, essa è stata determinata sulla brodo coltura dei lieviti cresciuti in YPD a 28°C utilizzando un substrato sintetico contenente un gruppo cromoforo, il para-nitrofenolo (il para-nitrofenil-β-D-glucopiranoside - pNPG). La rottura del legame glicosidico del substrato è stata monitorata seguendo la variazione dell'assorbanza a 400 nm dovuta al rilascio del para-nitrofenolo. Il saggio è stato condotto per 1h alla temperatura di 30°C in tampone citrato-fosfato ai due differenti valori di pH7 e pH3.4 con saggio bloccato. I dati sulla resistenza alla SO_2 sui ceppi isolati da Aglianico mostrano che la grande maggioranza di essi risulta resistente alle concentrazioni di MBK testate, rispettivamente 81% per 100 ppm di SO_2 e 66% per 250 ppm di SO_2. Anche in Fiano molti dei ceppi sono resistenti con percentuali del 78% per 100 ppm di SO_2 e del 49% per 250 ppm di SO_2. Dato interessante è che per entrambi i vigneti ed entrambe le concentrazioni di SO_2 testate, molti dei ceppi resistenti appartenevano ai generi non-*Saccharomyces*, dato del tutto in contrasto con quanto riportato in letteratura dove viene riportato un limite di resistenza per questi lieviti intorno a 10 ppm.
L'analisi dell'attività β-glicosidasica ha mostrato che questo enzima è assai poco presente nei ceppi isolati. Di quelli isolati solo tre hanno dimostrato di possederla: il ceppo AGWLR12, appartenente alla specie *Rhodotorula mucilaginosa*, il ceppo FWL66, appartenente alla specie *Hanseniaspora guilliermondii*, e il ceppo AGYP28, appartenente alla specie *Metshnikowia pulcherrima.* Questo risultato si discosta da quanto riportato in letteratura che invece indica questa attività enzimatica essere non rara tra le specie non-*Saccharomyces*. I tre ceppi nominati, insieme ai ceppi commerciali e a due ceppi di *S.cerevisiae* della mia ceppoteca scelti per il loro vigore fermentativo in presenza di SO_2 (FYP69 e AGYP37), sono stati usati per condurre delle vinificazioni in cantina.

Le prove pilota delle fermentazioni di Aglianico e Fiano sono state condotte in accordo al loro specifico ciclo tecnologico. Per quanto riguarda il ceppo AGWLR12, esso è stato utilizzato per condurre una vinificazione con i ceppi commerciali sia su Aglianico che su Fiano. Per quanto riguarda gli altri quattro ceppi, sono state approntate vinificazioni usando diverse combinazioni tra di loro e i ceppi commerciali. A intervalli regolari sono stati prelevati dei campioni e sono stati monitorati parametri quali zuccheri, pH, acidità volatile, acidità totale, grado alcolico e polifenoli totali per tutte le fermentazioni mentre per quelle effettuate con i ceppi AGYP28, AGYP37, FWL66 e FYP69 sono stati analizzati anche colore, intensità, flavonoidi totali, antocianine totali e colorate, attività antiradicalica e attività antiossidante. I risultati ottenuti indicano delle differenze tra le varie vinificazioni. La fermentazione in Aglianico condotta con *R. mucilaginosa* è stata particolarmente tumultuosa ed è terminata con una settimana di anticipo rispetto al controllo. L'acidità totale di molte delle fermentazioni condotte in Aglianico con i lieviti da me isolati, anche in combinazione col lievito commerciale, è risultata aumentata, con un aumento del 28% nel caso della fermentazione con *S. cerevisiae* autoctono (AGYP37) rispetto al controllo. In Fiano invece il quadro ottenuto è opposto con un controllo avente un'acidità totale leggermente superiore rispetto ai vini sperimentali. Per quanto riguarda l'acidità volatile, costituita essenzialmente da acido acetico, quasi tutte le sperimentazioni sperimentali non mostrano livelli più alti rispetto al controllo. E' un dato molto incoraggiante se si pensa che i lieviti autoctoni, soprattutto i non-*Saccharomyces*, vengono da sempre considerati grandi produttori di acido acetico. Il valore del pH, sia in Aglianico che in Fiano, rimane circa uguale nelle vinificazioni sperimentali in confronto ai rispettivi controlli. Neanche il grado alcolico varia significativamente comparando vinificazioni sperimentali e controlli. La determinazione degli zuccheri mostra un buon comportamento per tutti i lieviti sperimentali usati in Aglianico, con un livello di zuccheri residui intorno a 2 g/L. In Fiano invece i livelli di zuccheri residui a fine fermentazione tendono a essere più alti, arrivando fino a 5 g/L.

L'analisi dei polifenoli rivela che tutte le fermentazioni sperimentali in Aglianico effettuate riportano un considerevole aumento nel contenuto totale di polifenoli, con un aumento che va dal 15% al 25%. Nel Fiano invece il livello dei polifenoli è rimasto costante. Il contenuto in polifenoli è un dato molto importante poiché molte delle proprietà benefiche correlate all'assunzione moderata di vino (il cosiddetto paradosso francese) viene attribuita a questa classe di composti.

All'aumento di polifenoli totali corrisponde anche un aumento della classe dei flavonoidi mentre, nei vini rossi, le antocianine totali tendono a diminuire, con l'unica eccezione della vinificazione con lievito commerciale e *M. pulcherrima* in cui esse restano costanti. Ciò che invece aumenta nei vini Aglianico sperimentali, e in modo inversamente proporzionale alle antocianine totali, è il contenuto di antocianine colorate, fino all'aumento del 56% nella fermentazione con tutti e tre i tipi di lievito, a cui corrisponde un proporzionale aumento dell'intensità del colore. A tale proposito questa particolare fermentazione raggiunge un valore di intensità di colore pari a 14 D.O., contro invece il valore di solo 8.4 D.O. del controllo.

Per quanto riguarda l'attività antiradicalica, tutte le fermentazioni sperimentali non mostrano variazioni, eccetto che nel caso della vinificazione con i soli due lieviti autoctoni. In parte questo questo può essere dovuto anche all'aumento del contenuto di resveratrolo.

Per quanto riguarda l'attività antiossidante, le vinificazioni sperimentali in Aglianico mostrano un livello superiore al controllo, strettamente correlato all'aumento dei polifenoli, mentre per quelle in Fiano i livelli restano inalterati.
Nel tentativo di approfondire il discorso dell'influenza dei lieviti sui polifenoli, sono state effettuate delle analisi all'HPLC sui campioni del vino. Considerando la vendemmia 2007 i composti analizzati sono stati acido gallico, catechina, resveratrolo e quercetina, mentre per la vendemmia 2008 sono stati analizzati anche epicatechina, acido cumarico, acido cinnamico e acido clorogenico. Le analisi sono state condotte su colonna C-18 Hypersill Gold (Thermo).
Il dato sicuramente più interessante delle vinificazioni del 2007 sono gli elevati livelli di resveratrolo riscontrati nella vinificazione in Aglianico con *R. mucilaginosa,* assai più alti rispetto al controllo. Indagando le possibili cause di un tale aumento, è stato scoperto che la β-glicosidasi di AGWLR12 evidenzia la capacità di catalizzare efficientemente l'idrolisi del piceide per liberare resveratrolo, attività raramente riportata in letteratura.
Per quanto riguarda le vinificazioni del 2008, in Aglianico i livelli di acido gallico e catechina sono risultati comparabili o più alti rispetto al controllo, mentre i livelli di epicatechina sono rimasti costanti e il resveratrolo si è mostrato altalenante. Gli altri composti invece tendono a diminuire nei vini sperimentali. In Fiano, per tutte le vinificazioni, acido cinnamico e cumarico diminuiscono rispetto al controllo mentre la catechina aumenta. Gli altri composti invece rimangono stabili. La quercetina non risulta presente né in Aglianico né in Fiano, probabilmente perché essa si trova sotto forma dei rispettivi glucuronidi.
Sulle vinificazioni condotte con *R. mucilaginosa* AGWLR12 in Aglianico e Fiano sono state condotte analisi sulla frazione aromatica tramite tecnica SPME-GC-MS. Prima dell'analisi, il campione è stato raffreddato a 4°C per minimizzare la perdita di composti volatili. Si è usato l'isoottano come standard interno. La fibra usata per l'estrazione dei composti volatili è il Polidimetilsilossano (PDMS) 100 µm. Il rilevamento delle masse dei picchi è stato condotto con un detector a frammentazione elettronica, monitorando il rapporto degli ioni prodotti m/z da 29 a 300. I componenti sono stati identificati tramite comparazione degli spettri con le librerie NIST e Wiley. L' analisi della frazione volatile dei campioni di vino Aglianico e Fiano ha evidenziato notevoli potenzialità di questo lievito nella fermentazione del mosto. Sono state analizzate le cinque maggiori classi di composti responsabili dell'aroma del vino: alcoli, aldeidi, chetoni, esteri e terpeni.
Per quanto riguarda gli alcoli, il loro contenuto totale in vino Aglianico aumenta leggermente rispetto al controllo, mentre diminuisce di poco per quanto riguarda il Fiano. Per l'Aglianico l'incremento coinvolge principalmente quelli superiori. Nel Fiano il decremento riscontrato influisce su tutti i componenti alcolici.
Considerando le aldeidi, nell'Aglianico il loro contenuto totale diminuisce , tuttavia ciò è dovuto unicamente alla diminuzione dei livelli di 3-metilpentanale mentre tutte le altre aldeidi sono presenti in livello superiore rispetto al controllo. Addirittura, nel caso dell'esanale, esso è presente solo nella fermentazione sperimentale e non nel controllo, in concentrazione bene al di sopra del suo limite sensoriale. Nel Fiano invece il livello totale delle aldeidi subisce un decremento riguardante ogni componente di questo gruppo di composti.
Osservando la concentrazione dei chetoni si nota che nell'Aglianico l'unico composto a subire un notevole decremento è l'acetone rispetto al controllo mentre gli altri composti risultano pressocchè invariati. Nel Fiano il pattern di chetoni non subisce drastiche variazioni.

L'analisi degli esteri mostra che nell'Aglianico il loro contenuto totale rimane pressocchè invariato, ma è la concentrazione dei singoli composti a variare determinando un diverso pattern. Nel Fiano si ha un decremento a scapito di tutti i componenti.
I risultati più rilevanti sull'aroma del vino ottenuti utilizzando il ceppo AGWLR12 si hanno sulla frazione terpenica. Nell'Aglianico il contenuto totale di terpeni è più che raddoppiato, con particolare riferimento al β-damascenone e all'α-terpinolo . Nel Fiano il contenuto totale di terpeni aumenta addirittura di sei volte con particolare riferimento al linalolo e al citronellolo. Con ogni probabilità questo è dovuto all'azione della β-glicosidasi che questo lievito esprime in quantità elevata.
L'analisi sensoriale dei vini Aglianico e Fiano prodotti nel 2007 con *Rhodotorula mucilaginosa* AGWLR12 ha evidenziato che questi vini possiedono un'intensità odorosa e un sentore terpenico maggiore rispetto al controllo, raggiungendo rispetto a quest'ultimo un valore di impressione generale superiore. Anche per quanto riguarda i vini prodotti nel 2008 l'analisi sensoriale ha mostrato delle differenze tra i campioni, sebbene i risultati ottenuti siano preliminari.
L'ultima parte del mio dottorato ha riguardato la purificazione e la caratterizzazione di una β-glucosidasi del lievito AGWLR12. L'enzima è stato purificato a partire da una brodocoltura cresciuta in YPD a 28°C per 48h a 160 rpm. La purificazione è consistita in una prima fase di concentrazione, poi una separazione su colonne cromatografiche, prima per scambio anionico poi per gel filtrazione. Le analisi hanno mostrato che questa proteina è un omodimero di 120 KDa di massa. Sebbene il suo optimum di pH sia intorno a 6.5 e il suo optimum di temperatura sia di circa 60°C, questo enzima risulta abbastanza attivo alle condizioni del mosto (pH 3.3 e 25°C). Inoltre la sua attività non è seriamente inibita da alte concentrazioni di zuccheri e risulta invece essere esaltata dall'etanolo, contrariamente ad altre β-glicosidasi riportate in letteratura.
Uno studio sulla sua attività su substrati sintetici e su disaccaridi dimostra che esso ha una specificità stretta per i substrati glicosidici con legame β1-4. Nell'ordine seguono i substrati con legame α1-4 e α1-1 mentre c'è una scarsa o nessun'attività con il legame β1-6 e con il β1-4 gal-glu del lattosio. E' grazie all'attività di questo enzima che nelle fermentazioni effettuate con AGWLR12 il contenuto di terpeni nella frazione aromatica è fortemente aumentato, così come aumenta il contenuto di resveratrolo grazie all'attività sul suo precursore, il piceide, come già accennato precedentemente. Il sequenziamento dell' N-terminale della proteina non ha prodotto una sequenza unica, nonostante i ripetuti tentativi di purificazione.
Successivamente è stata tentata la purificazione di una β-glicosidasi dal lievito *Hanseniaspora guilliermondii* FWL66 che presentava interessanti caratteristiche quali optimum di pH intorno a 3, optimum di temperatura intorno a 25°C, alta attività specifica e bassa specificità di substrato, nonché una massa di soli 20 KDa. Purtroppo questo enzima è estremamente labile in condizioni di medio-alta concentrazione salina e non è stato possibile purificarlo per effettuare un suo sequenziamento.

Introduction

1- What wine is: chemical composition and production.

Wine is obtained from a total or partial alcoholic fermentation of grape must. The must is obtained after several steps:

a) *Mixed vintage*: choice of quality grapes. For example, the Chianti is a mixture of two grapes: the San Giovese and Trebbiano. However, there are also single-variety wines like the Campanian Falanghina and Aglianico;

b) *Removal of grape stalks*: this is made by special pressing and destemming machines thus obtaining the must or the juice of grape that provides the nutritional basis for the growth of various heterotrophic microorganisms.

The fresh must contains equal quantities of fructose and glucose, while the content of sucrose is much lower (10-12 g/l) and small amounts of pectin and pentosans are also present. Its main acids are L-tartaric acid and L-malic acid, the former being more abundant in good vintages. The content of nitrogenous compounds and lipids is very low while phenolic compounds (tannins) and pigments (anthocyanins) are more abundant and are mainly present in the grape stalks, in the peel and in the seeds. The quantity and quality of all of these substances, along with the aromatic components, are essential in giving the wine its specific character (79).
Although it is a complex medium , its pH (3- 3.5) is only compatible with yeasts, moulds, acetic acid bacteria, *Oenococcus oeni* and some other lactic bacteria such as *Lactobacillus spp*.. After having obtained the must, the simplest way to make wine is by spontaneous fermentation, i.e. without addition of an inoculum. In spontaneous fermentation, there is a sequential growth of indigenous yeasts already present on the surface of the grains and on the equipment of the cellar (21,73).
Following pressing and loading of the vats, the yeasts of the must, both aerobes and facultative anaerobes, consume the dissolved oxygen, thus creating a state of anaerobiosis. This condition inhibits the growth of moulds and acetic acid bacteria (obligate aerobes) and, therefore, after less than a day, the real fermentation begins. At this early stage, apiculate yeasts are chiefly responsible for the process; they are so named for their pointed shape which resembles that of a lemon (*apex*, tip in Latin). After three to five days, the alcoholic grade in the must reaches a value of approximately 3-4° and the non-*Saccharomyces spp.* have a low probability of surviving this ethanol concentration.
At this point, *Saccharomyces* yeasts take over, in particular *S. cerevisiae,* because they are more resistant to ethanol. Despite the large number of studies which have addressed wine and vineyard yeast ecology, the origin of *S. cerevisiae* remains somewhat controversial. Some authors claim that the primary source of this yeast is the vineyard, while others attribute it to the direct association with the cellar equipment and the fermentation plant (12,59).
If spontaneous fermentation reaches its conclusion, the alcoholic grade never usually exceeds 17-18° due on one hand to the inactivation of ferments, but sometimes also occasionally due to problems such as early stopping, growth of undesirable microorganisms, production of off-flavours etc. To avoid these unpredictable side effects, special precautions are used such as the addition to the fresh must of sulphur dioxide, often in the form of potassium metabisulfite (MBK) (14). Sulphur dioxide prevents the growth of the spontaneous microflora, not selected for an

adequate fermentation and which often results in the production of a large amount of unpleasant aromas like acetic acid. As a substitute for these native yeasts, selected strains of *Saccharomyces* yeasts (most of all *S. cerevisiae* or *S. bayanus*, *S. pastorianus* etc) are inoculated in must in order to ensure efficient completion of the fermentation process.
These strains are selected for specific parameters: resistance to SO_2 concentration, resistance to ethanol, low foaming power, no production of hydrosulphuric acid and sulfur dioxide, and rapid sedimentation at the end of fermentation. Eventually, they can possess the killer factor, or be resistant to it, and be film-forming or flocculent for specific types of winemaking (39).
After fermentation, the wine is subjected to an additional treatment with SO_2 that eliminates carbonylic compounds which have an unpleasant aroma, especially acetaldehyde. Other processes may follow fermentation, including ageing, a period of time during which a multitude of reactions confer the wine its final organoleptic characteristics. One of the most important reactions is the malolactic fermentation, a process accomplished by microorganisms such as *Pediococcus spp.* and *Lactobacillus spp.* that convert the malic acid in lactic acid and reduce the wine acidity.
The chemical composition of the wine is extremely variable, being influenced by many environmental factors such as climate, meteorological conditions, terrain, type of yeast, production method, ageing etc. Analysis of wine is based on the determination of the content of alcohols, glycerol, tannins, sugars, ashes, pigments, nitrogenous and aromatic compounds but, due to the presence of significant amounts of sensitive components, wine valuation and classification is possible only through the combination of chemical analysis and organoleptic tests.

Ethanol and other alcohols

The ethanol content varies considerably: it is normally between 55 and 110 g/L, up to 130g/L for Southern European wine, but a level higher than 144g/L is considered as index of ethanol addition. Instead, methanol is present at very low concentrations (38-200 mg/L) but its content in the fermented grape seed is much higher due to pectin hydrolysis, so it can be 1-2 % of distillates. In addition to ethanol, the most abundant alcohols present in wine are propylic, butyl and amyl alcohols, which are responsible for the ethereal aroma of wine. A remarkable quantity of sugar derived glycerol (6-10g/L) that gives the wine its so called body is also present.

Organic acids

The organic acids derived from grapes are tartaric acid, malic acid, citric acid, while succinic, lactic and carbonic acid (CO_2) along with some volatile acids derive from fermentation. The red wines have a lower acidity in comparison with white wines, and Mediterranean wines tend to have relatively low acidity. The presence of acetic and propionic acid, as well as an unusual quantity of acid lactic, are an indication of contamination.

Phenols, anthocynins and nitrogenous compounds

Phenolic compounds (tannins) originate from the chlorogenic acids and are structurally similar to the derivates present in most of the fruits. They are esters

formed by hydroxybenzoic acids, such as salicylic acid (2- hydroxybenzoic), the 4-hydroxybenzoic acid and gallic acid (3,4,5 hydroxybenzoic), and sugars such as glucose. The tannins are responsible for the astringent proprieties of wine and in red wines they tend to polymerize during ageing, thus reducing the degree of astringency. The color of red wine is essentially due to the pigments, the anthocyanins, that are present in the grape peel. Nitrogenous compounds are largely metabolized by yeasts during fermentation therefore only small quantities of free amino acids (200-800 mg/L) remain upon completion of fermentation,

Aromatic substances

More than a thousand volatile compounds exist within winds, among these the following can be distinguished:

- Varietal aromas: these are mainly attributable to the grape variety used and to its botanical characteristics, but it can also depend on the land (for example the slope of the hill), on cultivation techniques and climate. They are mainly formed by terpene alcohols and some esters.
- Pre-fermentative aromas: these depend on the conditions of the grape and therefore on its maturity, physiological quality ('health') and integrity. They are formed through enzymatic transformations of precursors present in the pulp and in the peel and consist of some alcohols, lactic and organic acids and some phenyl derivatives.
- Fermentative aromas: these develop in the must after alcohol and malolactic fermentation by some microorganisms. It is important to underline that the yeast strain leaves its mark on the wine and helps to define its quality. The contribution of the strain cannot be comparable to that given by the vine and the quality of the grape, but there is no doubt that fermentation with different yeast strains yields different products that can be distinguished by sensory and chemical analysis.
- Post-fermentative aromas: these are formed as a result of the ageing process. They can come directly from the wood (generally durmast) of the barrels in which the wine is stored or may originate from the metabolism of some lactic bacteria and yeasts of constituents of the wood, such as lignin and/or its derivates (the phenyl-propenoic acids). These bacteria and yeasts are always present in the wine, even after racking and malolactic fermentation. A group of compounds may be a result of thermal degradation of the wood (mainly hemicellulose) that occurs during the phases of its processing: these are the furfural, the 5-methyl-furfural and the furfuryl alcohol.

2 - The vine and wine: yesterday and today

2.1 History

The vine is generally classified as belonging to the genus *Vitis* which includes two subgenera, *Euvitis* and *Muscadinia*, the former including most of the species of *Vitis*. Only one species of *Vitis*, that is *Vitis vinifera*, originated in Europe while more than thirty species are from China and approximately thirty-four species are from Northern and Central America. The wine can be obtained by grapes of *Vitis vinifera* or from a crossbreed between this and other species, for instance, *Vitis lambrusca*, *Vitis*

rupestris etc, but in Italy grapes belonging to the species *Vitis vinifera* alone are used for the production of wine. The scientific evidence for the origins of various cultivars is fragmented, but it is generally accepted that the species *Vitis vinifera*, the most cultivated among the species of genus *Vitis*, includes approximately five thousands cultivars.
Archeological discoveries suggest that the earliest production of wine obtained by grape fermentation took place in Georgia and Iran, around 6,000 B.C., in areas where the European species *Vitis vinifera* grows naturally. In 2003, an archeological study indicated the possibility that the grape was used with rice to produce a mixed fermented beverage in China around 4,000 B.C.
Some Neolithic terracotta jars, found near Jiahu, in the Chinese province of Henan, contained traces of tartaric acid and other organic compounds commonly present in wine, though other local fruits of that region, such as the hawthorn, cannot be excluded as ingredients in the making of these beverages (45). In case grapes were used, these are likely to have belonged to any of twelve species of *Vitis* growing in that region, rather than the species *Vitis vinifera*, which was introduced into China only 6,000 years later. In Europe, the oldest evidence of wine production dates back to 4,500 B.C. and comes from archeological sites in Greece. The same site contains the earliest evidence of squashed grapes. In ancient Egypt, six of thirty-six wine amphora found in the tomb of Pharaoh Tutankhamen bear the name "Kha'y", the chief imperial wine maker. Five of these amphoras came from the King's personal estate and the sixth came from the royal house of Athens (36). Traces of wine dated 2,000-1,000 B.C were also found in the Chinese region of Xinjang.
The Roman Empire gave a further boost to wine production, which evolved from an elite product into an everyday beverage. In that period, the cultivation of vines spread all over the empire and, with increased production, the consumption grew too. However, the wine being produced in those days was very different from the drink we know today. This was because of conservation techniques, especially boiling, which meant the wine was a syrupy beverage, very alcoholic and sweet and it was necessary to dilute it with water and add honey and spices to get a more palatable taste. With the decline of the Roman Empire, vine cultivation declined significantly and only in the Middle Ages it recovered because of the Cistercian and Benedictine monks. In his decree, Saint Benedict affirmed:

"*Although we read that wine is by no means a drink for monks, yet, since in our days they cannot be persuaded of this, let us at least agree not to drink to satiety, but sparingly: because wine causes even the wise to fall away*."

Most of the cultivation techniques known today developed during the Middle Ages, and they remained unchanged until the eighteenth century, when production developed a modern feature. This was due to stabilization of quality and taste of wines and the introduction of glass bottles and corks. In the nineteenth century, the mildew and the vine-pest, vine diseases coming from America, destroyed huge quantities of vineyards. Farmers were forced to cross the surviving vines with the American vines (*Vitis labrus*) which were more resistant to these parasites, and regularly used products such as sulphur to protect the plants. In the twentieth century, France introduced production regulation (guaranteed origin, definition of production territories etc.) which led to an increase in wine quality at the expense of quantity.

2.2 Regulation

Regulation (EC) n.479/2008 of 29 April 2008, on the organization of the common wine market, establishes the vine cultivars allowed to be used for the production of wine in the member states; these cultivars must belong to the species *Vitis vinifera* or must originate from a cross between this and other species of the genus *Vitis*. This regulation also controls the oenological practices allowed in the European Union, based in particular on the practices recommended by the International Organization of Vine and Wine (OIV).
The Member States may impose more restrictive rules for wines produced in their territory to preserve the essential characteristics of wines with a denomination of protected origin or geographical indication, or of sparkling and strong sweet wines. In Italy, in compliance with law n.116 of 1963, the DPR 930/1963 which regulated the productive characteristics of various wines was issued. Subsequently, in 1992, in an effort to make the best wines more clearly identifiable, necessary corrections to the DPR 930/1936 were made with the new law n. 164 that identifies the wines as:

- table wines;
- table wines of typical geographic origin (IGT – “Indicazione Geografica Tipica”);
- quality wines produced in specified regions (VQPRD – Vini di Qualità Prodotti in Regione Determinata);
- wines of simple denomination (DOS – Vini a Denominazione Semplice);
- wines of controlled origin (DOC – Vini a Deneminazione Controllata);
- wines of controlled and guaranteed denomination of origin (DOCG – Vini a Denominazione di Origine Controllata e Garantita);
- quality wines produced in specified regions (VQPRD, DOC, DOCG) with indication of the sub-area (municipality, neighborhood, farm, vineyards).

The product specification, established by the above mentioned laws, rules the production of DOC, DOCG and VQPRD wines, the conditions required to fall into those categories and the precise production characteristics to ensure quality. Those characteristics are the designation of origin, the soils of grape production, the maximum yield per hectare, the minimum alcohol content and the physical-chemical properties. Such wines are subject to quality control of chemical-physical and organoleptic properties over the entire production process and, for the DOCG wines, even for bottling. Then, committees of experts examine the product and decide whether it qualifies for the specific label.
There are currently over 260 DOC wines (constantly increasing) from which over fifteen hundred varieties are derived. In addition, with respect to the DOC and DOCG wines the sub-area of production may also be indicated. In Italy, the wine market contributes to a considerable percentage of the gross domestic product (GDP) and the main source of income in the food sector, both in terms of production and export. It is therefore not surprising that wine production and related practices are strictly regulated. In the bill n.475 of 2006 presented to the Senate, article 1 stresses the importance of this sector:

"*The vine and wine are inseparable elements of the history, culture, landscape, social life and economy of the Italian nation that recognizing such role regulates and protects the vineyards, the territory in which they are present and the products thereof according to the European legislation”*.

2.3 Wine in the modern market

It is broadly agreed by economists, sociologists and other intellectuals that the rapidly changing society we are witnessing at the beginning of this third millennium will lead it to being unrecognisable within a period of twenty to thirty years (54). Like any other industry, the wine industry must continue asking questions about how best to face the next market developments in the near future. Although rightly considered traditional, the wine sector is no stranger to change. At the beginning of the seventeenth century, wine was considered as the only healthy beverage, while today's consumers consider it as the universal beverage for moderate use, an essential lifestyle ingredient.

Today this sector is rapidly evolving to meet new consumer needs and has to ensure that its marketing strategies remain targeted. In order to fully understand this statement, one must take into account the competitiveness of the global economic system, which has seen the rise in the wine sectors of new countries and regions such as Argentina, Australia, Chile, California, Canada, New Zealand and South Africa. These new producers account for 20% of world market production, whilst countries considered to be classic wine producers such as Italy, France, Spain, Portugal and Germany account for 50% (54). The approximately twenty-seven billion litres of wine that are produced each year, come from eight million hectares of vineyards around the world and are about five billion litres in excess of what the market can absorb. This surplus, which each year fluctuates between 15% and 20% of total production, has given rise to fierce competition for every little piece of the market. This surplus is mainly present in the wholesale wine category, a wine with no particular features, which indicates that the consumer has shifted its preference to wines of high and very high quality. Moreover, the consumer is becoming more aware of his/her health and the environment and often chooses products with offer a good quality to price ratio. Finally, one needs to bear in mind that the global consumption of wine tends to decrease (57).

All these factors have contributed to the development in the wine industry, which is shifting from a product to a market oriented approach. In this way, the surplus of production can no longer be considered as a productive problem. It is a problem of management by the producers that remain unaware that it is the consumer and no longer the manufacturer that determines the degree of quality that products need to reach in order to end up in his/her basket. However, consumer choice is still influenced by factors such as the style of wine, the purity, the uniqueness and the diversity.

It is clear that technological innovation is one of the milestones to which the wine industry points for its success in global competition to meet consumer needs in the 21st century without losing the sense of tradition, which still remains a very important factor. The improvements that can be implemented relate to every phase of wine production, from the methods of cultivation of vines to harvest, from fermentation to bottling and even the labelling. Biotechnology, by its nature an interdisciplinary science, is well suited to improvement of many of these phases. Currently, there are two main objectives to improve wine production, which are subject of biotechnological research: the genetic improvement of grapevine and of wine yeast (55).

3 - Biotechnology in the wine industry

3.1 - Improvement of the vine

In recent years, the study of the grape vine has achieved important goals, the most famous of which was the complete sequencing of the genome through collaboration between the company Genoscope, the Institut National de la Recherche Agronomique (INRA) in France, several universities and the Istituto di Genomica Applicata (IGA) in Italy (34). Thus the vine has become part of a small group of plants whose genome has been sequenced, including *Arabidopsis thaliana*, rice and poplar. The selected inbred line was derived from Pinot Noir variety, which has a genome of approximately 480 Mb. This was the first example of a sequence of a plant cultivated both for its fruit and the derived drink, and it is crucial for a better genetic characterization of natural variation, its role in conferring phenotypic variety and for applied projects aimed at the development of new vines.
The comparison between the genome of the vine and that of *A. thaliana*, of rice and poplar has already revealed the ancestral nature of the vine in comparison with other plant species, and has shed light on how it could be the genome of the progenitors of flowering plants. The genome of the vine is not new to manipulation since it has been a very important crop since ancient times both for agriculture and for religious activities. It has been subjected to selection (the simplest form of biotechnology) over many centuries, evolving from a bushy plant to a climber. The vine has been easily tamed, giving rise to eight million acres of vineyards scattered around the world today.
The techniques currently available in agricultural biotechnology have allowed further genetic manipulation, such as the insertion of genes and regulatory elements into the genome in order to confer beneficial traits. Techniques such as transformation mediated by *Agrobacterium tumefaciens* or by biolistic delivery have allowed changes to be made to the plant DNA, though these changes have often caused complications such as the silencing of the transgene. Significant progress has been made using embryonic cell lines as targets for transformation, a technique that since 1989 has led to a stable production of genetically modified vine lines from different laboratories, both private and public (81,82). These have focused mainly on three targets: the improvement of the health of the vine, its cultivation and its quality.
As for the health of the vine, different approaches have been used to allow the plant to defend itself against pathogens such as fungi, bacteria, viruses and insects, but almost all are based on the natural interaction between host and pathogen. This interaction is complex and changeable, because the host and pathogen co-evolve in the struggle for survival. Most of the transformation strategies involve the insertion of a gene with anti-pathogenic activities, either as multiple copies, or in an inducible conformation in order to improve the defence of the plant (Table 1). The other main approach to improving disease resistance in grapevine, as in other plants, is to have the plant express a gene derived from the pathogen either at strategic time points, in an altered form, or in unfit quantities, so preventing the onset of infection. Many antiviral strategies are based on this pathogen-derived resistance (PDR) (70).
Biotechnological research has also addressed the improvement of grapevine growing, developing plants capable of adapting to adverse climatic conditions. Knowledge on stress tolerance in plants, as well as aspects of growth and

Table 1. Targets for the genetic improvement of grapevine cultivars and rootstocks (54)

Desirable properties	Focus area	Examples of current and potential target genes
Improved disease resistance		
Fungal tolerance	Grapevine defence and defence signalling in response to fungal pathogens; pathology of the various fungal pathogens; innate resistances (molecular basis) of various species towards fungal pathogens	Glucanase- and chitinase-encoding genes from fungi, yeast and plants; ribosome inactivating proteins (RIPs); thaumatin-like protein (Vvtl1); antifungal peptide encoding genes from plants and insects; PGIP (polygalacturonase-inhibiting protein); encoding genes from plant species, stilbene phytoalexins (stilbene synthases: stsy, vst1, vst2); phenylalanine ammonia lyase: pal) CuZnSOD (putative CuZn superoxide dismutase; detoxification enzyme-producing genes (NADPH-dependent aldehyde reductase, Vigna radiata-Eutypine reducing enzyme)
Bacterial tolerance	Grapevine defence and defence signalling in response to bacterial pathogens; pathology of the various bacterial pathogens; innate resistances (molecular basis) of various species towards bacterial pathogens	Anti-microbial peptides (lytic peptide, Shiva-I, defensins); dysfunctional import and integration protein encoding gene (virE2delB) from Agrobacterium
Viral tolerance	Epidemiology of virus infections and vectors; molecular biology on infecting virus; pathogen-derived resistance strategies (coat-proteins; movement proteins)	Virus coat proteins (translatable, anti-sense, non-translatable); virus movement proteins (anti-sense); replicase (RNA-dependent RNA polymerase), proteinases; 2,5 oligoadenylate synthase.
Improved stress tolerance		
Resistance to water stress	Aquaporins; isolation of root-specific promoters	TIPs (tonoplast integral proteins); PIPs (plasma membrane integral proteins)
Oxidative damage	Carotenoid biosynthesis and control (several putative genes and promoters have been cloned); anaerobiosis	Carotenoid biosynthetic genes; Adh (alcohol dehydrogenase) genes; SODs (cystosolic CuZnSOD, chloroplast-residing CuZnSOD, mitochondrial-residing MnSOD)
Osmotic stress and other abiotic stresses	Proline accumulation; polyamines and their role in stress	Vvp5cs (Δ^{1}-pyrroline-5-carboxylate); Vvoat (δ-ornithine aminotransferase); FeSOD, glycine betaine, antifreeze genes from Antarctic fish (freezing tolerance)
Improved quality factors		
Colour development	Ripening related processes and signals, anthocyanin biosynthesis and control (several genes and some promoters have been cloned); isolation of berry-specific promoters	ufgt (UDP-glucose:flavanoid 3-O-glucosyltransferase) and/or regulatory sequences of ufgt; production of pelargonidin-based anthocyanins for novel berry colour; anthocyanin methyl-transferases
Sugar accumulation and transport	Phloem loading/unloading; invertases; sugar transporters; isolation of berry-specific promoters	Invertases from plants and yeast to study phloem loading/unloading; sucrose transporters (Vvsuc11, Vvsuc12, Vvsuc27)**; h**exose transporters (Vvht1, Vvht2)
Reduced browning (table and dried grapes)	Oxidation reactions	Silencing of polyphenol oxidase
Seedlessness (table grapes)	Seed-formation; isolation of seed-specific promoters	Baranase gene

development has proved useful in this field. The distribution of carbon sources, the translocation of sugars, water transport and the role of aquaporins as well as the regulation of these processes are targets on which research has focused in order to improve this aspect of the industry (81). However, they are complex processes that are regulated by pathways and signals that are equally complex. More studies are required to obtain concrete results as they cannot be achieved simply by the insertion of one or more genes. Especially in this field, proteomics and metabolomics are likely to become increasingly important tools in the path to success.

The third major objective of genetic vine manipulation regards the improvement of its quality. The parameters that determine the quality vary depending on the market under consideration. In the wine industry, a measure of quality is an optimal ratio of sugars, acids and polyphenols, while in the fruit industry (grapes), factors such as bunch size and its appearance will have priority.

Molecular biology is currently involved in the study of berry maturation to influence processes like the production of colour and the development of the sugar content and flavours (81,82). In this regard, the study of the complete genome of the vine has revealed the existence of large families of genes related to the aroma of wine, which are present in a very high copy number.

This is the case, for example, for genes encoding stilbene synthase, an enzyme that drives the synthesis of resveratrol, the compound proposed as one of those responsible for the health benefits associated with the moderate consumption of wine, the famous French paradox (63). A similar situation was found for other families of genes involved in the synthesis of terpenes and tannins, the major components of aromas, resins and essential oils. The ultimate goal of this research is to affect the metabolic pattern of the biochemical pathways to increase the formation of products related to wine quality.

3.2. - Improvement in yeast

As mentioned previously wine yeasts are also subject of considerable research. Most of the research conducted on yeasts has concerned itself with the species *Saccharomyces cerevisiae* due to its dominant role during fermentation and to the fact that genome sequence is well characterized. It has a relatively small genome (13000 Kb), a large number of chromosomes (16 linear chromosomes that range in length from 200 to 2200 Kb), a small number of genes (6000 genes coding for proteins), not an extended repetitive DNA and few introns (27). Through genetic engineering of this organism, the improvement in both the economy of the process and the quality of the wine has been attempted (table 2) (54,56). Research targets have included the efficiency of fermentation, wine processing, control of microbiological contamination, as well as the healthiness and sensory characteristics of wine.

With regards to the efficiency of the fermentation process, attempts have been made to optimize the yeast in order to prevent incomplete fermentation, caused mainly by non-optimal utilization of carbon sources (glucose and fructose) and nitrogen. The main objective was therefore to regulate the flow of the glycolytic pathway through insertion of alleles effective on hexoses (mainly fructose) and capable of abating the nitrogen catabolite repression in the assimilation of proline and arginine, which account for 30% to 65% of the amino acid content of grape juice (79). Attempts have also been made to improve the uptake of substances such as sterols, the production

Table 2. Targets for the genetic improvement of wine yeast strains (54)

Desirable properties	Focus areas	Examples of potential target genes
Improved fermentation performance		
Improved general resilience and stress tolerance	Stress response, sterol, glycogen and trehalose accumulation	Modification of glycogen or trehalose metabolism [for example acting on GSY1 and GSY2 (glycogen synthase), TPS1 (trehalose-6-phosphate synthase), TPS2 (trehalose-6-phosphate phosphatase)]
Improved efficiency of sugar utilisation	Hexose transporters, hexose kinases	Overexpression and modification of HXT1-HXT18, SNF3, FSY1 and use of heterologous transporters and kinases
Improved efficiency of nitrogen assimilation	Improved utilisation of less efficient N-sources	Proline catabolism [PUT1 (proline oxidase) and PUT2 (pyrroline-5-carboxylate dehydrogenase)] and use of heterologous catabolic genes
Improved ethanol tolerance	Sterol formation, membrane ATPase activity	Modification of the expression of PMA1 and PMA2 (ATPase), sterol anabolic genes
Increased tolerance to antimicrobial compounds	Resistance to killer toxins, sulphur dioxide, agrochemicals	Inclusion of KIL2 (zymocin and immunity factor), overexpression of CUP1 (copper chelatin)
Reduced foam formation	Cell surface proteins	Deletion of FRO1 and FRO2 (froth proteins)
Improved processing efficiency		
Improved protein clarification	Proteases	Overexpression of PEP4 (protease A) and secretion of other proteases
Improved polysaccharide clarification	Glucanases, pectinases, xylanases, arabinfuranosidases	Overexpression of END1 (endoglucanase), EXG1 (exoglucanase), CEL1 (cellodextrinase), BGL1 (β-glucosidase, cellobiase), PEL5 (pectate lyase) and PEH1 (polygalacturonase), XYN1-5 (xylanases), ABF2 (arabinofuranosidase)
Controlled cell sedimentation and flocculation	Flocculins	Late expression of flocculation genes (FLO1, FLO5, MUC1/FLO11) under control of promoters (HSP30) imparting desired expression
Controlled cell flotation and flor formation	Cell wall hydrophobic proteins	Late expression of MUC1/FLO11 under control of promoters (HSP30) imparting desired expression pattern
Improved biological control of wine spoilage microorganisms		
Wine yeasts producing antimicrobial enzymes	Lysozyme, glucanases, chitinases	Expression of HEL1 (hen egg white lysozyme), CTS1 (chitinase), EXG1 (exoglucanase) and other antimicrobial enzymes
Wine yeasts producing antimicrobial peptides	Bacteriocins	Expression of PED1 (pediocin), LCA1 (leucocin) and other heterologous bacteriocin and zymocin genes
Wine yeasts producing sulphur dioxide	Sulphur metabolism and SO_2 formation	Overexpression of MET14 (adenosylphosphosulphate kinase) and MET16 (phospho adenosylphosphosulphate reductase), and deletion of MET10 (sulphite reductase)
Improved wine wholesomeness		
Increased production of resveratrol	Stilbene synthesis	Expression of 4CL9/216 (co-enzyme A ligase), VST1 (stilbene synthase)
Reduced formation of ethyl carbamate	Amino acid metabolism, urea formation	Deletion of CAR1 (arginase) or expression of URE1 (urease)
Reduced formation of biogenic amines	Bacteriolytic enzymes, bacteriocins	Expression of HEL1 (hen egg white lysozyme), PED1 (pediocin), LCA1 (leucocin) and other bacteriocins

Decreased levels of alcohol	Carbon flux, glycerol metabolism and glucose oxidation	Overexpression of GPD1 and GPD2 (glycerol-3-phosphate dehydrogenase), modification of FPS1 (glycerol transport facilitator), expression of GOX1 (glucose oxidase)
Improved wine flavour and other sensory qualities		
Enhanced liberation of grape terpenoids	Glycosidases, glucanases, arabinofuranosidases	Overexpression of END1 (endoglucanase), EXG1 (exoglucanase), CEL1 (cellodextrinase), BGL1 (β-glucosidase, cellobiase), PEL5 (pectate lyase) and PEH1 (polygalacturonase), ABF2 (arabinofuranosidase)
Enhanced production of desirable volatile esters	Esterases	Modified expression of ATF1 (alcohol acetyl transferase) and other alcohol transferases, IAH1 (esterase) and other esterases
Optimised fusel oil production	Amino acid metabolism	Deletion of the ILE, LEU and VAL genes
Enhanced glycerol production	Glycerol metabolism	Overexpression of GPD1 and GPD2 (glycerol-3-phosphate dehydrogenase), FPS1 (glycerol transport facilitator), and deletion of ALD6
Bio-adjustment of wine acidity	Maloethanolic and malolactic fermentation, lactic acid production	Expression of MAE1 (malate permease), together with MAE2 (malic enzyme) or mleS (malolactic enzyme), or LDH1 (lacticodehydrogenase)
Optimisation of phenolics	Phenolic acid metabolism	Modified expression of PAD1 (phenyl acrylic acid decarboxylase), pdc (ρ-coumaric acid decarboxylase), padc (phenolic acid decarboxylase)
Reduced sulphite and sulphide production	Sulphur metabolism, hydrogen sulphide formation	Deletion of MET14 (adenosylphosphosulphate kinase) and MRX1 (methionine sulphoxide reductase)

of trehalose and glycogen and the over-expression of aquaporins, all factors involved in the increasing the resistance of *S. cerevisiae* to stress (53). Yeast can also affect the efficiency of the wine process when it is subjected to fining (addition of compound adsorbent followed by precipitation) and clarification (sedimentation, centrifugation, filtration, etc.) after fermentation. Novel yeasts strains have been created which are capable of secreting polysaccharolytic and proteolytic enzymes that degrade proteins numbing the wine, and polysaccharides obstructing the membranes used in filtration, or which are able to precipitate or flocculate at the end of fermentation, simplifying the clarification process (47).

Moreover, yeast strains have been created which can secrete antimicrobial enzymes (lysozyme, chitinases, endoglucanases) and peptides (zymocines and bacteriocines), allowing a reduction in the dose of chemical preservatives such as sulphur anhydride, dimethylcarbonate, benzoic acid, fumaric acid and sorbic acid, whose excessive use damages the quality of wine.

The metabolism of yeast can be manipulated so as to reduce the presence of undesirable compounds (suspected carcinogens such as ethylcarbamate or neurotoxins such as biogenic amines), modulate the concentration of ethanol or produce beneficial substances (resveratrol, carnitine, etc.).

Last but not least, organoleptic quality (appearance, aroma and taste) is a parameter which is highly influenced by yeast. Strains of *S. cerevisiae* have been engineered to secrete enzymes that can enhance flavour and colour (pectinase, glycosidase,

glucanase) and foreign-modifying enzyme activities (alcohol acetyltransferases, esterases, isoamyl acetate hydrolases etc.) to produce optimum quantities of glycerol, higher alcohols, phenolic acids and organic acids (79).

3.3 Limits to the application of Biotechnology

Despite the scientific progress and accumulation of knowledge described here relating both to yeast and grapes, the 21st century wine industry is yet to adopt, on an industrial-scale, a vine cultivar that is genetically modified or a recombinant yeast strain, and for very specific reasons (58).
The legislation is very often reluctant to allow the use of genetically modified organism (GMO) in agribusiness, and if they are eligible, they must meet the requirement of "substantial equivalence" regarding food and beverages. Furthermore, they must provide the obvious guarantees such as the full definition of the integrated DNA, removal of any sequence which is not essential to expression, the absence of any selective advantage of GMO that could confer dominance in a natural habitat, no risk for human health or environment and demonstrate a clear benefit for both the producer and the consumer.
It is important to consider that many tools used by genetics (DNA sequences, promoters, markers and vectors) as well as the patent that protects the protocols, may be obstacles to the commercialisation of modified vines and yeast. On the other hand, depending on the laws of each country, the vines and yeasts that are genetically modified have to be protected by their developers through patents or other strategies (82). The impact on the market is another factor to consider: key to the marketing strength of a wine is the strong identity of its product, based on the name and prestige of a few selected cultivars. It is the name of the grapevine that is used to distinguish the wine and it is the main source of information on the label for the consumer, so the wine industry is reluctant to introduce new varieties. There is uncertainty about how to identify new varieties: whether to use the same name of the original unmodified cultivar or to give them new names. This, along with the consumer's preference for specifically labelling products obtained through gene technology, leads to hesitation among producers. In this context, they fear that genetics could accelerate the trend towards standardization of wines to suit the tastes of the average consumer in the global market.

4- Indigenous yeasts: the egg of Columbus?

It is clear that the world of wine is currently subject to the opposing stresses of a transition phase: on the one hand, the need for the industry to implement new strategies in a near saturated market juxtaposed to the need to retain the richness of tradition. On the other, the modern consumer is more conscious and tends to prefer products of high quality and with characteristics of typicality and originality. Biotechnology has tried to build a link between these two poles through genetic engineering both of vine and yeast, but the results often clash with the mistrust of the

consumer and the prohibition of laws, in the alimentary sector, that is traditionally conservative.

How can the needs of both parties be combined? The answer lies, as often happens, before our eyes. It is well known that the world's best wines are almost all produced with spontaneous fermentation, using only the yeasts already present in the grapes. Look at the case for instance in Italy of Chianti or Barolo, or in France of Château Lafite-Rothschilds, Château Margaux and the other well-known Premier Cru wines. The complexity of the aroma of these great wines is due to the diversity of the spontaneous microflora, but this type of fermentation is not a standardised process. It is rather erratic. This is why there are good years as well as bad vintages.

In spontaneous fermentation there is a sequential growth of yeasts, in addition to the previously mentioned *Saccharomyces spp.*, belonging to the non-*Saccharomyces* genera of *Kloeckera spp.*, *Hanseniaspora spp.*, *Candida spp.*, *Metschnikowia spp.*, *Pichia spp.*, *Rhodotorula spp.* that predominate in the initial stages, until the ethanol reaches the 3-4° mark. These types of yeasts are known as major producers of acetic acid and off-flavour, and that is why they are industrially removed and replaced with selected strains. The successive stages of a spontaneous fermentation, as mentioned above, see the dominance of *S. cerevisiae*, known universally as "wine yeast" (12,13).

Today, research has shown that indigenous yeasts have been ignored for too long and with them, their biodiversity. In recent years, some researchers have offered evidence how wine quality can be improved (18,22,35,59,67,76,77). These studies are based on the dated idea that the best yeasts capable of fermenting the wine are on the grapes. Time, as well as environmental factors in the vineyards, would influence the selection of microorganisms suitable for this purpose.

Regarding indigenous yeasts of the genus *Saccharomyces spp.*, the collective literature regards the parameters usually considered in the selection of an industrial strain (fermentative power, resistance to SO_2, etc.), their persistence in the cellar and their interaction with industrial strains. Yeasts have also been studied for the presence of unwanted enzymatic activities, the production of phenolic off-flavour (POF) as the 4-vinyl guaiacol, 4-vinylphenol and 4-ethylphenol, or other metabolites (73). Further analyses have been conducted on non-*Saccharomyces* yeasts. The enzymatic potential of this heterogeneous group of yeasts, which used to be unknown, have been subject of significant research. The data obtained so far show the presence of enzymatic activities in far greater number than *Saccharomyces spp.,* and that these, among others include proteases, pectinases, lipases, glycosidases, and esterases (20,41). In addition, these yeasts, unlike *Saccharomyces spp.,* are often able to secrete the enzymes that they produce, simplifying their characterisation and subsequent cloning.

These enzymes, derived from microorganisms adapted to the conditions of the must, may well possess features such as low pH optimum, resistance to high concentrations of solutes and ethanol, good activity at room temperature, all qualities that may be useful in fields other than oenology. Some studies have also investigated the influence of non-*Saccharomyces spp*. on the aroma of wine or on sensory qualities, focusing on the production of acetic acid, ethyl acetate, acetone, glycerol and ethanol, alone or in conjunction with *Saccharomyces* yeasts, but they have not carried out in-depth analyses (73,77,83,84,85). Finally, some authors have reported that these yeasts may be responsible for the unique features that bind a wine to its growing region (68). The study of these indigenous yeasts, as previously mentioned, could lead to a way to respond to market demand without rejecting tradition, rather

rediscovering it. Moreover, this will minimise legal obstacles and allow each area to maintain its own strongest typicality.

In this study, two vines have been selected from Irpinia, a region possessing a well established wine craft. The first variety is Aglianico, a red grape variety of ancient Greek origin, characterised by good vigour, abundance and constant productivity. It prefers hilly lands of volcanic origin, but adapts well to different soil types. Its grapes have small and medium sized berries, spherical with pruinose skin, blue-black coloured, with little consistency and medium thickness. They are used exclusively for winemaking, preferably alone.

Fig.1: The Aglianico vineyard at the ATI "F. De Sanctis", in Avellino

It is from this grapevine that Taurasi is obtained, an important DOCG wine, described as having an intense ruby red colour, a dry, full, harmonious taste and a distinctive ethereal and pleasant smell with hints of chocolate and plum. Given the high concentration of tannins, the Taurasi is aged for at least three years before being put on sale.

The second variety selected is the Fiano, a white grape variety derived from the ancient "Vitis Apicia", imported from the Greeks. The name "Vitis Apicia" or " Apina" derives from the typical characteristic of this grape: it has such a sweet fragrance that it attracts swarms of bees in vineyards. The berries of the grapes are medium sized, ellipsoidal, with resistant golden yellow peel, and slightly pruinose. It has good vigour and gives excellent results in soils of volcanic origin. It produces the wine Fiano di Avellino, DOCG too, a dry white wine, with straw yellow colour, more or less intense. It tastes harmonious, balanced, rich in nuances of dried fruit and has a pleasant smell with a predominant hint of toasted hazelnut.

The aim of the research described here is to study the microbial biodiversity of these two vines, especially of the indigenous yeasts, belonging both to the genera *Saccharomyces spp.* and non-*Saccharomyces spp.*, isolated from grapes and must samples. Primarily, the study concerns the creation and characterisation of an appropriate strain collection and subsequent characterisation of parameters that are industrially useful. Secondly, the study examines the influence of some of these yeasts on wine fermentation, focusing on a biochemical characterisation of final

products of fermentation, with particular emphasis on polyphenols and aromas, using the wines produced by industrial strains as a basis for comparison. Attention will be also drawn to the influence of these yeasts on organoleptic properties. Finally, a further aim has been to explore and characterise the enzymatic potential of selected yeasts for their possible use in the oenological field.

Materials and Methods

1- Grape and must sampling from Aglianico and Fiano

Sampling of grapes and musts (19) was carried out using equipment belonging to the Agricultural Technical Institute "F. De Sanctis' in Avellino. The Aglianico vineyard is situated in the “Cappuccini” area in Avellino, on a hilly terrain located 350 meters above sea level, exposed to the south-west. The terrain has a sandy and silty texture, is deep, and has a moderately coarse, well-drained soil skeleton. The density is 3,000 vines per hectare and the simple Guyot farming system is employed. The Fiano vineyard is located close to that of the Aglianico at “Le Torrette” on a very similar type of soil, albeit exposed to the southwest-west, cultivated using the same methods and with the same stocking density.

Grape sampling was carried out in 2007 and in 2008, in the period from June to September, in order to obtain a sufficiently representative range of the various states of fruit ripeness, from fruit setting (initial fruit development stage which occurs after flowering) to the stage of fruit ripening during which the colour of the epicarp changes, up until the ripe grapes immediately prior to harvest. Sampling was carried out in order to have a sample that was representative of the plot investigated. The clusters were cut so as to cause no external contamination, were placed in sterile plastic bags and stored in refrigerated containers prior to transportation to the laboratory for analysis. As for the must, both the Aglianico and Fiano were collected at the ATI "F. De Sanctis” experimental winery. Sampling was carried out following the pressing of the grapes, from the filled vats that had not been inoculated with yeast or during the fermentation. Once extracted, the samples were collected in sterile containers and kept cold prior to analysis in the laboratory.

2- Yeast isolation

Once in the laboratory, grape samples were cut into branches and berries, with scissors previously dipped in alcohol, and placed in sterile bags. The Ringer solution (8.5 g/l NaCl and 1 g/l peptone) was then added to each (10g) sample at a ratio of 1:2 and the sample homogenized for 30s at 230 rpm by way of a Stomacker homogenizer (Seward, UK).

The sample then underwent various dilution tests in order to reach a concentration suitable for counting and 0,1 mL was spread onto plates with different media (Oxoid, Hampshire, UK) :

• **YPD**: a complete medium which allows growth of yeasts, moulds and bacteria (dextrose 20 g / L, peptone 20 g / L, yeast extract 10 g / L, agar 20 g / L).

• **WL nutrient medium**: complete medium allowing growth of the main species of yeast and other microorganisms, but the colonies develop specific colours allowing an initial differentiation (yeast extract 4 g / L, tryptone 5 g / L, glucose 50 g / L; potassium bisphosphate 0.55 g / L, potassium chloride 0.425 g / L, calcium chloride 0.125 g / L, magnesium sulphate 0.1125 g / L, ferric chloride 0.0025 g / L; manganese sulfate 0.0025 g / L; bromocresol green 0.0022 g / L, agar 15 g / L)

• **Lysine medium** is a selective medium because it contains nitrogen as the only form of lysine and therefore should not allow growth of the *Saccharomyces* spp. genus (dextrose 44.5 g / L, monoacidic potassium phosphate 1.78 g / L, magnesium

sulphate 0.89 g / L, calcium chloride 0.178 g / L, sodium chloride 0.089 g / L; adenine 0.00178 g / L, DL-methionine 0.000891 g / L, L-histidine 0.000891 g / L , DL-tryptophan 0.000891 g / L, boric acid 0.0000089 g / L, zinc sulphate 0.0000356 g / L, ammonium molybdate 0.0000178 g / L, manganese sulphate 0.0000356 g / L, ferrous sulphate 0.0002225 g / L; lysine 1 g / L; inositol 0.02 g / L, calcium pantothenate 0.002 g / L; aneurine 0.0004 g / L, pyridoxine 0.0004 g / L; acid p - aminobenzoic 0.0002 g / L, nicotinic acid, 0.0004 g / L; riboflavin 0.0002 g / L; biotin 0.000002 g / L; Folic acid 0.000001 g / L, agar 17.8 g / L).

The plates were incubated at 28 °C for 5 days, the colonies counted and the randomly isolated yeasts were picked and stored on YPD slants at 4°C.

3-Yeast Identification

3.1-Biochemical and morphological identification

The strains of yeast isolates were classified according to colony colour and morphology on WL, as well as by their microscopic and biochemical characteristics. The yeast cultures were inoculated on YPD broth and incubated at 28°C for 24 hrs. They were used to prepare the slides for observation at the microscope (Olympus optical BX40, Japan) to determine the shape of the cells.
As far as the microscopic characteristics are concerned, the following parameters were analyzed:

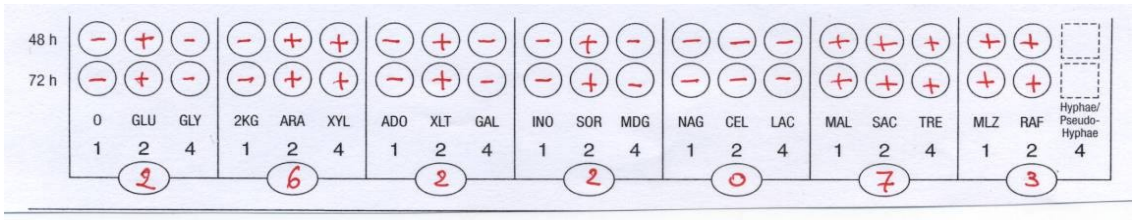
- Morphological analysis (apiculate, ellipsoid, coccoid)
- Multiplication method (division or budding)
- Ability to sporulate
- Number of spores per ascus

Sporulation capacity was tested on sodium acetate agar medium (4 g sodium acetate / L, dextrose 1 g / L, agar 15 g / L). An amount of yeast cells was spread on this solid medium and were incubated at 28°C for 15 day.
For the analysis of biochemical characteristics, API 20 C AUX kits supplied by Bio-Merieux were used, in which identification was made based on the ability of the microorganism to metabolize specific substrates represented by the different types of sugars in the table 3. The gallery consists of 20 strips containing dehydrated substrates for the execution of 19 assimilation tests. The strips were filled with minimal semi-agarised medium which had been previously inoculated with the microorganism, and the growth achieved was verified on the basis of the substrates that the yeast was able to use. The growth achieved was verified by comparison with the control growth (containing no substrate), while the identification was undertaken using an analytical index. The strips were filled with an inoculum obtained by transferring a small quantity of a colony from the YPD medium plate and resuspended in 3 ml of saline to a vial containing the C API medium to achieve a level of turbidity of 2 on the McFarland scale (0.5 OD at 625 nm). The gallery, placed in a container closed by a lid, was incubated at 28 °C in an incubator for 48-72 hours.

Test	Substrate	Quantity (mg/cup)
0	None	1.2
GLU	D-glucose	1.2
GLY	Glycerol	1.2
2KG	2-cheto calcium gluconate	1.2
ARA	L-arabinose	1.2
XYL	D-xylose	1.2
ADO	Adonitol	1.2
XLT	Xylitol	1.2
GAL	D-galactose	1.2
INO	Inositol	1.2
SOR	D-sorbitol	2.36
MDG	alpha-methyl glucopyranoside	1.2
NAG	N-acetyl glucosamine	1.2
CEL	D-cellobiose	1.2
LAC	D-lactose (of bovine origin)	1.2
MAL	D-maltose	1.2
SAC	D-sucrose	1.2
TRE	D-trehalose	1.2
MLZ	D-melezitose	1.2
RAF	D-raffinose	1.9

Table 3: Different substrates in the API 20 C AUX kit (Bio-Merieux)

After 48 hours of incubation (or 72 hours, if tests, particularly glucose, were inconclusive), growth of yeast compared to a negative control was assessed. A more turbid strip than the control indicated a positive reaction, which was then recorded on a results card. Microorganism identification was achieved by constructing a numerical profile on the results sheet. On the latter, test results were reported which were separated into groups of three, with each test being awarded a value of 1, 2 or 4. By addition of test values corresponding to positive reactions within each group, a 7-digit profile number was obtained. This profiling allowed the genus and species of each yeast species of interest to be categorized according to an analytical index. The following is an example of the card:



3.2-Molecular identification of yeasts

In order to unambiguously determine the yeasts belonging to a particular species, the D1/D2 region of 26S rDNA gene consisting of a 600 bp region was sequenced. This was achieved by PCR using yeast cells obtained directly from a colony on YPD medium plates. The standard primers used, commonly referred to as NL1 and NL4 in the literature (4,17), have the following sequences:

NL1 5'-GCATATCAATAAGCGGAGGAAAAG-3'

NL4 5'-GGTCCGTGTTTCAAGACGG-3'

The PCR reaction was conducted using the EuroTaq polymerase (Euroclone) with a Mastercycler Personal thermocycler (Epphendorf, Hamburg, GE). The reaction mix was as follows:

73.8 µl H2O
5.0 µl dNTP (10 mM)
3.0 µl $MgCl_2$ (50 mM)
2.5 µl primer NL1
2.5 µl primer NL4
10.0 µl 10X PCR buffer
1.2 µl ETaq
Total volume 98.0 µl

The PCR reaction was performed using the following parameters:

Stage	Number of Cycles	Temperature	Time (minutes)
1	**1**	**94 ° C**	**15**
2	**40**	**94 ° C** **55 ° C** **72 ° C**	**1** **2** **2**
3	**1**	**72 ° C**	**10**

The first extended cycle was included in order to lyse the cells, and the polymerase was added immediately prior to stage 1 completion. Sequencing was carried out using the Sanger method with a 3730 Genetic Analyzer DNA sequencer (Applied Biosystems).

4- Selection of yeasts

4.1-Selection for resistance to sulfur dioxide

The strains of yeast isolates were tested for resistance to two concentrations of sulfur dioxide, 100 ppm and 250 ppm (14,39,62). The tests were conducted on the 2007 vintage Fiano musts pasteurized for 30 ' in tubes stoppered with cotton wool. Prior to inoculation, strains were grown in YPD at 28 °C under agitation at 160 rpm in a shaker incubator (New Brunswick Inc., USA) until early exponential phase (A_{600}nm = 0.4-0.6) and the inoculum was added at a concentration of 1% of the total (10 ml) . Potassium metabisulfite (MBK) was used as the source of sulphur dioxide.

4.2-Selection for the secretion of a β-glucosidase

The yeast strains were grown for 24 hours in YPD liquid medium at 28 °C with agitation at 160 rpm in a shaker incubator. Cultures were then centrifuged at 13000 rpm for 15 min and the supernatant isolated. A test for the presence of β-glucosidase activity was performed using a colorimetric assay consisting of para-nitrophenyl-β-D-

glucopyranoside (PNPG) as a substrate; in the presence of β-glucosidase, it releases para-nitrophenol as a chromophore, which can be measured by spectrophotometry at 400nm. The test was conducted in 30 mM phosphate-citrate buffer at pH 7 and pH 3.4, at a temperature of 30 °C. The reaction mix was prepared as follows:

600 µl 50 mM phosphate citrate buffer (pH 7 or pH 3,4)
200 µl pNPG (4.5 mg / ml)
200 µl supernatant
1 ml Total volume

The assay was carried out by allowing the reaction to progress for a period of 1 hour and then blocked by addition of 200 µl of 1.2 M Na_2CO_3. The readings were carried out at 400nm and water instead of supernatant was used in the blank.

5-Technological cycle of wine production

5.1-Aglianico

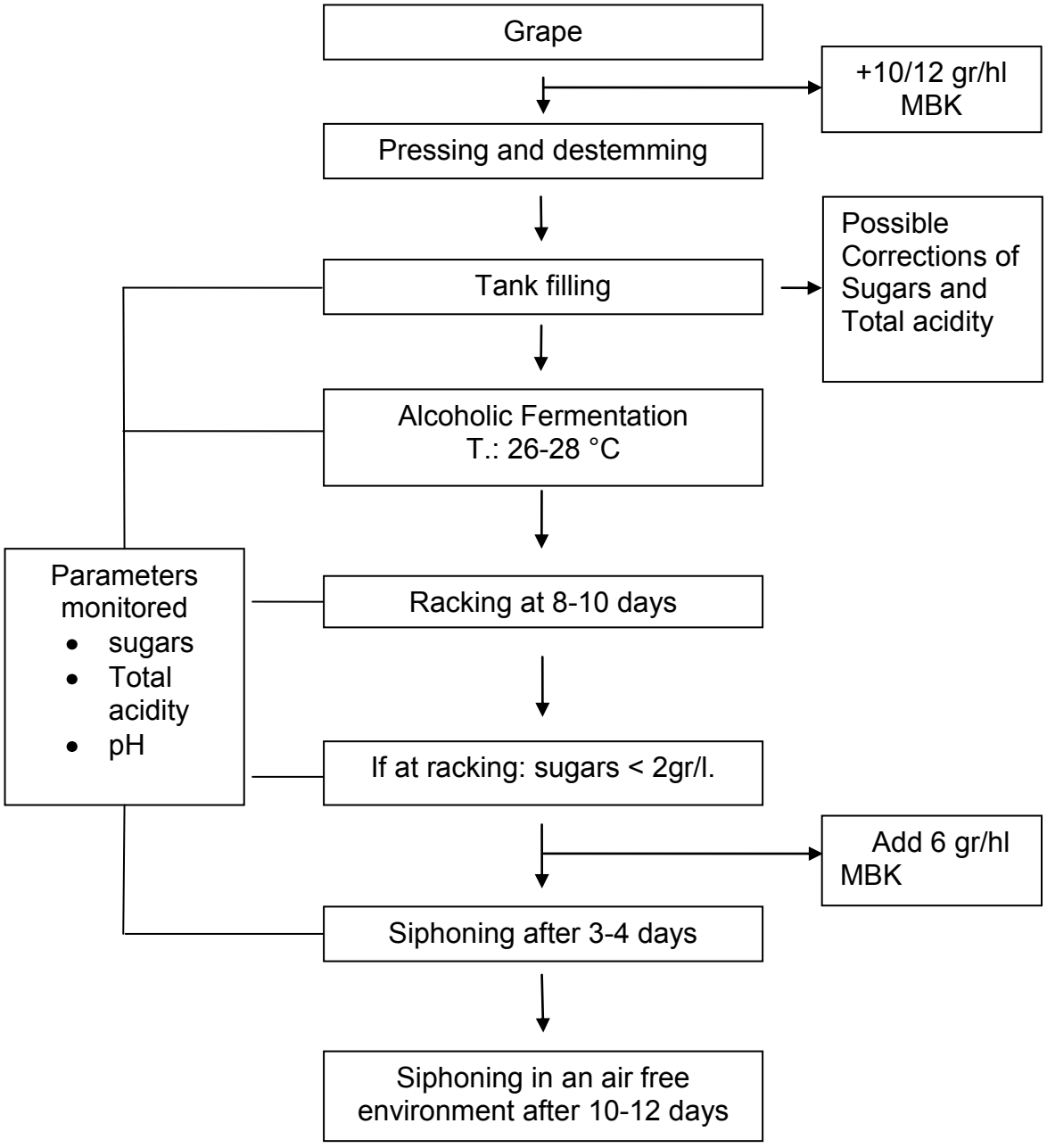
The grapes harvested at the vineyards of the Agricultural Technical Institute "F. De Sanctis' in Avellino in the Cappuccino area, were, immediately after harvest, transported to the experimental winery of the institution and, through destemming machines, the musts were separated from the stems. 10 g / hl of MBK was then added and the tanks gradually filled. Once the tanks had been filled, parameters including reducing sugars, total acidity, pH, free sulfur dioxide, and total sulfur dioxide were monitored and then inoculated with 20g/hl reactivated dried *Saccharomyces cerevisiae* (var. bayanus) yeast and Oenoferm Freddo® (Erbslöh Geisenheim AG, Geisenheim, Germany). For experimental fermentations, selected indigenous yeasts (each at 20 g / hl) were also added. Seven fermentations were carried out for the Aglianico:

- Aglianico 2007 vintage inoculated with commercial yeast
- Aglianico 2007 vintage inoculated with commercial yeast and *Rhodotorula mucillaginosa* AGWLR12 experimental yeast
- Aglianico 2008 vintage inoculated with commercial yeast
- Aglianico 2008 vintage inoculated with *Saccharomyces cerevisiae* AGYP37 experimental yeast
- Aglianico 2008 vintage inoculated with commercial yeast and *Metshnikowia pulcherrima* AGYP28 experimental yeast
- Aglianico 2008 vintage inoculated with experimental yeast and Saccharomyces *cerevisiae* AGYP37 and *Metshnikowia pulcherrima* AGYP28 experimental yeasts
- Aglianico 2008 vintage inoculated with commercial yeast, and *Saccharomyces cerevisiae* AGYP37 and *Metshnikowia pulcherrima* AGYP28 experimental yeasts

After inoculation, alcoholic fermentation was conducted at a temperature of 26-28 °C with three fullings per day. Fulling is the resubmersion of the marc ‘hat’ that floats in

the vats and its aim is to enhance color extraction from the skins and to micro-oxygenate the reservoir. Residual sugar, total acidity and pH were monitored during the fermentation. 8-10 days after the initiation of the process the must was separated from the grape skins (by racking) and, if the residual sugar was found to be less than 2,5 g / L, 6 g / hl of MBK was added, or fermentation left to progress until this value was reached. After 3-4 days of having carried out an initial racking the wine was siphoned off in order to prevent the accumulation of averse odours and grape residues, and after a further 10-12 days, it was siphoned again in an air free environment in order to remove the sediment. Each experiment was conducted in triplicate. The protocol for Aglianico winemaking is illustrated in figure 2.

Fig. 2: Protocol of winemaking 'Aglianico



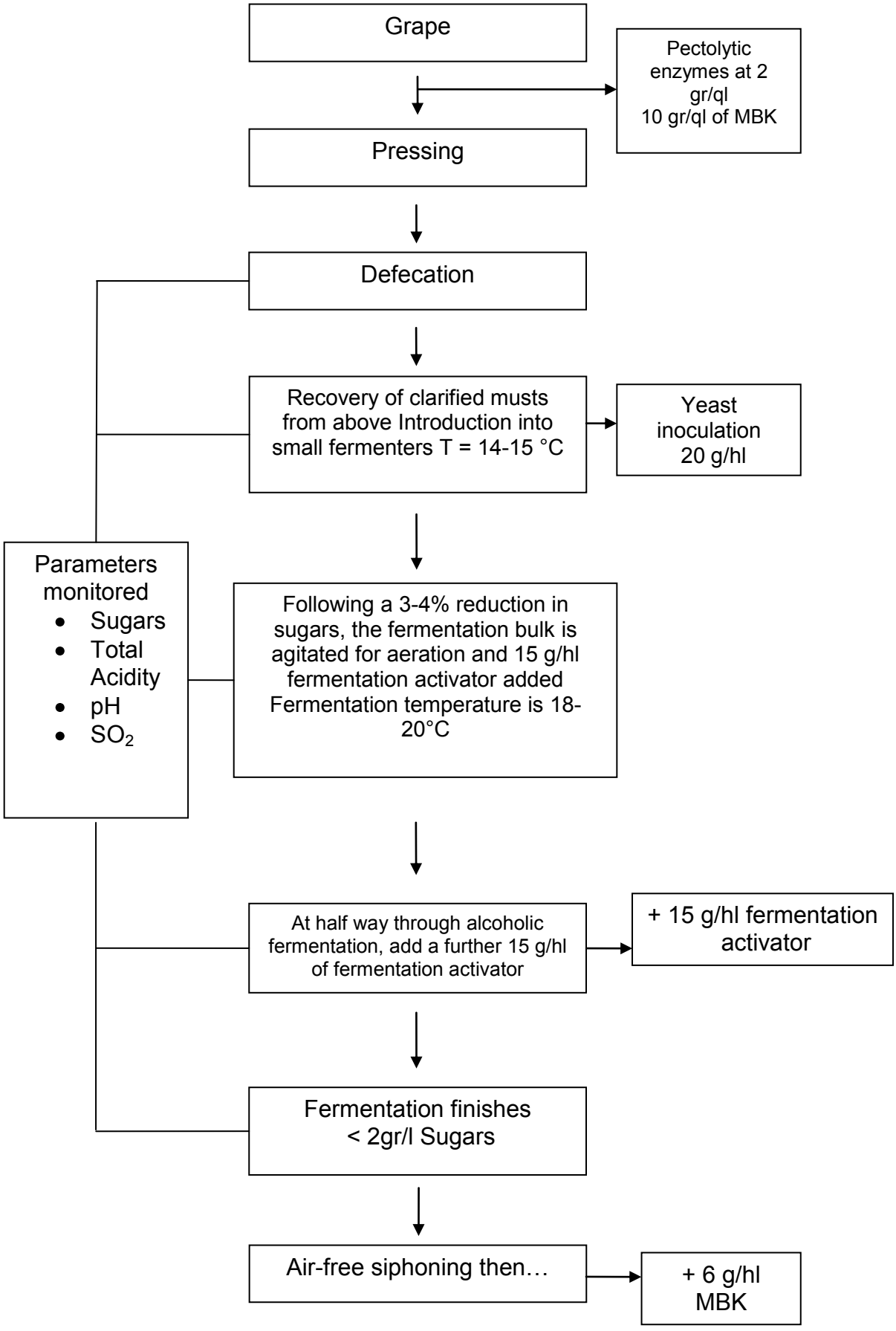
5.2-Fiano

Grapes were harvested at the vineyards of the Agricultural Technical Institute "F. De Sanctis' in Avellino in the "Torrette" area and, immediately after harvest, transported to the experimental winery of that institution. After pressing, pectolytic enzymes (Trenolin Opti® DF pectolytic enzyme, AG Erbslöh Geisenheim, Geisenheim, Germany) and 10 g / hl of MBK were added, and the must was then left to defecate. Following clarification, it was recovered from the defecation reservoirs and introduced into the vats with an inoculum of 20 g/hl reactivated dried yeast *Saccharomyces cerevisiae* (var. bayanus) and Oenoferm® Freddo (AG Erbslöh Geisenheim, Geisenheim, Germany). For experimental fermentations, selected indigenous yeasts (each at 20 g/hl) were also added. Seven fermentations were carried out for the Fiano:

- Fiano 2007 vintage inoculated with commercial yeast
- Fiano 2007 vintage inoculated with commercial yeast and *Rhodotorula mucillaginosa* AGWLR12 experimental yeast
- Fiano 2008 vintage inoculated with commercial yeast
- Fiano 2008 vintage inoculated with *Saccharomyces cerevisiae* FYP69 experimental yeast
- Fiano 2008 vintage inoculated with commercial yeast and *Hanseniaspora guilliermondii* FWL66 experimental yeast
- Fiano 2008 vintage inoculated with *Saccharomyces cerevisiae* FYP69 and *Hanseniaspora guilliermondii* FWL66 experimental yeasts
- Fiano 2008 vintage inoculated with commercial yeast, *Saccharomyces cerevisiae* FYP69 *Hanseniaspora guilliermondii* FWL66 experimental yeasts

The fermentation was conducted at an initial temperature of 14-15 °C but, following a 3-4 % decrease in sugars, the must was aired, 15 g / hl of fermentation activators added (VitaDrive®, Combi® Vitamon, Erbslöh AG Geisenheim, Geisenheim, Germany) and the temperature raised to 18-20 °C. During this process, the levels of sugars, total acidity and pH were monitored and when the fermentation was approximately half way through its course, a further 15 g/hl of fermentation activators were added. When the sugar content was less than 2,5 g/l, an additional 6 g/hl of MBK was added and the wine siphoned off in an air free environment. Each experiment was conducted in triplicate. The protocol of Fiano winemaking is illustrated in figure 3.

Fig. 3: Protocol of wine Fiano



6-Analysis of wine samples

6.1-Analysis of sugars

The analysis of sugars on the must and wine samples was carried out using two methods: by densitometry and by an enzymatic method. Densitometric analysis was conducted using a Babo mustimeter. A sample of must or wine stripped of grape skins was placed in a glass cylinder. The Babo mustimeter is slowly immersed into the solution and allowed to float. The value of the scale at the flotation level, allows determination of the amount of sugar (w/w) which, multiplied by the value of the density given by the mustimeter, gives the sugar content (w / v). As for the enzymatic analysis of sugar residues, this was undertaken using a glucose and fructose determination kit (Paramedical Ltd.), according to the manufacturer's instructions. In short, in the presence of ATP, NADP and G6PDH (glucose-6P-dehydrogenase) and HK (hexokinase), glucose produces NADPH. The intensity at 340 nm is proportional to the concentration of glucose in the sample.
The hexokinase may also phosphorylate fructose. By addition of PGI (phosphoglucoisomerase), new NADPH is produced by the conversion of fructose to glucose-6P. The resulting increase in UV at 340nm is proportional to the concentration of fructose in the sample. The reaction was carried out at 37 °C. The tests were repeated in duplicate. The determination of the concentration of glucose and fructose was performed by interpolation on a calibration curve obtained using solutions of known glucose concentrations (25-400 mg / L, R^2 = 0.9948, y = 0.0008 x + 0.005).

6.2- Determination of alcohol content

The alcohol content of all the wines was determined by means of a Malligand ebulliometer (Tecnolab, Belpasso, CT, Italy).

6.3-Analysis of total acidity

Total acidity was measured using a titrimetric method in which 25 ml of sample was diluted 1:2 with dd H_2O and, under constant stirring, 4N NaOH was added until the pH value reached 7. The number of NaOH milliequivalents required to neutralize the solution, compared to that required for a one litre volume, equates to the titratable acidity in milliequivalents of tartaric acid per litre (mEq/l). Milliequivalents can be converted into grammes, by considering that a tartaric acid equivalent weighs 75g.

6.4- Analysis of acetic acid

Acetic acid in wine samples was determined with a special kit (Paramedical srl). In short, in the presence of acetic acid, ATP, NAD and CS (citrate synthase) and ACS (acetyl-CoA synthetase) enzymes, NADH is produced and can be measured by absorbance at 340 nm. The reaction temperature is 37 °C. The tests were repeated in duplicate. The determination of the concentration of glucose and fructose was performed by interpolation on a calibration curve obtained with solutions of known

concentrations of acetic acid (50-500 mg/L, y = 0,0007 x + 0,0265, R^2=0,9935).

6.5-Analysis of total polyphenols

Total polyphenol content of wines was determined using the Folin-Ciocalteu method adapted to a micro scale (43). 0.79ml distilled water, 0.01ml appropriately diluted sample, and 0.05ml Folin-Ciocalteu reagent were combined in a 1.5ml polypropylene tube and vortexed. Aglianico and Fiano wine samples were diluted 20 and 3 times respectively with dd H2O. After exactly 1 min, 0.15ml of sodium carbonate (20%) was added, the mixture vortexed and allowed to stand at room temperature in the dark for 120 min. The absorbance was read at 750 nm, and the total polyphenol concentration was calculated from a calibration curve using gallic acid as standard (60-500mg / L, R^2 = 0,9918, y= 0,001x + 0,0253). Results were expressed as mg / L gallic acid equivalents (GAME).

6.6-Total flavonoid assay

Total flavonoid content was measured by the aluminium chloride colorimetric assay (42). Samples of Aglianico wine were diluted 1:9 with methanol (final concentration 80 %) while the Fiano samples were diluted 1:4. An aliquot (1 ml) of diluted sample or standard solution of catechin (10, 40, 80, 120, 150 and 200 mg/L in 80 % MeOH) was added to 10 ml volumetric flask containing 4 ml of dd H_2O. 0.3 ml 5% $NaNO_2$ was then added to the flask. After 5 min, 0.3 ml 10 % $AlCl_3$ was added. After 6 minutes, 2 ml 1 M NaOH was added and the total volume was made up to 10 ml with dd H_2O. The solution was mixed thoroughly and the absorbance measured against a blank prepared at 510 nm. The results were corrected for dilution and expressed as mg/l of catechin.

6.7-Determination of total and coloured anthocyanins

Measurements were performed using a well-established spectrophotometric method (3,42,74). The wine sample was placed in a 0.2-cm path length quartz cuvette, and the absorbance measured at 520 nm (A_{520}). Following this, 0.02ml of a 20 % sodium metabisulphite solution was added, the sample was mixed thoroughly, and after 1 min the absorbance read at 520nm (A_{520} SO_2). 12 % ethanol was used as a blank. All measurements were corrected to a 1.0 cm path length. Further, wine (0.02 ml) was mixed with 0.98ml 1 N HCl solution (dilution 1:50) in a 1.5-ml Eppendorf tube, vortexed, and allowed to stand for 180 min at room temperature. The absorbance was read at 520nm (A_{520}HCl) using a 1.0 cm path length cuvette. For the blank, 0.02 ml of 12 % ethanol was used instead of wine. The concentration of total anthocyanins (TA) and coloured (ionized) anthocyanins (AC) was calculated as follows:

$$TA\ (mg\ L\text{-}1) = 20 \times [A_{520}HCl - (5 / 3) \times A_{520}\ SO_2]$$

$$CA\ (mg\ L\text{-}1) = 20 \times (A_{520} - A_{520}\ SO_2)$$

6.8-Measurement of the antiradical activity

All samples were diluted 1:10 with MeOH immediately prior to analysis. A 0.025ml aliquot of diluted sample was added to 0.975 ml $DPPH^{\bullet}$ solution (60 mm in MeOH), vortexed, and the absorbance read at t = 0 and 30 min (3,42). Results were expressed as Trolox equivalents (TRE mM) using the following equation:

$$AA\ (mM\ TRE) = 0.018 \times A_{515} + 0.017\ \%$$

as determined from linear regression, after plotting $\%\Delta A_{515}$ of known solutions of Trolox against concentration (0.02-1.28mM, $r^2 = 0.9935$), where

$$\%\Delta A_{515} = [(A_{515}(0) - A_{515}(30) / A_{515}(0)] \times 100.$$

6.9-Measurement of the reducing power

For the determination of the reducing power of wines a protocol based on the ferric reducing / antioxidant power (FRAP) assay was developed with modifications (3,38). Wine (0.05 ml), diluted 1:10 with distilled water, and 0.05 ml of ferric chloride (3mm in 5 mM citric acid) were mixed thoroughly in a 1.5 ml Eppendorf tube, and incubated for 30 min in a water bath at 37 °C. The mixture was then added to 0.90 ml of 1mm TPTZ solution in 0.05 m HCl, and vortexed. After exactly 10 min the absorbance was read at 620 nm. PR was calculated from a calibration curve established by plotting known amounts of ascorbic acid against A_{620}. Results were expressed as ascorbic acid equivalents (AAE mM) using the following equation:

$$RP\ (mM\ AAE) = (0.679 \times A_{620} - 0.008) \times F_D$$

where F_D is the dilution factor. For the blanks, distilled water was added instead of wine. One blank was prepared for each wine tested.

6.10-Analysis of intensity and hue

For the analysis of the intensity, Aglianico wine samples were diluted 1:10 with dd H_2O and the same for Fiano samples. The absorbance of the samples was read against a water blank at 420nm, 520 nm and 620 nm (42,74). The intensity is the sum of absorbance at these three wavelengths. For red wines alone, the hue is given by the ratio between the absorbance at 420nm and 520nm.

6.11-Analysis of polyphenols by HPLC

To assess the polyphenol content by HPLC, the sample was centrifuged at room temperature for 10 minutes at 13000 rpm. The supernatant was removed and then filtered through a membrane with a 0.45 µm cut-off (Millipore). The sample was then loaded onto C-18 column Hypersill Gold (Thermo) previously equilibrated with eluent A (2 % acetic acid) flow 1 ml/min., and eluted with eluent B (0.5 % acetic acid in water / acetonitrile 50:50) applying the following gradient:

Time	Eluent A (%)	Eluent B (%)
0	90	10
35	45	55
40	0	100
45	0	100
50	90	10

The peak area obtained is compared to that of runs made with standard known concentrations of polyphenols allowing quantitative determination. The HPLC system used is a Finnigan Surveyor LC Pump with SpectraSystem detector RI-150. The wavelengths analyzed are 280nm, 320 nm and 370 nm.

6.12-Determination of Wine Aroma

6.12.1-Sample preparation for HS-SPME-GC analysis

Prior to analysis, wine samples were cooled to 4 °C to minimize the loss of highly volatile compounds. About 20 ml of each wine was placed into a 100 ml glass vial containing 3 g of NaCl (saturation level) and 0.5µl isooctane as an internal standard. The vials were subsequently sealed with PTFE-silicone septa (Supelco, Bellefonte, PA, USA) and mixed by magnetic stirring. Each analysis was undertaken in duplicate using different vials. 100mm polydimethylsiloxane (PDMS) (5) was the fibre used for the extraction of the volatile components, in headspace condition. The sample vials were equilibrated for 30 min at 40 °C in a thermostated bath followed by fibre exposure to the headspace for 20 min. The exposure was performed by inserting the stainless steel needle through the vial septum and pushing the fibre into the sample headspace to collect the analytes. The fibre was then drawn into the needle and the SPME device removed from the vial and inserted into the injection port of the GC for thermal desorption apparatus. The analytes from the fibre removal was carried out in the splitless mode at 240 °C for 5 min. During the injection process the fibre was maintained for 10 min in splitless mode.

6.12.2-Gas chromatography-mass spectrometry

All aroma standards were purchased from Sigma (St. Louis, MO, USA) and were of the highest purity available. The analyses were performed using a Agilent 6890 GC

Agilent 5973 gas chromatograph coupled with mass spectrometry (MS) detector and a DB-WAXetr column, 30 mm x 0.25 mm id, 0.25 µm film thickness (J & W Scientific, Folsom, CA, USA) was employed . Helium was used as the carrier gas with a flow rate of 1.5 ml/min, the injector temperature was 250 °C and the oven temperature was programmed from 40 °C (held for 6 min) to 180 °C at 5 °C min^{-1} (held for 3 min), then at 7 °C min^{-1} to 240 °C (held for 5 min). Detection was by mass spectrometry on the total ion current obtained by electron impact at 70 eV and the masses were scanned from 29 to 300 m/z. The components were identified by comparison with the spectra of the NIST and Wiley library (11,25,26).

6.13- Sensory analysis

6.13.1-Sensory analysis of 2007 wines

Sensory analysis was carried out on final winemaking products as FIA W2 / Fiano control and AGL W2 / Aglianico control to explain the sensorial profile. The analysis was performed by an expert sensory panel composed of eight members previously trained in the sensory analysis of wine. Nine main attributes to describe the wine samples were formally selected by the panel. Six of these characteristics were common to both wines (general impression, acidity, freshness, intensity, sweetness, terpenic flavour) while the other three were typical for Fiano (exotic fruit, hazelnut, fruity) and Aglianico (red fruit, tannin balance, full flavoured). The highest rating assigned by the panel to the range plot was set as nine.

6.13.2- Sensory analysis of 2008 wines

For the sensory analysis of wine samples of the 2008 vintage, the parameters monitored were expanded upon by taking into consideration visual, olfactory, gustatory-tactile, retro-olfactory and after swallowing sensations. Quantitative descriptitve analysis was used to define the sensory profiles of the wine samples (48). Each sample was evaluated three times in different sessions. The bottles were opened one hour prior to evaluation and 50ml of wine were poured at 20°C into ISO glasses for evaluation. The 12 members of the test panel evaluated 3 different wine samples during each session, and each sample was blinded but identifiable by way of a three number code. Wine was presented to each panel member in a different sequence in order to prevent evaluation errors induced by the wine's position. Each panel member recorded the mark on a scorecard.

6.14-Statistical analysis

The statistical analysis was carried out using the Microsoft Excel software package (Microsoft Corp.)

7-Purification of a β-glucosidase from *Rhodotorula mucillaginosa* AGWLR12

7.1-Growth in liquid medium

The yeast cultures were inoculated in flasks containing YPD liquid medium sterilized in an autoclave at 120 °C for 15 minutes. The flasks were placed in a shaker incubator at 28 °C, under agitation of 160 rpm. Yeast growth was followed by measuring the absorbance at 600 nm with a spectrophotometer (Cary 50 Bio UV-Visible, Varian, USA) and samples collected at different times. At the end of the stationary phase of growth (after about 46 hours of culture) cells were harvested by centrifugation at 8000 rpm, 4 °C for 15 minutes (Sorvall RC5C). The biomass was stored at -20 ° C and the supernatant at 4 °C after addition of 0.1 mM PMSF (phenyl-methyl-sulfonyl-fluoride), a protease inhibitor (64).

7.2- Determination of the enzymatic activity

A kinetic colorimetric assay was used in order to determine the number of enzyme units present in the supernatant of AGWLR12 liquid culture (6). The reaction mixture used (1 ml) contained:

200 µl of 15 mM pNPG dissolved in 100 mM citrate-phosphate buffer pH 5 or 7
20 µl of enzyme (from 0.6 to 1.2 micrograms of enzyme)
780 µl deionized H_2O

Prior to addition of the sample to the reaction mix, the expected change in OD_{400}, due to a slight substrate autolysis, was allowed to reach zero. Following addition of the sample, absorbance at 400 nm versus time was continuously recorded for approximately 5 minutes after the beginning of the assay. The slope of the curve obtained was also calculated. This value represents the initial velocity V_0 of the enzymatic reaction and is expressed in ΔOD / min. The enzyme units were calculated with the following formula:

$$\text{Units / ml} = \frac{\Delta \text{ OD / min x assay volume x dilution}}{\text{pNP } \varepsilon \text{ x sample volume}}$$

pNP ε = molar extinction coefficient of para-nitro-phenol whose values are: 14:13 x 10^3 M^{-1} cm^{-1} at pH 5 and 9 x 10^3 M^{-1} cm^{-1} at pH 7. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 µmol of para-nitrophenol in one minute at the temperature and pH of the assay. The specific activity was calculated as the number of enzyme units per milligram of protein.

7.3-Determination of protein concentration

The Bradford method was used for quantification of proteins (10), the principle of which is based on Coomassie Brilliant Blue binding to the peptide bond: the method has a sensitivity of less than 2.0 µg/ml. A Bio-Rad kit was used for determination of the protein concentration (Hercules, CA, USA) according to the manufacturer's instructions and using bovine serum albumin as standard.

7.4-Concentration of the β- glucosidase

Enzyme was purified from 2 litres of culture medium using the supernatant separated from the biomass by centrifugation (Sorvall RC5C, 20 min, 8000 rpm, 4 °C). The supernatant was concentrated in an ultrafiltration membrane module from PM 10 (Pellikon - Millipore) to a final volume of 400 ml. The sample obtained was further concentrated by YM10 (Amicon - mod. 8400) ultrafiltration membrane to a volume of 60 ml. To this end, the concentrated sample was then dialyzed against 10 mM Tris-HCl pH 8 (2,83).

7.5-Anion-exchange chromatography on DEAE Fast Flow

The dialyzed sample, consisting of a volume of 60ml, was loaded onto a 30ml anion-exchange chromatography column (DEAE Sepharose XK16 Fast Flow (Amersham Pharmacia Biotech) and balanced in the same dialysis buffer. After absorption of the sample, and washing with 10 mM Tris-HCl pH 8, proteins were eluted in a linear gradient of NaCl from 0 to 500 mM at a flow rate of 0.5 ml/min, and collected in 2 ml fractions. The collected fractions were tested for enzyme activity using the standard assay and protein concentration by measuring the absorbance at 280 nm. The active fractions were pooled and subjected to a subsequent purification step (9,15,29).

7.6-Chromatography on Superdex 200 XK 26/60

The active fractions were pooled and concentrated on a YM10 membrane (Millipore) by ultrafiltration. The concentrated sample was loaded onto a 320ml Superdex 200 XK 26/60 FPLC column (Amersham Pharmacia Biotech) for preparative gel filtration, a column capable of fractionating globular proteins in a range from 10 to 500 kDa. The column was balanced in 10 mM Tris/HCl pH 8 + 150 mM NaCl buffer at a flow of 1 ml/min. After absorption of the sample (volume 10 ml), elution of proteins was performed with the same buffer, flow of 2 ml/min and 4 ml fractions collected. Enzyme activity was once again detected by the standard assay and the combined active fractions were concentrated on a YM10 membrane (Millipore) by ultrafiltration. The concentrated enzyme was used for all characterization experiments.

8-Characterization of the β-glucosidase from *Rhodotorula mucilaginosa* AGWLR12

8.1-Determination of optimum pH

The activity of many enzymes changes according to pH. This is because both substrates and active sites often contain acidic or basic functional groups whose ionization state is influenced by pH. The pH dependence of β-glucosidase was determined at pH values ranging from 3.5-8.0 using 200 mM citrate phosphate buffer made up to different pHs at room temperature and using pNPG as substrate. Activity was measured at 30 ° C at a final concentration of 7.5 mM pNPG (43).

8.2-Effects of temperature on enzymatic activity: thermostability

Thermal stability of β-glucosidase was analyzed by incubating the enzyme (at a concentration of 0.1 mg/ml) in citrate-phosphate buffer pH 7.0 at 25, 40, 50 and 60 °C. At predetermined time intervals (1, 2, 4 and 24 hours), aliquots of pre-incubated enzyme were taken from the incubation mixture and assayed at 30 °C under standard conditions.

8.3-Effect of temperature on enzymatic activity: thermophily

The speed of most chemical reactions increases with increasing temperatures and enzymatic reactions are no exception. If the initial velocity of an enzyme reaction is plotted as a function of temperature and conditions remain unchanged, an initial increase in the velocity is observed followed by a decrease and passing by a maximal rate defined as the optimum reaction temperature, The dependency of β-glucosidase on temperature was ascertained by performing the standard enzyme assay in citrate phosphate buffer at pH 7, at temperatures ranging from 25-80° C.

8.4- Purity of β-glucosidase and determination of molecular weight

The molecular weight of β-glucosidase was determined both in denaturing conditions on SDS-PAGE (polyacrylamide gel electrophoresis with sodium dodecyl sulfate) and by native analytical gel filtration on a Superdex 200 HR 10/30. SDS-PAGE was also used to verify the purity level reached following several purification steps and the degree of homogeneity reached thereafter. The SDS-PAGE gel was performed using 10 % (w/v) prepared with the reagents at the concentrations described in the following table and in agreement with the discontinuous system of Laemmli . The (Bio-Rad) SDS-PAGE running buffer consisted of the following: 192 mM glycine, 0.1 % (w/v) SDS, 25 mM Tris / HCl pH 8.3. Samples were mixed with loading buffer consisting of 62.5 mM Tris / HCl pH 6.8, 20% glycerol, 2% SDS and 5% β-mercaptoethanol containing bromophenol blue as tracer (BFB).

	Separating gel (ml)	Stacking gel (ml)
ddH_20	2.005	3.05
polyacrylamide	1.67	0.670
1.5 M Tris/HCl pH 8.8	1.25	-
0.5 M Tris/HCl pH 6.8	-	1.25
APS	0.025	0.025
SDS 10 %	0.05	0.05
TEMED	0.002	0.005

This buffer allows visualization of the electrophoretic migration front and confirms the passage of current in the circuit and through the gel.
The samples were then denatured (along with protein standards) by heating at 95 °C for 5 minutes. The gel was run at 150 Volts for about 1 hour and the proteins were then stained with Coomassie blue G-250. Pharmacia standard molecular weight protein markers were used: Phosphorylase b, 97 kDa, bovine serum albumin, 66 kDa, ovalbumin, 45 kDa, carbonic anhydrase, 30kDa, trypsin inhibitor, 20.1 kDa. The molecular weight of β-glucosidase was determined by charting the log molecular weight versus the migration distance of the protein standards. A curve fitted to the distance migrated by the known protein standards allowed determination of the molecular weight of β-glucosidase.

The molecular weight was also determined by analytical gel filtration on a Superdex 200 HR 10/30 column. The column was balanced in 50 mM Tris/HCl pH 8.4 containing 1 mM β-mercaptoethanol , loaded with 0.1 ml of purified β-glucosidase (60 micrograms of protein) and eluted with 0.5 ml/min flow rate with the same buffer. The molecular weight was determined using a calibration curve obtained by placing in abscissae the retention volumes of proteins with known molecular weight standards (bovine thyroglobulin 670 kDa, 158 kDa α-globulin, ovalbumin 44 kDa, myoglobin 17 kDa, Vitamin B12 1 35 kDa) and in the ordinate the values of logarithm of their molecular weights.

8.5- Kinetic constants

The calculation of kinetic constants (K_m and V_{max}) were made under optimum conditions of pH, ionic strength, temperature and at time intervals which did not lead to enzyme inactivation. The Michaelis-Menten equation was used for analysis, it describes the kinetic behaviour of an enzyme as a function of substrate concentration using the following equation:

$$v = kcat \cdot [E_0] \cdot [S] / (Km + [S])$$

When the substrate concentration tends to infinity we obtain:

$$Vmax = Kcat \cdot Etot$$

which is the maximum velocity reached by the reaction with the specific enzyme concentration. Since this velocity is independent of the concentration of substrate, using the definition of V_{max}, the equation can be rewritten as:

$$v_0 = V_{max} \cdot [S] / (K_m + [S])$$

The reciprocal Michealis-Menten equation allows the establishment of a correlation between 1/V and 1/S, which is represented by the Lineweaver-Burk plot. The 1/S interception is equal to -1/Km and the 1/V interception is equal to 1/Vmax. The main advantage of the double reciprocal plot is that it allows accurate determination of V_{max}, which can only be evaluated approximately with a graph of V_0 as a function of [S]. The K_m of the β-glucosidase assessed using pNPG as substrate was determined at 30 ° C in 50 mM citrate-phosphate buffer pH 7 using increasing concentrations of substrate from 3.75 mm to 60 mm and a fixed amount of enzyme equivalent to 1 µg.

8.6- Specificity of β-glucosidase for synthetic substrates and disaccharides

To assess the substrate specificity of β-glucosidase, assays were carried out using para-nitrophenylglucoside synthetic and natural disaccharides as substrates. For synthetic substrates, the test standards already described were carried out while the enzymatic hydrolysis of disaccharides was evaluated by measuring the glucose released using a coupled enzymatic assay (Kit - Glucinet - Sclavo). The principle of this test is to determine the amount of glucose present in the sample using the reagent "Glucinet" consisting of: phosphate buffer pH 7.5 150 mM, glucose oxidase (*A. niger* ≥ 18 U / ml), peroxidase (horseradish ≥ 1 U / ml), 4-aminoantipyrine (0.4 mM) and Na-hydroxybenzoate (10 mM). The intensity of colour developed is directly proportional to the amount of glucose present in the sample. Disaccharide hydrolysis with subsequent release of glucose was measured as follows:

Reagents :

- Substrate (disaccharide) 150 mM (200 µl)
- 200 mM citrate-phosphate buffer pH 7 (75 µl)
- Enzyme 25 µl

The reaction is incubated at 30 ° C and 10 µl samples are removed at 10, 30 and 60 minutes intervals and added to the Glucinet reagent. The absorbance is measures at 510 nm against a blank. The amount of glucose is calculated using a calibration curve (2-10 µg/ml glucose, R^2=0.9976, y=0.0278 x +0.0048)

8.7-Effect on enzymatic activity of ethanol and sugar

The effect on β-glucosidase of ethanol and sugars (glucose and fructose) is evaluated by adding various substances at concentrations ranging from 5 to 20% at the reaction mix by running a test standard.

8.8 – Effect of β-glucosidase activity on piceid hydrolysis

The effect of β-glucosidase on piceid hydrolysis is assessed on a reaction mix containing:

- 300 µl buffer citrate-phosphate pH 7, 50mM
- 200 µl of trans-piceid 15 mg/ml
- 500 µl of purified enzyme (60 U) and dd H_20

A blank reaction mix is prepared in which the enzyme is replaced with dd H_2O .
The reaction is carried out at 45°C and the products are checked at different times by HPLC analysis (See Materials and Methods 6.10).

Results and Discussion

1- Isolation of yeast strains

A collection of 442 yeast strains was created by isolation from grape samples, must and wine of Aglianico and Fiano grapevines. The size of the yeast collection ensures faithful correspondence of the actual microbial biodiversity of the two vineyards. Analysis of the morphological and biochemical features of the yeasts highlights some differences between microbial populations of Aglianico and Fiano.
In Aglianico, the isolated microorganisms belong to the genera *Saccharomyces spp.* (65.1%), *Kloeckera spp.* (27.3%), *Candida spp.* (5.7%) and *Metchnikowia spp.* (1.9%) (Fig6). On the grapes, only the genera *Candida spp* and *Kloeckera spp* were detected. In the must, at the start of fermentation, *Saccharomyces* yeasts was already predominant; at this stage *Candida* disappeared, on the contrary *Kloeckera* reproduced easily and a small percentage of *Metchnikowia* yeasts was also present. During subsequent stages of fermentation, only *Saccharomyces spp* are resistant to the rising concentration of ethanol until completion of fermentation (Fig.7) (8). This is in agreement with scientific literature which reports that non-*Saccharomyces* yeasts are able to tolerate an ethanol content of up to 3-4°(16,31,32,65,72).

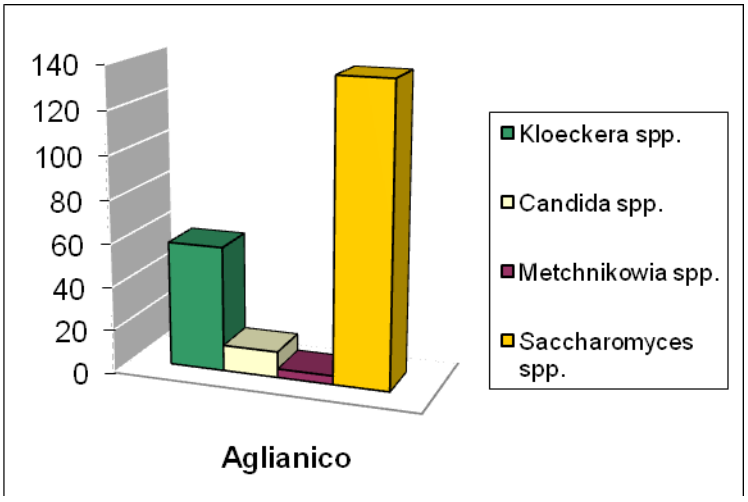


Fig.6

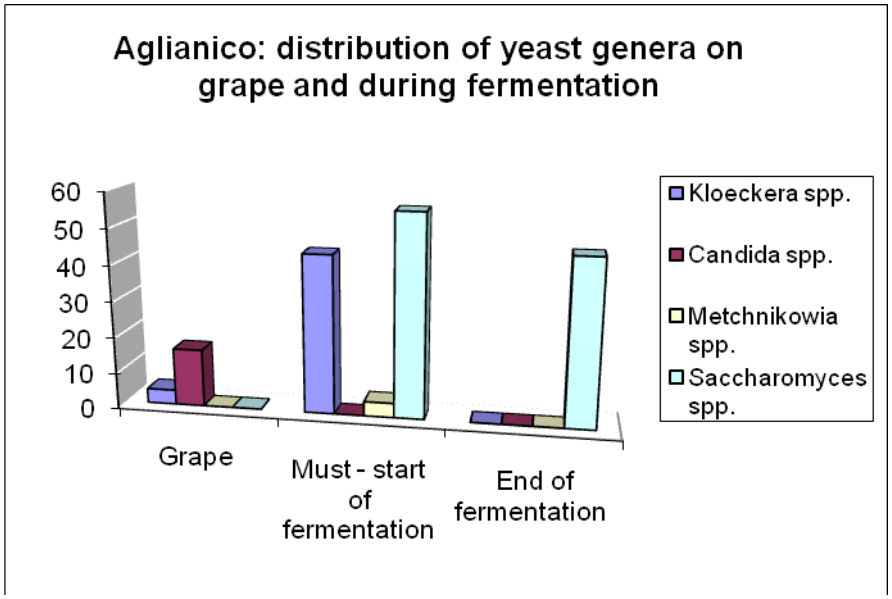


Fig.7

In Fiano the collected data show that the isolated yeast belong to *Saccharomyces spp* (51.3%), *Kloeckera spp*. (22.6%), *Metchnikowia spp* (15.7%) and *Candida spp*. (10.4%) (Fig.8). They are the same yeast genera as Aglianico but have a different distribution. As for Aglianico, non-*Saccharomyces* yeasts are predominant on Fiano grapes but there is also a high percentage (19%) of *Saccharomyces* yeasts.

The presence of *Saccharomyces spp* is reported in the literature by some authors as being a consequence of grape damage because a high concentration of sugars, such as that found in grape juice, promotes the growth of these yeasts (32). Damage to Fiano grapes alone is probably due to their skin, which is thinner and more fragile than that of the Aglianico cultivar. *Metchnikowia spp* were detected on grapes as well,. As in Aglianico, *Candida spp* are no longer detectable at the start of the Fiano fermentation process, unlike *Kloeckera spp* and *Metchnikowia spp.,* and the *Saccharomyces spp* are already predominant across all other genera. However at the end of fermentation, *Saccharomyces* yeasts are not the only surviving species, because *Kloeckera* spp. is still detectable (fig.9). As reported previously by other authors (13,33,46,61), the lower temperature (18-20°C) allows this yeast to endure increasing concentrations of ethanol.

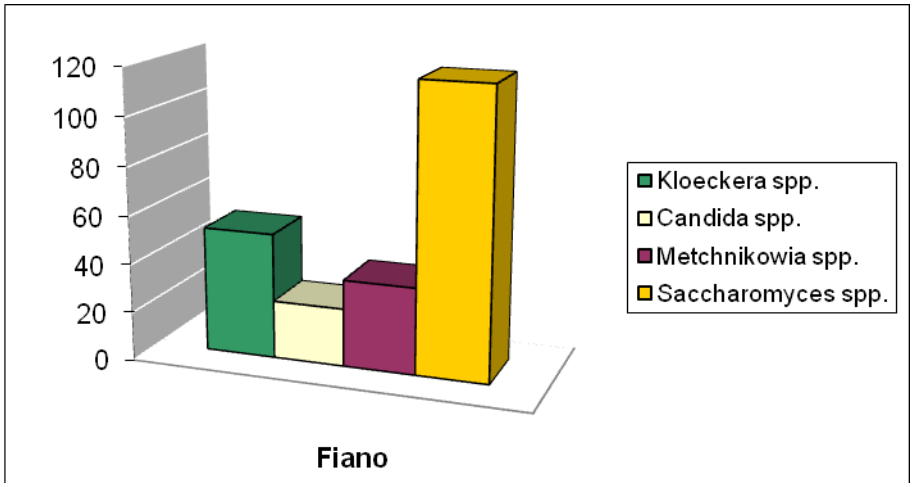


Fig.8

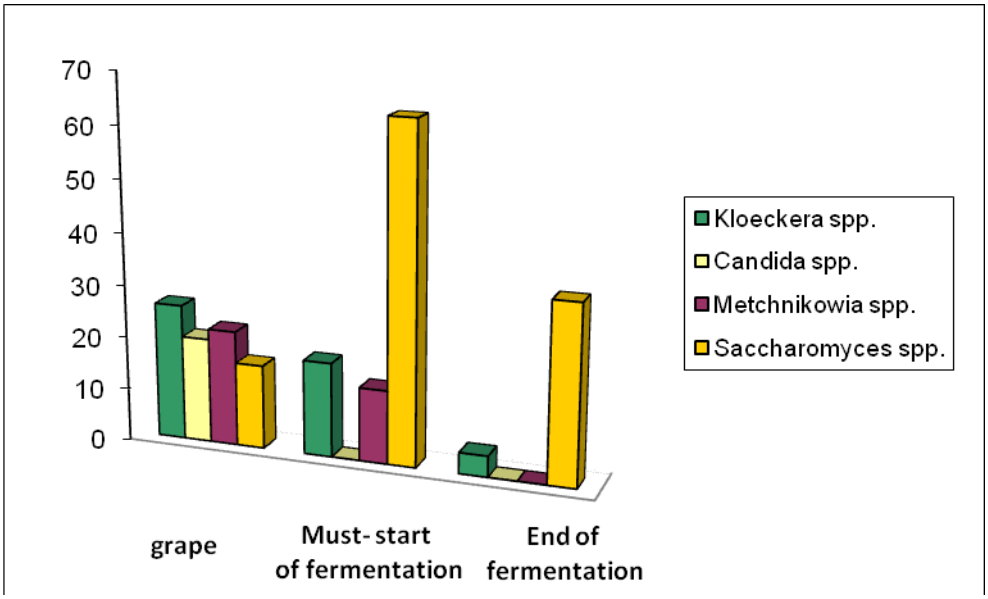


Fig. 9

It is interesting to note that the same four genera alone were isolated, both in Aglianico and in Fiano. In the literature it has been reported by a number of authors that the indigenous yeasts belong not only to the genera *Saccharomyces spp*, *Candida spp*, *Kloeckera spp* and *Metchnokowia spp* but also to *Aureobasidium spp, Auriculibuller spp, Brettanomyces spp, Bulleromyces spp, Cryptococcus spp, Debaryomyces spp, Hansenula spp, Issatchenka spp, Kluyveromyces spp, Lipomyces spp, Pichia spp, Rhodosporidium spp, Rhodotorula spp, Sporidiobolus spp, Sporobolomyces spp, Torulaspora spp, Yarrowia spp, Zygoascus spp*, and *Zygosaccharomyces spp* (32).

2- Sulphur dioxide resistance

A subsequent selection on our collection was carried out in order to ascertain resistance of the indigenous yeasts to sulphur dioxide. Sulphur dioxide is the most widely antiseptic agent used in wine cellars to control the growth of spontaneous microflora during fermentation, though this is not its sole function.

It combines with dissolved oxygen, thus preventing the chemical oxidation of wine, in particular of certain polyphenols and some aromatics. It inhibits the effect of oxidases and sometimes leads to their destruction, protecting the mash from pre-fermentative oxidation. When in contact with grape skin, sulphur dioxide promotes the diffusion of partially polymerized coloured compounds contained in the vacuole by means of small holes in the cell walls, thus promoting the release of anthocyanins. A calibrated use of SO_2 enhances the qualities of smell and taste of wine, because this preservative combines with unpleasant compounds, such as acetaldehyde and pyruvic acid, making them no longer perceptible to taste or smell. Finally, SO_2 has a mild clarifying activity, since it promotes the coagulation of colloidal substances, increasing the phenomenon of spontaneous precipitation of the lees (16).

The resistance to this chemical compound is therefore a very important feature for an industrial wine yeast. The current Italian legislation sets the threshold at 160 mg/L for red wines, 210 mg/L for white wines and 400 mg/L for the sweet wines. Instead, the biological discipline provides the threshold of 60 mg/L for red wines, 80 mg/L for white wines, and 120 mg/L for sweet wines, although the recommended amount is less than 20 mg/L. For our selection, we checked the resistance at two different concentrations of sulphur dioxide, 100 mg/L and 250 mg/L. 100 mg/L is the most common concentration used in wine cellars. MBK was used as a source of SO_2. The data collected are shown in table4.

Table 4: Resistance of indigenous yeast strains to SO_2 100 mg/L and 250 mg/L

Cultivar	100 mg/L SO_2 (+)	100 mg/L SO_2 (-)	250 mg/L SO_2 (+)	250 mg/L SO_2 (-)
Fiano	180	50	112	118
Aglianico	172	40	140	72

The results of the resistance assay demonstrate that most of the collected yeasts are resistant to SO_2 100 mg/L, more precisely 81.1% of yeasts isolated from Aglianico and 78.3% of yeasts isolated from Fiano. These values decrease when a

concentration of 250mg/L of SO_2 is employed (66% of yeasts isolated from Aglianico and 48.7% of yeasts isolated from Fiano), but they remain remarkable.
The most interesting data were derived from the analysis of genera of resistant strains. In Aglianico, 17.9% of strains resistant to SO_2 100 mg/L and 11.4% of those resistant to SO_2 250 mg/L belong to non-*Saccharomyces* genera. In Fiano 30.8% of strains resistant to SO_2 100 mg/L and 23.2% of those resistant to SO_2 250 mg/L belong to non-*Saccharomyces* genera. This is somewhat impressive because the scientific literature commonly reports that these genera are very sensitive to SO_2: the growth of *Kloeckera apiculata* was reported to be totally inhibited by 5.7 mg/L SO_2, *Pichia vini* by 13.1 mg/L SO_2 and *Candida vini* by 31.2 mg/L SO_2 (16). However, these strains were isolated from a non-sulphited must and then stored for many years in collections without being in contact with the antiseptic reagent before the inhibition experiment.
Our data are explicable only by a development of resistance of indigenous yeasts, at levels higher than that permitted by legislation. The resistance to sulphur dioxide could be caused by a “selective pressure” of sulphur levels used on grapevines throughout the years, which could in turn explain why so few yeast genera were isolated from grapes and mash: only those showing increased resistance have survived. It can also be concluded that a significant number of indigenous yeasts, even non-*Saccharomyces spp*, continue to grow undisturbed even after the addition of the usual dose of SO_2 until they die of ethanol intoxication after three or four days from the beginning of fermentation.

3- Presence of β-glycosidase activity

Another selection was made on the yeast collection to assess the presence of β-glycosidase activity. β-glycosidases are enzymes widely used in wine industry because they can liberate a group of odorous molecules usually linked to sugar moieties in the grape juice and wines, showing no olfactive characteristics, mainly monoterpenes. At present, about 50 monoterpene compounds are known, of which the most important are linalool, geraniol, nerol, citronellol and α-terpineol, but others are likely to be present in particular grape varieties. In general, most monoterpenes are present as the aglycone part of a glycoside and, unfortunately, their flavour potential remains quite stable during winemaking and in young wine (1,30,37).
Terpene glycosides can be hydrolysed not only by enzymes but by acids as well, however under acidic conditions, rearrangements of the monoterpenes may occur, whereas with enzymatic hydrolysis, the changes in the natural distribution of these compounds are minimal, resulting in a more natural flavour.
A β-glycosidase is also present in grape skin, but this enzyme has low activity and is quite unstable at grape juice and wine pH values. In general, β-glycosidases of plant origin show a low activity on terpene monoglucosides possessing a tertiary alcohol group (linalool, α-terpineol) and are only capable of hydrolysing terpene monoglucosides possessing a primary alcohol group (geraniol, nerol, citronellol) (37). Regarding fungal glycosidases (mainly from *Aspergillus niger*), these enzymes are present only in low quantities in the majority of commercial enzyme preparations and are strongly inhibited by glucose. These commercial fungal preparations sometimes show undesirable activities (production of vinyl-phenols) and generate oxidation artefacts during the hydrolysis of glycosides (37). Yeast glycosidases have not been

widely studied yet, especially those of oenological origin, so they could be a new promising source of enzymatic activities.
The screening on our collection of 400 strains has show that only two yeasts from Aglianico, AGWLR12 and AGYP28, and just one from Fiano, FWL66, possess an extracellular β-glycosidase activity. The analysis of the sequence of region D1/D2 of 26S rDNA revealed that AGWLR12 belong to the *Rhodotorula mucillaginosa*, FWL66 belongs to *Hanseniaspora guilliermondii* and AGYP28 is a *Metschnikowia fructicola* strain. However, the presence of a reddish pigment suggests that this is a *Metschnikowia pulcherrima* strain, a species which is closely related. The *R. mucillaginosa* AGWLR12 strain is the only representative of its genus found in our yeast collection. Our screening revealed that β-glycosidases are poorly expressed in epiphytic microflora of Aglianico and Fiano grapes and musts. It is quite surprising that only 0,75% of our strains express this enzyme, particularly as the literature reports that β-glycosidase is expressed by many wild strains of *Candida spp* and *Kloechera spp,* thus a higher percentage was expected. It is also true that glycoside precursors of odorous monoterpenes are present in flavouring grapes between 6.5 and 28 mg/L, so they cannot be an essential carbon source for indigenous yeasts. Many studies have reported that non-*Saccharomyces* yeasts are a source of a wide range of enzyme activities but, even in wild strains isolated from grapes, the percentage of yeasts possessing β-glycosidase can change considerably (7,18,20,68).

4- Fermentation with indigenous yeasts

4.1- 2007 vintage

In order to assess the influence of indigenous yeasts on industrial and biochemical parameters of Aglianico and Fiano wine, we conducted several fermentations using different combinations of commercial yeast strains, the yeast strains previously cited (AGWLR12, AGYP28, FWL66) and two other strains, identified by the sequence of their region D1/D2 of 26S rDNA as *Saccharomyces cerevisiae* and chosen for their high fermentative capability even at high SO_2 concentration (250 mg/L). The fermentations were carried out on two different vintages. On the first, in 2007, two fermentations in Aglianico and two in Fiano were executed with the only commercial yeast strain usually used, and then in combination with the strain *R. mucillaginosa* AGWLR12. This was carried out in order to ensure the influence of the same indigenous yeast on two different wines, and to determine phenotypic differences in the strain during the process. On the second vintage, in 2008, we carried out ten fermentations using the commercial strain and the indigenous strains in several combinations (see Materials and Methods 5.1 and 5.2). The fermentations by the non-*Saccharomyces spp.* alone were not considered because in previous experiments they were not able to complete the process, resulting in a stuck fermentation (data not shown). The different combinations allow us to study the interaction among these different strains and their influence on the final quality of wine (22). With regards to the 2007 vintage, very interesting data were obtained with both the fermentations. In Aglianico that was particularly tumultuous using the non-*Saccharomyces* AGWLR12. The course of this fermentation is described in fig. 10.

As can be seen, the fermentation with *R. mucillaginosa* AGWLR12 is complete following just 12 days from the start. The final values of sugar content, total acidity, pH and alcohol volume remained constant through time. The control fermentation by the only commercial strain *Saccharomyces cerevisiae* (var. *bayanus*), Oenoferm® Freddo has a normal course and ended after 18-19 days from the start. Analysis of the main oenological parameters (fig.11) of the two fermentations of Aglianico must shows that they were both completed, because the concentrations of residual sugars are around the value of 2 g/L , thus inferior to the critical threshold of 2,5 g/L, at which value the wine may be contaminated with acetic acid bacteria.

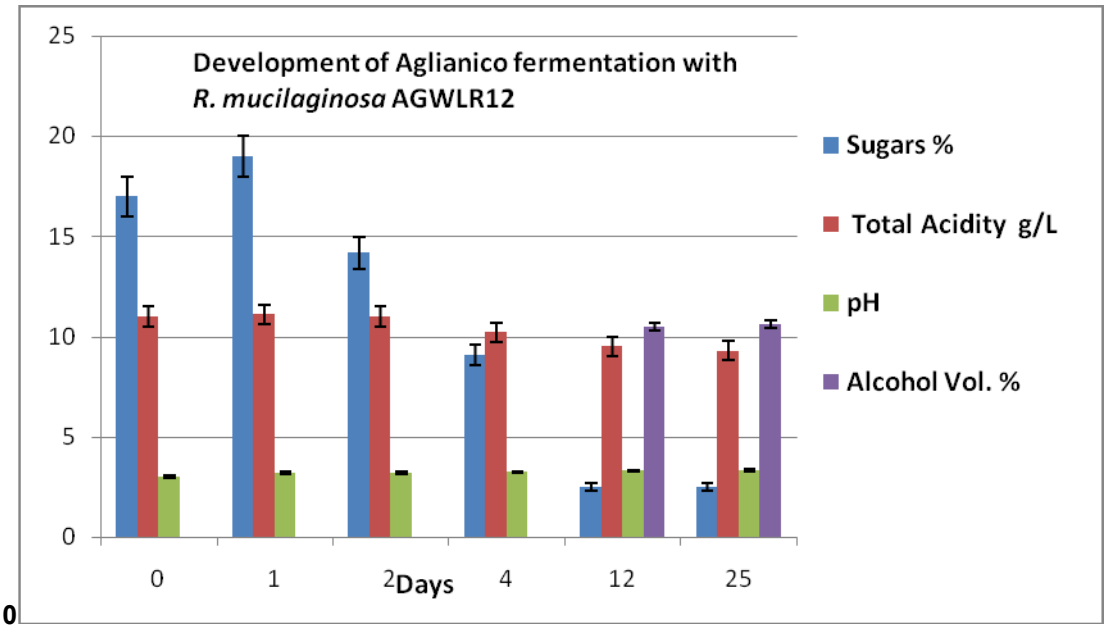


Fig.10

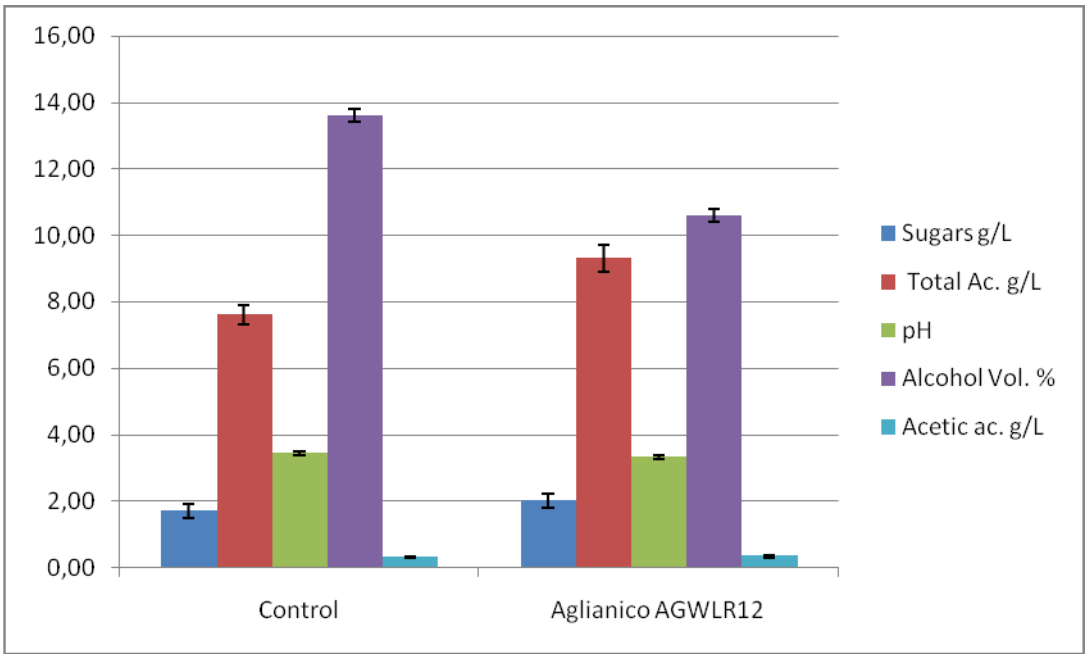


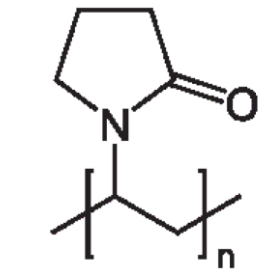
Fig.11

The completion of the experimental process therefore demonstrates a good compatibility between AGWLR12 and the commercial yeast. Using a mixed culture is sometimes problematic because of nutrient depletion (nitrogen, oxygen), secretion of killer toxins or production of lytic enzymes that cause a stuck fermentation (50). Regarding the total acidity, it was 1,7 g/L higher in AGWLR12 fermentation compared to the control, which is a good property for a wine. Indeed the total acidity consists predominantly of tartaric acid, a compound present at higher levels in good vintages, where it acts as a flavour enhancer and confers wine freshness. The value of volumetric alcohol with AGWLR12 is lower than the control, and this was expected as this non-*Saccharomyces* yeast is an obligate aerobe and the sugars that it consumes are not fermented to ethanol, as can be seen in fig.10, regarding the first stage of fermentation. This yeast therefore died as a result of the ethanol produced by the commercial strain and the combination of these two yeast strains could be suitable for the production of low-alcohol wine. The experimental fermentation in Aglianico differed from the control for a greater quantity of lees too. Lees are a deposit of solids on the bottom of the fermentation tanks, consisting of potassium bitartrate, calcium tartrate, proteins coagulated with tannin, pectic substances, yeast cells and bacteria. At the end of alcoholic fermentation and/or malolactic fermentation, the wine is more or less opalescent or turbid. To prevent extended contact with the particles in suspension (yeast, mucilage, curdled protein, bacteria) that could make the wine's taste or aroma strange, increase the volatile acidity and other alterations, immediate separation of these substances is critical. Natural precipitation of these compounds is too slow and takes many months, so fining agents have to be used. They are usually gelatine, bentonite, casein, egg albumin, carbon, sparkolloids (derived from alginic acid from algae), silica sol and arabic gum (47). Sparkolloids and arabic gum have a polysaccharide structure similar to the polysaccharides excreted by *Rhodotorula mucilaginosa*. In fact, the name *mucilaginosa* refers to the mucilaginous envelope surrounding the colonies of this yeast. In fig.12 the structures of alginic acid, arabic gum, carboxymethylcellulose, polyvinylpyrrolidone and the hypothetical structure suggested for an exocellular polymannan isolated from the strain *Rhodotorula mucilaginosa* YR2 (75) are shown. These polysaccharides of yeast origin could be the cause of the greater precipitation of lees as they may act as fining agents on suspended particles present in wine during fermentation. The polysaccharides from *Rhodotorula* may also be responsible for the increase in total acidity in the experimental fermentation in Aglianico. They could act like arabic gum which acts as a protective colloid: its action is similar to that of metatartaric acid, and prevents the growth of tartrate crystals and their successive precipitation (47). Finally, even if these yeast polysaccharides are present in wine, they did not hinder the successive filtration step. The use of a strain of *Rhodotorula* could be a useful way to supplement the must with unsaturated fatty acids, of which the genus *Rhodotorula* is reported to be rich in, especially linoleic (C18:2, n-6) and α-linoleic (C18:3, n-3) acids (52). Furthermore, these yeasts synthesize carotenoids or carotenoid like pigments which give colonies a red colour, and which may be of interest as vitamin precursors and effective antioxidants (40). Finally, the siderophore rhodotorulic acid demonstrates activity in food preservation against *Botritis spp*(72).

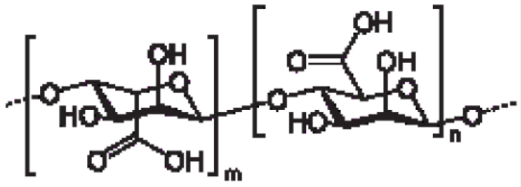
In Fiano the fermentation with *Rhodotorula mucilaginosa* AGWLR12 gave very similar results except for the duration of fermentation which remained the same. (fig.13) Both control and experimental fermentations reached completion, as the residual sugars are under the value of 2 g/L. As in Aglianico, in the experimental wine the total acidity is higher and the alcoholic grade is lower but the increase in total

acidity is much higher than in Aglianico (+36% in Fiano and +22% in Aglianico) whereas the decrease in alcoholic grade is much lower (-7% in Fiano and -22% in Aglianico). Furthermore, in Fiano AGWLR12, the pH value decreases significantly compared to the control, and a more acidic pH is helpful in containing microbiological contamination.

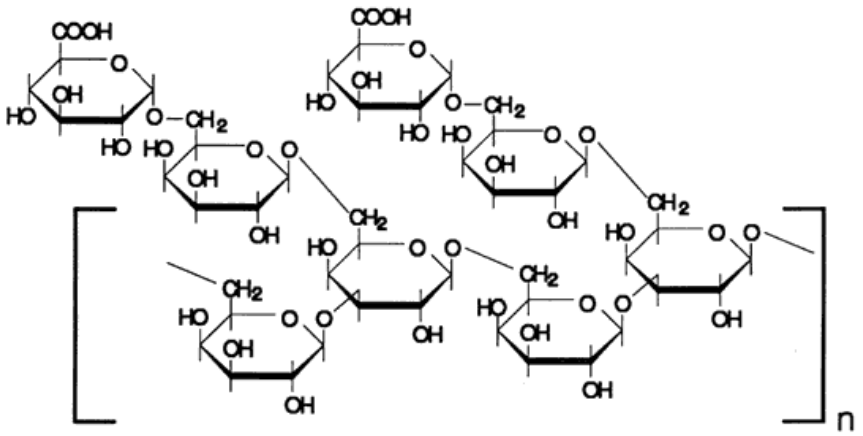
Fig.12



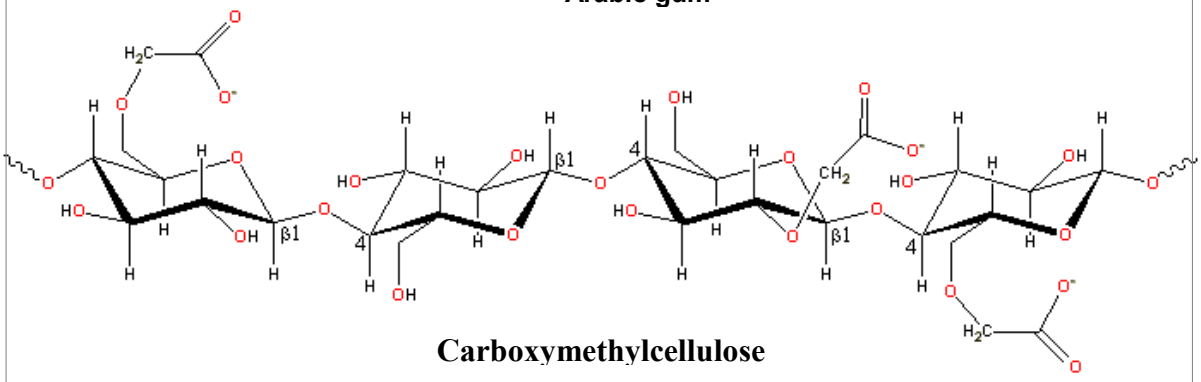
Polyvinylpyrrolidone



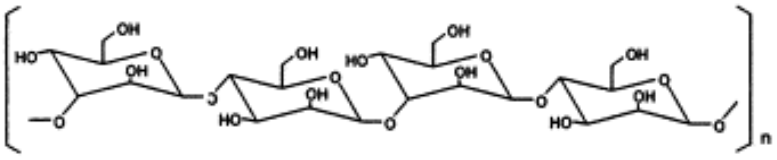
Alginic acid



Arabic gum



Carboxymethylcellulose



Hypothetical structure of polymannan from *R. mucilaginosa* YR2 (75)

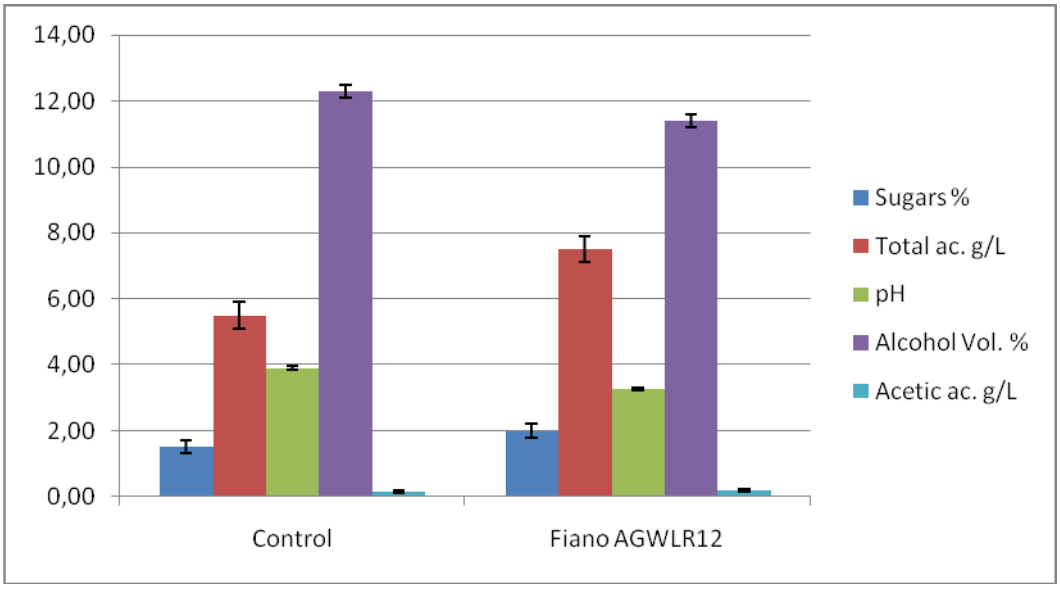
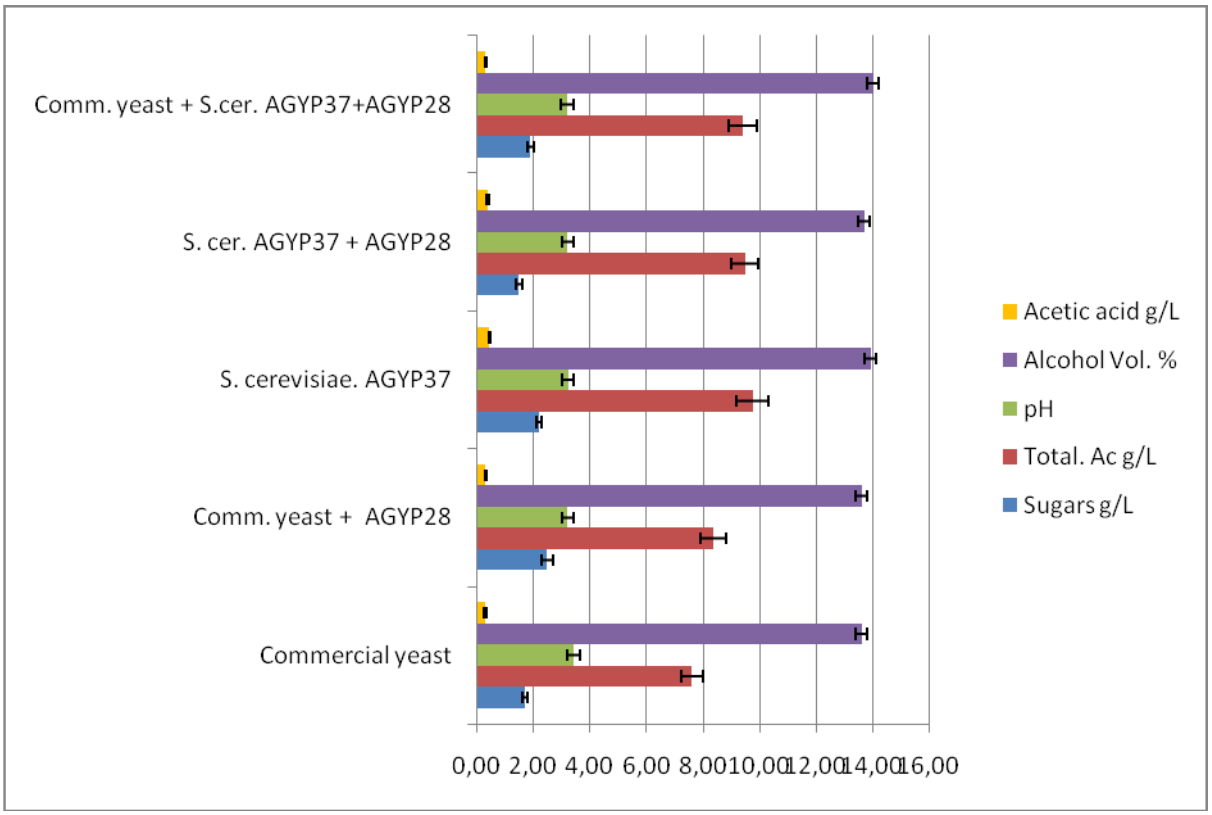


Fig.13

4.2- 2008 Aglianico vintage

During the 2008 vintage, as the majority of our yeast strains had just been isolated, we had enough yeast strains to carry out several fermentations using both *Saccharomyces* spp. and non-*Saccharomyces spp*. in different combinations; each isolated yeast strain was used to ferment the must of origin alone. The volume of must used for each process was 30 litres. Furthermore, in this second series of experiments, not only were the usual oenological parameters (residual sugars, total acidity, acetic acid, pH and alcohol volume) checked, but also the quantity of total polyphenols, total flavonoids, total and coloured anthocyanins, antiradical activity, reducing power, colour intensity and shade (only for red wines), which are parameters that greatly affect the wholesomeness and the appearance of wine and consequently the impact on consumers. This further analysis provides a deeper insight into the reciprocal interactions of native and commercial strains. In Aglianico, after 18 days all the fermentations were completed, with no further sugar consumption and carbon dioxide production. The values of residual sugars, total acidity, acetic acid, pH and alcohol volume are shown in fig.14 and reported in table 5. All the fermentations in Aglianico were completed, as the residual sugars in final wines were present at levels of up to 2,5 g/L or less. It is interesting to note that the combination of native yeasts AGYP37/AGYP28, selected only for their SO_2 resistance and β-glycosidase activity, showed the same results as the commercial yeast, a strain belonging to *S. cerevisiae* var. *bayanus*, naturally more alcohol resistant than a normal *S. cerevisiae,* and specifically selected to complete efficiently the fermentation. In fact, it is usually used to ferment high sugar content musts. The yeast *S. cerevisiae* AGYP37 is responsible for most of the sugar consumption, but, when used alone, it stops the fermentation at a level of residual sugars higher than the commercial strain, as shown by statistical analysis ($p<0,001$, 2,2 g/L vs. 1,5 g/L of residual sugars) so part of the efficiency of the AGYP37/AGYP28 combination is due to *Metschnikowia pulcherrima*.

Fig.14: Oenological parameters in 2008 Aglianico fermentations



Commercial yeast: *S. Cerevisiae* var. *Bayanus*; AGYP28: *Metschnikowia pulcherrima*

Table 5

Yeast strain	Sugars (g/L)	Total acidity (g/L)	pH	Alcohol vol. %	Acetic acid (g/L)
Commercial yeast	1,7±0,1	7,61±0,40	3,44±0,22	13,6±0,2	0,30±0,02
Comm. + AGYP28	2,5±0,2	8,36±0,45	3,23±0,20	13,6±0,2	0,33±0,03
AGYP37	2,2±0,1	9,75±0,55	3,24±0,20	13,9±0,2	0,45±0,04
AGYP37+AGYP28	1,5±0,1	9,49±0,48	3,20±0,21	13,7±0,2	0,33±0,03
Comm.+AGYP37+AGYP28	1,9±0,1	9,41±0,49	3,23±0,22	14,0±0,2	0,39±0,03

Along with the commercial *Saccharomyces* strain, this same non-*Saccharomyces* strain fails to improve the efficiency of sugar uptake, indeed making it worse ($p<0,001$, 2,5 g/L).The interaction among different strains seems to be important in determining the final sugar level, even if all wines can be classified as dry. Regarding total acidity, all experimental fermentations using the autochthonous yeast strain AGYP37 had a value significantly higher than that of the control, with an

improvement shifting around + 25%. The greater total acidity is probably due to a reduced precipitation of tartaric acid, but further research is required to provide an explanation to this phenomenon. The variations in pH and alcoholic content were not significant. When *M. pulcherrima* AGYP28 is present, it did not affect the alcohol content in the same way as *Rhodotorula mucilaginosa* AGWLR12, and it could be explained partly because it is not an obligate aerobe but a facultative aerobe, thus it can produce small amounts of alcohol, meaning favourable conditions for *Saccharomyces spp*. to propagate and become the dominant species. *S. cerevisiae* AGYP37 appears to be very alcohol tolerant and efficient at producing alcohol, as seen for the commercial strain specifically selected. The comparison between these two could lead to the hypothesis that there has been a natural selection in the vineyard or in the cellar and that AGYP37 could be optimized to metabolize the Aglianico must. The last of the classical oenological factors analysed was the presence of acetic acid. As expected, the acetic acid present in wines made by the commercial yeast was low, as this has been specifically chosen for these properties. Surprisingly, none of the experimental wines exceeded the threshold of 500 mg/L: the maximum concentration was reached by *S. cerevisiae* AGYP37, with 450 mg/L, well below the threshold of 1,2 g/L established by law for red wines. No high production of acetic acid was detected in presence of *M. pulcherrima* AGYP28, in contrast with what is reported in the literature: in many studies, *M. pulcherrima* has been shown to be a high producer of volatile acidity during wine fermentation (32). Our experiments show instead that not only its production of acetic acid is insignificant but that *M. pulcherrima* AGYP28 contributes to its decrease, as in the case of fermentation alongside AGYP37. This may be a hypothesis for the change in acetic acid production seen with AGYP37. It has been proposed that one possible reason for the production of acetic acid by wine yeast is the lack of fatty acids and sterols in must. In brief, the presence of easily oxidizable polyphenols and oxidising enzymes causes the consumption of all the oxygen, an essential reagent for synthesising the C16 and C18 unsaturated fatty acids, and for the cyclization of squalene. The yeast is therefore in a critical metabolic situation, and is forced to consume the acetyl-CoA surplus derived from the pyruvic acid of glycolysis by producing acetic acid (79). *M. pulcherrima* AGYP28 could affect this equilibrium providing an alternative source of fatty acids and sterols, as this yeast species is well known to accumulate lipids. Indeed, in addition to its red pigment, another typical morphological feature of *Metschnikowia pulcherrima* is the presence of large fat globules in cells appearing refractile under light microscopy (the name *pulcherrima*, which means “beautiful”, refers to this). As previously mentioned, in 2008 vintage we performed an analysis on wine which examined the amount of polyphenols, flavonoids, anthocyanins, antiradical and reducing power, and finally colour. The results are illustrated in fig.15, 16, 17 and 18. As expected, the use of native yeasts affects these parameters as well as the classical oenological features. The total polyphenol content is significantly higher ($p<0.001$) when using at least one native strain compared to the only commercial *S. cerevisiae* (fig.15), and each native yeast seems to contribute in a different manner, as the increase of the polyphenolic content changes using one or the other of these strains. The increase in polyphenols is higher in the presence of *S. cerevisiae* AGYP37 and does not subsequently increase, even in the presence of the other native yeast and of commercial yeast.

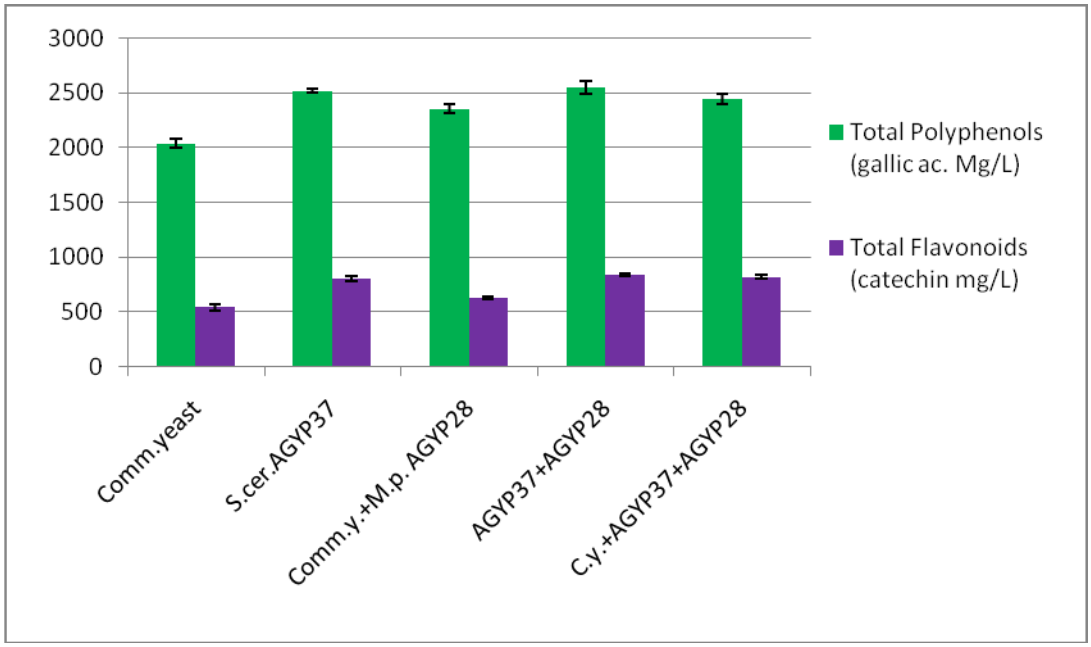


Fig.15

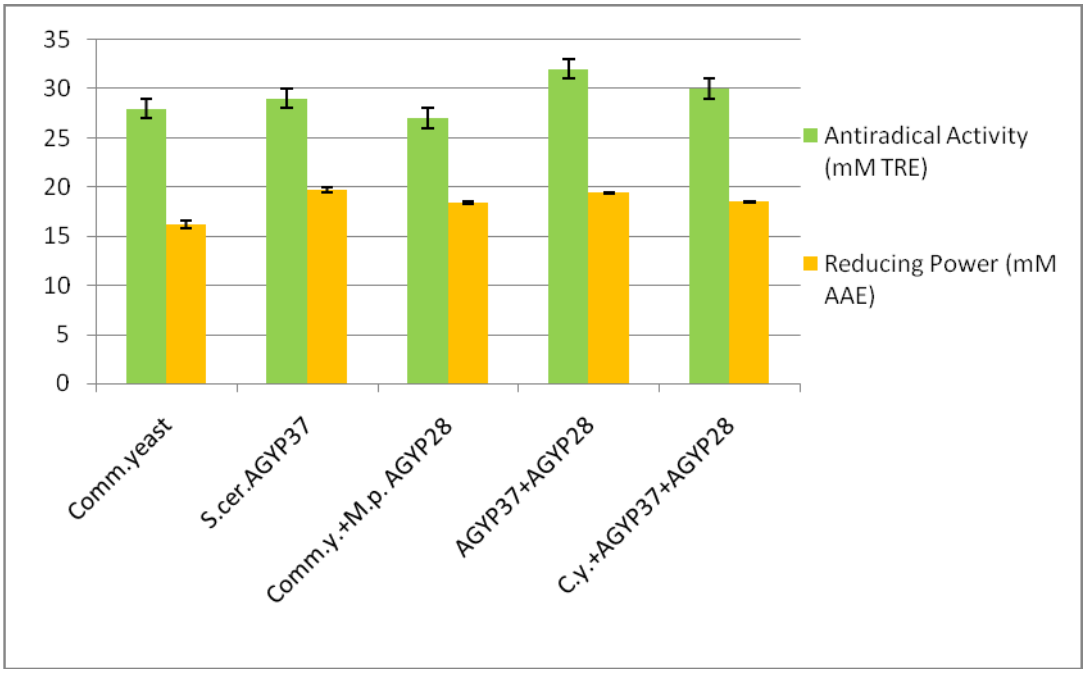


Fig.16

The presence of a higher polyphenol concentration provides an additional health benefit as these compounds have been.shown to be effective against reactive oxygen species (ROS), compounds involved in many diseases including cardiovascular diseases, certain types of cancer, neurological disorders, chronic kidney disease, inflammation hypertension and diabetes. They are normally generated in during cell metabolism. However, different external agents (cytokines, toxins, drugs, radiation, etc.) can also trigger ROS production. This can be largely counteracted by antioxidant defence systems, which can be enzymatic (superoxide dismutase, catalase, glutathione peroxidase), non-enzymatic (glutathione, paraoxonase), or dietary (antioxidant vitamins A, C, and E, and polyphenols).

Phenolic compounds are secondary antioxidants included in the category of free radical terminators. Phenolic antioxidants are excellent hydrogen or electron donors, and their phenoxy radical intermediates can stabilize the unpaired electrons by resonance delocalization around the aromatic ring, minimising the number of suitable sites for attack by molecular oxygen (47). The increase in polyphenol content is accompanied by a proportional increase in flavonoids and antioxidant activity, and antiradical activity remains unchanged, except in the fermentation with the combination of native yeast AGYP37/AGYP28, where it shows an increase, probably due to it possessing the highest content of resveratrol (see below).

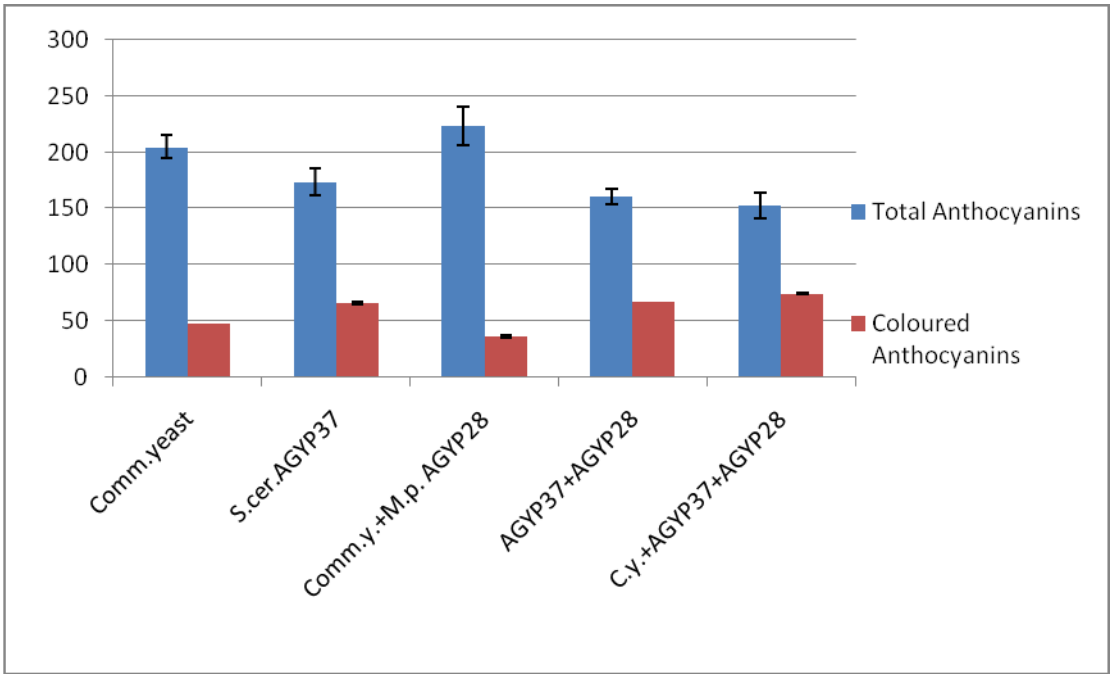


Fig.17

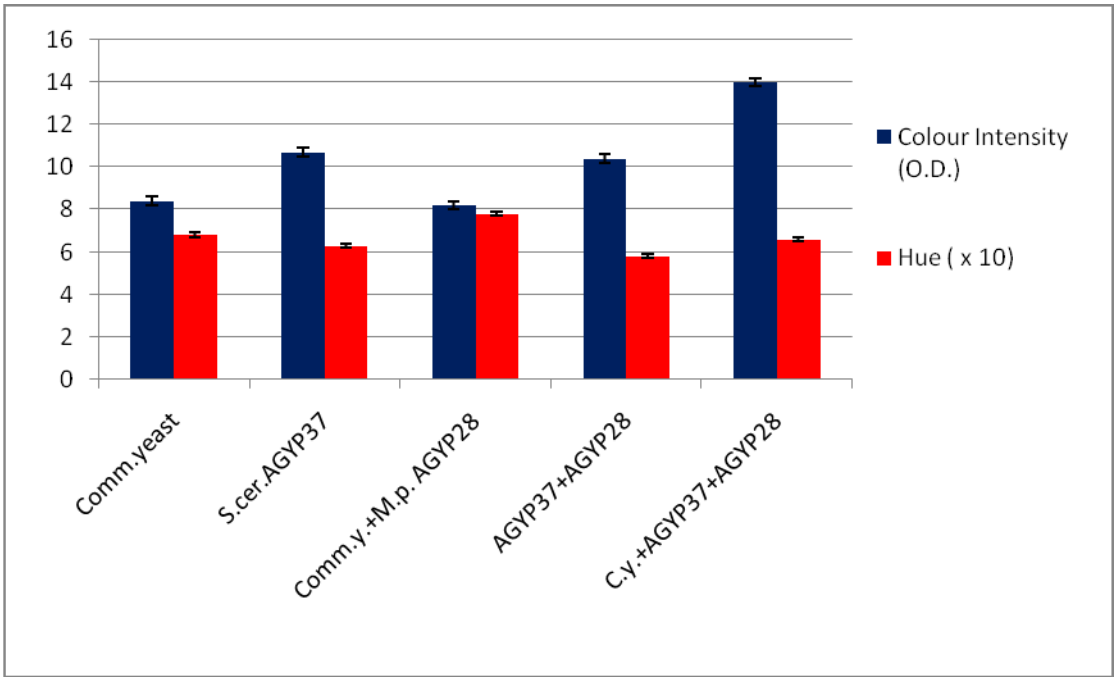


Fig.18

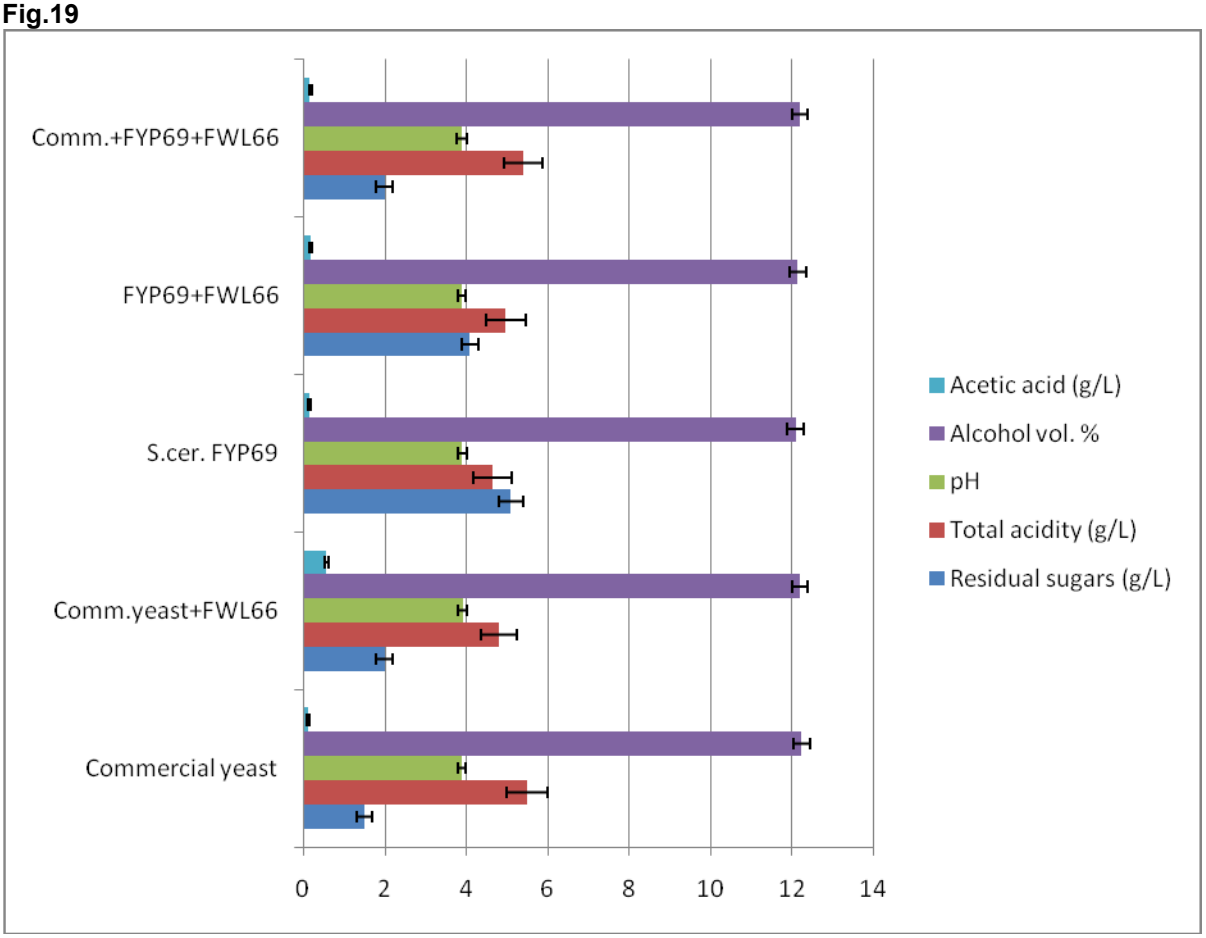
Anthocyanin analysis reveals the presence of two distinct groups: one with and one without Saccharomyces AGYP37. In the group where it is present, the total anthocyanin content is lower, with a decrease of 15 to 25% compared to control. Statistical correlation analysis shows that their content is inversely proportional to that of the colored anthocyanins (r =- 0.99) and that, contrary to those reported by some authors, it is not related to antioxidant activity (r = -- 0.52), but is attributable instead to the presence of polyphenols (r = 0.98) and flavonoids (r = 0.86). The increase of coloured anthocyanins in all fermentations conducted using AGYP37, corresponds to an obvious increase in the intensity of color, that reaches the highest OD value of about 14 using the combination of all three yeasts (commercial, AGYP37, AGYP28) compared to the OD value of about 8 seen in the control. The analysis of the hue of the color shows that all wines possess a value ranging from 0.5 and 0.7, a value which is quite normal for young wines that therefore do not show any signs of early oxidation, and a typical ruby red colour.The fermentations with AGYP28 could have an added value as *Metschnikowia pulcherrima* is reported to be useful in microbial preservation of food by means of its pigment, the pulcherrimin, which appears to act by chelating iron in the culture medium, albeit in a different way to other siderophores such as rhodotorulic acid, as it is retained within the cells.

4.3- 2008 Fiano vintage

Fermentations of Fiano took about 1 month as the sugar content was higher than in Aglianico must and the fermentation was carried out at a lower temperature (18°C as opposed to 26°C). With regards to the main oenological parameters, the commercial strain demonstrated high efficiency in consuming sugars in must, even in combination with the native strains (residual sugars≤ 2g/L). The use of autochthonous yeasts increased the concentration of residual sugars at the end of fermentation given that, in two cases, the threshold of 4 g/L was reached (FYP69/FWL66: 4.1 g/L) and exceeded (FYP69: 5.1 g/L), and the final product could therefore be attacked by acetic acid bacteria. All the experimental fermentations showed a very similar pH value and alcohol volume to the control, this latter likely due to the same reasons as for the Aglianico i.e. a part of the sugars consumed by the non-Saccharomyces strain FWL66 is converted into alcohol. As expected from preliminary data of the strain selection, the yeast *Hanseniaspora guilliermondii* FWL66 survived until the end of the process (fig.4). Despite a handful of previous reports, no growth inhibition of this yeast by *S. cerevisiae spp*. was detected during the tests. The total acidity tends to be constant in experimental wines. Finally the analysis of acetic acid shows that, with the exception of the Comm.y./FWL66 fermentation (acetic acid 580 mg/L), all the wines have levels inferior to 200 mg/L, well below the threshold of 1,08 g/L established by law for white wines. The potential production of high quantities of acetic acid by *Hanseniaspora guilliermondii* FWL66 seems to occur in combination with the commercial strains alone, whereas it is less significant in other cases. The literature is rife with articles that advise against the use of this species for wine fermentation, although it is reported that the production of acetic acid varies considerably among different strains of the same species. Analysis of the polyphenolic wine fraction shows that the total polyphenol and flavonoid content does not undergo significant changes compared to the control; antiradical activity is also unchanged (fig.20-21). Regarding the antioxidant activity, it remains unchanged compared to the control except in the case of fermentation with yeast

FYP69 alone where it undergoes a decrease, shown by the increased absorbance value at 420nm , which for white wines is an index of the oxidation state of the wine (fig.22). Given that it is white wine anthocyanins are absent.

Fig.19



Commercial yeast: *S. Cerevisiae* var. *Bayanus*; FWL66: *Hanseniaspora guilliermondii*

Table 6: Values of oenological parameters of Fiano fermentations.

Yeast strain	Residual sugars (g/L)	Total acidity (g/L)	pH	Alcohol volume %	Acetic acid (g/L)
Commercial yeast	1,5±0,2	5,50±0,50	3,90±0,10	12,3±0,2	0,11±0,03
Commercial yeast+FWL66	2,0±0,2	4,80±0,45	3,92±0,11	12,2±0,2	0,58±0,04
S.cerevisiae FYP69	5,1±0,3	4,65±0,47	3,91±0,12	12,1±0,2	0,15±0,03
FYP69+FWL66	4,0±0,2	4,98±0,50	3,90±0,10	12,2±0,2	0,19±0,03
Comm.yeast+FYP69+FWL66	2,0±0,2	5,40±0,48	3,89±0,12	12,2±0,2	0,17±0,03

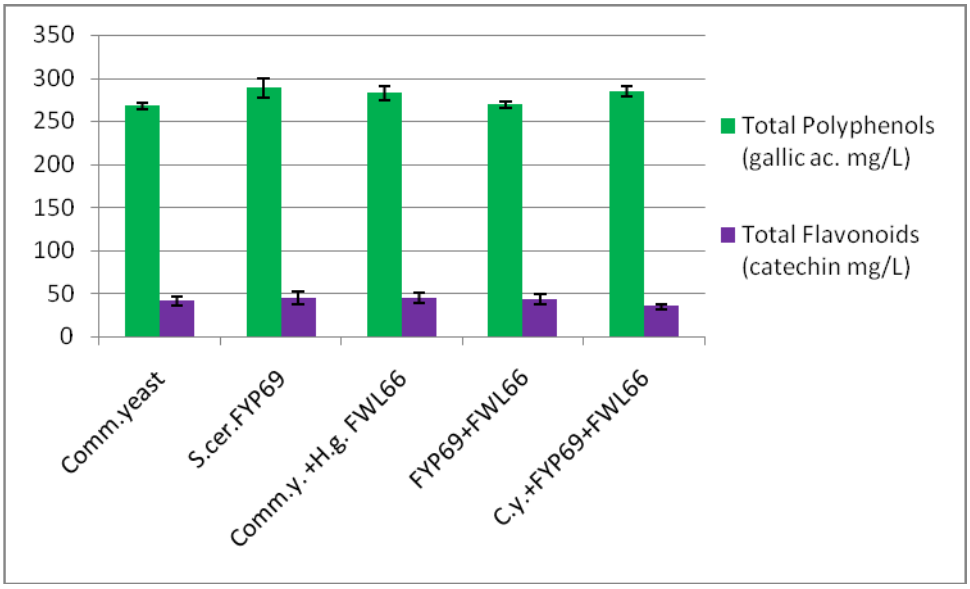


Fig.20

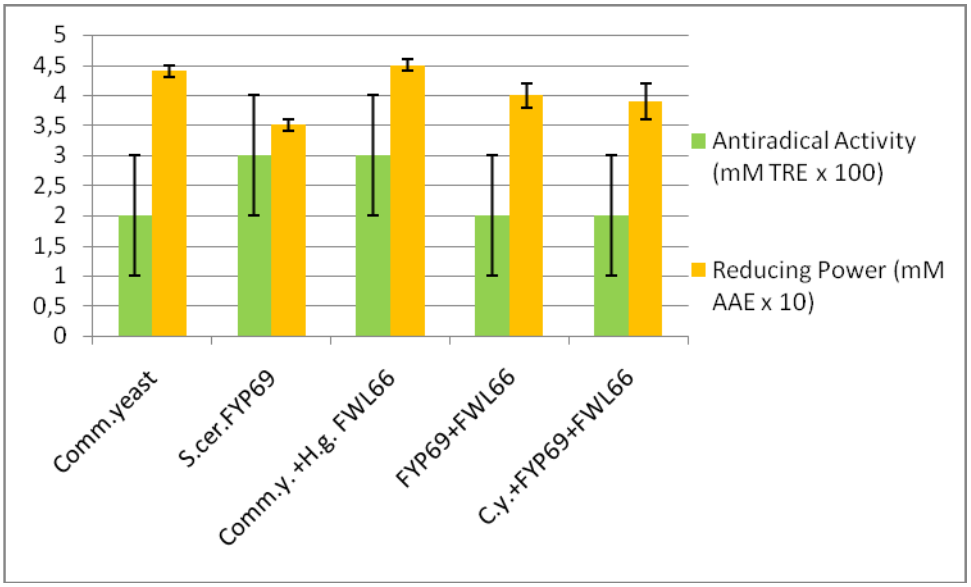


Fig.21

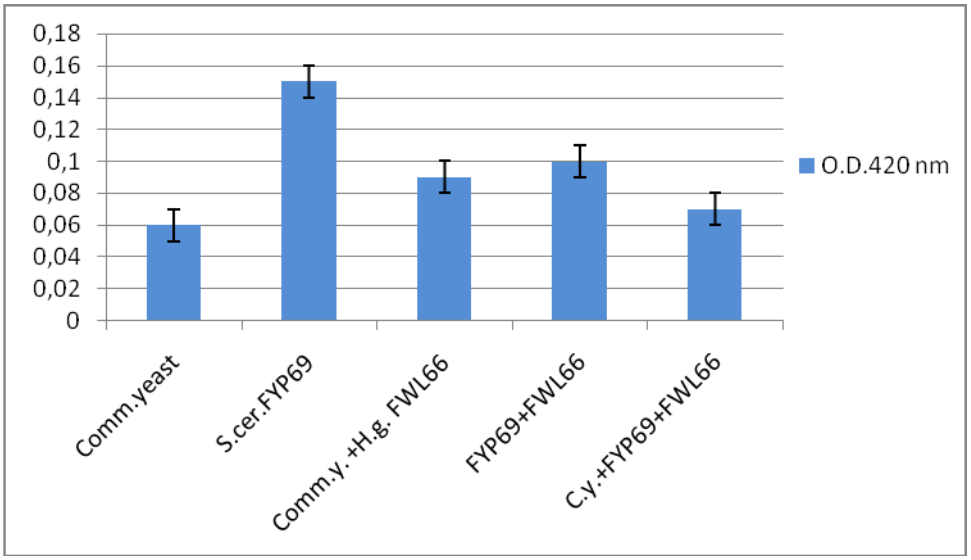


Fig.22

5- HPLC analysis of polyphenols

5.1- 2007 vintage

HPLC analysis of polyphenols in the 2007 vintage wines was conducted by analyzing four compounds: gallic acid, catechin, resveratrol and quercetin. The results obtained show that the Aglianico fermentation carried out with *R. mucilaginosa* AGWLR12 differs from the control with regards to the large increase in resveratrol. Since this compound is a phytoalexin which is only produced in plants, the only way in which its content can be increased in the must is by hydrolysis of its precursor, trans-piceide. The analysis of the β-glucosidase activity of AGWLR12 showed a high efficiency of this enzyme in catalyzing the reaction (see Characterization of β-glucosidase activity of AGWLR12 described below).
Also worthy of note is the absence in both the control and experimental wine of quercetin, a compound reported in the literature as always present in wine. The most likely explanation for this is that most or all of the quercetin is present as quercetin glucuronides, other forms in which this compound is present.
The experimental fermentation of Fiano however, shows only a slight decrease in catechins compared to the control.

5.2- 2008 vintage

HPLC analysis for samples obtained from the 2008 harvest was further expanded upon through analysis of the concentrations of epicatechin, cinnamic acid, p-coumaric acid, and chlorogenic acid. Results obtained for the Aglianico wine are reported in table 7 and shown in figure 23. The fermentation experiments with native yeasts show a gallic acid content that is higher than the control ($p<0,001$), except in the fermentation with commercial yeast and the two native strains. The presence of a greater quantity of gallic acid may be a consequence of hydrolysis of gallotannins acid, esters of gallic acid with glucose. In three of the four fermentations carried out with indigenous strains, the level of catechins increased by around 25%, whereas epicatechin remains more or less constant except in the fermentation with all three yeasts, where it decreases considerably. Coumaric acid and cinnamic acid always undergo a substantial decrease in the presence of native yeast. The resveratrol content in different wines does not differ significantly from the control except in the case of fermentation with the combination of the two native yeasts AGYP37/AGYP28, where it is present in greater quantities, and in the case of fermentation with all yeasts where it decreases.
As observed for the 2007 vintage, even in this case the quercetin was absent, which is likely to be a characteristic of the Aglianico grape. Chlorogenic acid is also nearly always absent in this combination, but is present in significant concentrations (36 mg / L) in the case of fermentation with autochthonous *Saccharomyces*. The absence of this compound, which is usually reported as being ubiquitous in wine, may signify the presence of an enzyme activity capable of splitting the ester-linkages between cinnamic and quinic acid. One explanation may be that this activity is repressed in the presence of AGYP37 only. A notable fermentation characteristic of the latter yeast include the highest levels of gallic acid, catechin and chlorogenic acid, as well as the highest levels of resveratrol compared to the control when it is in combination with AGYP28.

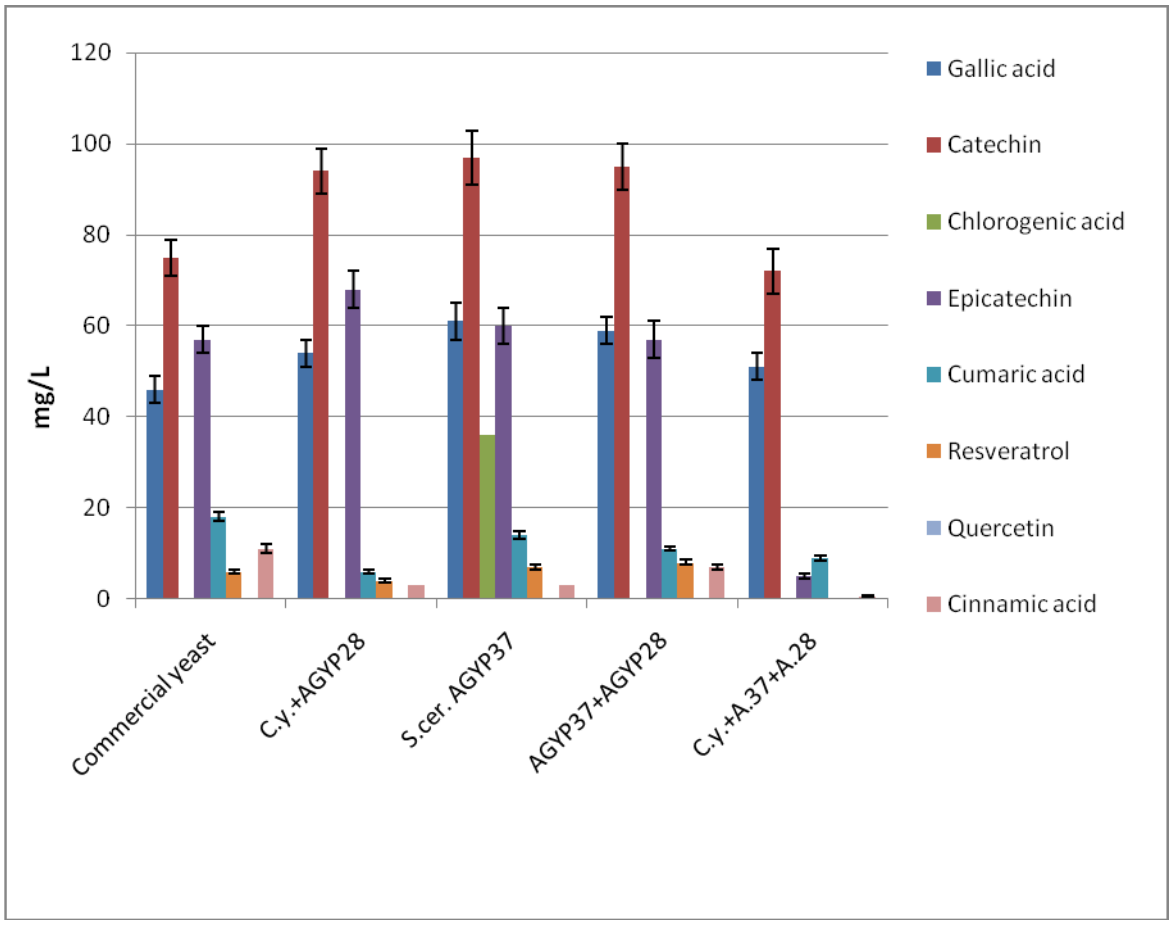


Fig.23

Table 7

Standard Polyphenols (mg/L)	Aglianico COMM. YEAST	Aglianico COM. Y.+ AGYP28	Aglianico AGYP37	Aglianico AGYP37+ AGYP28	Aglianico COM.Y.+ AGYP37+ AGYP28
Gallic acid	46.1±2.0	54.2±2.1	61.3±4.2	59.2±3.2	51.1±3.0
Catechin	75.3±4.4	94.2±5.2	97.1±6.1	95.3±5.1	72.2±5.2
Chlorogenic acid	/	/	36.3±2.4	/	/
Epicatechin	57.3±3,4	68.4±4.3	60.2±4.1	57.1±4.2	5.1±0.3
Coumaric acid	18.0±1.0	6.1±0.4	14±0.9	11±0.9	9±0.6
Resveratrol	6.0±0.5	4.3±0.4	7.0±0.5	8.1±0.5	0.4±0.1
Quercetin	/	/	/	/	/
Cinnammic acid	11.0±1.1	3.1±0.1	3.0±0.1	7.3±0.5	0.5±0.1

Instead, the combination of the two *Saccharomyces* strains appears somewhat detrimental since their co-fermentation causes the disappearance of resveratrol, epicatechin and cinnamic acid.

With regards to the fermentation in Fiano, the use of native yeast yielded more consistent results between them than observed for Aglianico. The amount of cholorgenic acid (here) and epicatechin remains constant. Gallic acid remains almost constant, unlike Aglianico. Coumaric acid and cinnamic acid instead always decrease in the fermentation experiments. The only compound to increase in the test is catechin, which shows an increase of between 14% and 24%. Overall polyphenol analysis suggests that no particular combination of yeasts used in the fermentation of Fiano appears to be better than the control.

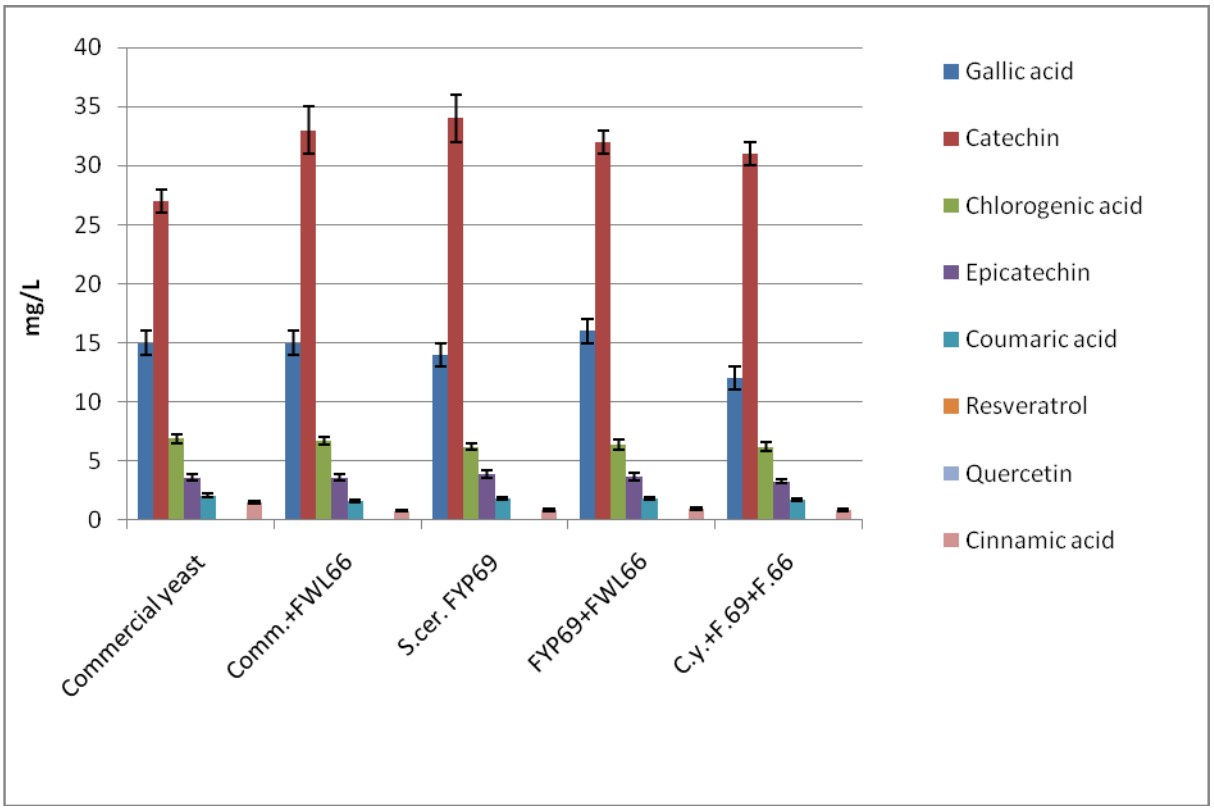


Fig.24

Table 8

Standard Polypehnols (µg/ml)	Fiano COMM	Fiano COMM + FWL66	Fiano FYP69	Fiano FYP69 + FWL66	Fiano COMM + FYP69 +FWL66
Gallic acid	15.3±0,7	14.7±1.0	14.1±0.9	15.7±0.7	12.0±1.0
Catechin	27.3±0,9	32.8±1.2	34.0±1.6	32.3±1.2	31.2±0.9
Chlorogenic ac.	6.9±0.4	6.7±0.3	6.2±0.3	6.4±0.4	6.2±0.4
Epicatechin	3.9±0.3	3.6±0.3	3.9±0.3	3.7±0.3	3.3±0.2
Coumaric ac.	2.1±0.2	1.6±0.1	1.8±0.1	1.8±0.1	1.7±0.1
Resveratrol	/	/	/	/	/
Quercetin	/	/	/	/	/
Cinnammic ac.	1.5±0.1	0.8±0.1	0.9±0.1	1±0.1	0.9±0.1

6- Analysis of aromatic profiles by GC-MS

For the assessment of the impact of native yeasts on the aromatic profile of wine, we focused on yeast strain *Rhodotorula mucilaginosa* AGWLR12, which is able to produce β-glucosidase enzyme of benefit to the winemaking processes. The purified enzyme showed a good activity in the specific pH, sugar and ethanol concentration conditions required for must fermentation. Application of β-glucosidase to our winemaking process lead to the release of a significant amount of glycosidically bound aromatic molecules contained in grape juice (30,44,69,80).
The aromatic profile of a wine consists of a qualitatively and quantitatively complex chemical pattern. Flavour production is affected by several factors such as soil, climate, vines, ripeness, yeast strain, winemaking process and ageing effect. More than 1000 compounds have been identified as contributors to wine aroma; these include a wide range of compound classes such as hydrocarbons, alcohols, esters, aldehydes, ketones, acids, ethers, lactones, sulphur and nitrogen compounds. In recent years, a new group of compounds, the nor-isoprenoids (Figure 25), have been subject of significant interest as they have been shown to make a substantial contribution to wine aroma, despite the fact that they are present at very low concentrations, i.e. within the parts per trillion (ppt or ng/L) range.

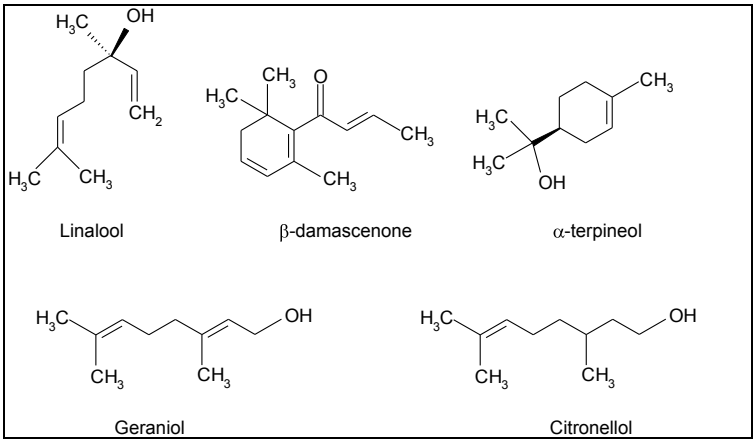


Fig.25: The most abundant nor-isoprenoids in Aglianico and Fiano

The volatile fractions of *Aglianico* and *Fiano* wines, obtained both with the autochthonous strain of *Rhodotorula* AGWLR12 and commercial *S. cerevisiae* yeast strains as control, were characterized by several components; the most important of them, a total of 38 compounds were selected and reported in Tables 9-13. Compounds were selected if present in both *Fiano* and *Aglianico* samples and according to quantity. Following winemaking, four samples of wine were withdrawn at several interval times for *Aglianico* and *Fiano* vines in 120 day ranges and for each one 3 aliquots were gathered. The samples were named: *Aglianico* grape, AGL M1 (must 9 days after harvest), AGL M2 (must 12 days after harvest), AGL W1 (wine collect 30 days after harvest), AGL W2 (wine collect 120 days after harvest), *Fiano*

grape, FIA M1 (must 9 days after harvest), FIA M2 (must 12 days after harvest), FIA W1 (wine collect 30 days after harvest), FIA W2 (wine collect 120 days after harvest). Two controls of *Aglianico* and *Fiano*, obtained through *Saccharomyces cerevisiae* and classic winemaking processes, were used as comparators to the samples studied.

Alcohols. The development of alcoholic fractions of volatile compounds is shown in table 9. In Aglianico AGWLR12, the concentration of alcohols increases in must during the fermentation, then decreases in wine 12 days after the end of the fermentation, and finally increases again. The methanol strongly decreases and the higher alcohols are more abundant than in the control but they don't exceed the threshold of 400 mg/L, over which they have negative repercussions on final wine quality. These alcohols are responsible for the ethereal aroma of wine. In Fiano AGWLR12, the development of alcohols is constant and all the compounds occur at lower concentrations than in the control.

Aldehydes and ketones. Table 12 shows the results obtained for acetaldehyde, isobutanal, 3-methylbutanal, 2-methylbutanal, 3-methylpentanal, hexanal and 2-hexanal. Acetaldehyde is the earliest metabolic sub-products of fermentation, but within the examined groups, no significant differences can be observed. In this case the selected yeast strain inoculated did not have any significant influence. In fact, the concentrations seen for *Aglianico* and *Fiano* vines were similar both in musts and in wines. The only difference observed compare to the control, is the exclusive presence of hexanal in Aglianico AGWLR12. Its presence can be important as this aroma (*grassy*) has a very low sensory threshold (0,05 µg/L). More significant are the differences between wines regarding ketones: the *Aglianico* grapes demonstrated a more significant ketone content compared to *Fiano* grapes. It is interesting to observe that during the fermentations with *Rhodotorula spp.,* ketones of both *Aglianico* and *Fiano* musts and wines decreased by 94,3 and 84,7% respectively. Furthermore, in Aglianico AGWLR12 acetone concentration decreased significantly (table 10).
.
Esters. Table 11 shows that the ester content in final products is lower for *Aglianico*, (255,59 µg/L), compared to that observed for *Fiano* wine (361,46 µg/L); these values are similar to those of relative controls (266,61 and 398,44 µg/L respectively). Ethyl acetate was the predominant ester, and its development was similar in the *Aglianico* and *Fiano* wines. In both cases, the concentrations of this ester were around 200 µg/L, meaning no detrimental impact on the quality of the wine, as also demonstrated by the Panel test described below. In fact, it was generally observed that a greater concentration of ethyl acetate in the wine resulted in an "acidic taste" which was of an unpleasant taste. Isoamyl acetate, displaying a characteristic banana-like odour, developed in a different way in each one of the groups studied. During fermentation, it reached a maximum of 17,27 µg/L (AGL W2) in *Aglianico* samples, and a higher value of 81,3 µg/l in *Fiano* samples (FIA W2), but both were lower than the control. Instead, an increase of etylformate (*raspberry*) in Aglianico was detected.

Table 9. Concentration of alcohols (mg/L)

Compounds	Aglianico Grape	AGL M1	AGL M2	AGL W1	AGL W2	Aglianico control	Fiano Grape	FIA M1	FIA M2	FIA W1	FIA W2	Fiano control
methanol	13,05	10,52	77,99	26,31	30,59	80,020	8,90	13,60	27,88	25,69	20,62	22,729
ethanol	159,50	106,70	831,30	657,00	806,50	808,583	59,60	54,83	680,10	683,60	1059,90	1168,328
1-propanol	2,46	0,08	6,16	3,91	11,54	6,209	0,00	0,06	3,42	1,08	8,84	9,744
isobutanol	0,79	11,02	41,64	26,71	84,05	44,405	0,52	1,62	10,18	2,14	39,38	43,409
3-methyl-1-butanol	5,42	92,99	24,23	9,40	123,40	26,892	2,58	32,21	1,59	0,90	107,10	118,056
2-methyl-1-butanol	20,03	52,43	22,84	19,12	85,00	32,616	15,09	28,19	14,51	0,36	74,58	82,210
Σ Alcohols	**201,25**	**273,74**	**1004,16**	**742,45**	**1141,08**	**998,723**	**86,69**	**130,51**	**737,68**	**713,77**	**1310,42**	**1444,476**

Table 10. Concentration of ketones (µg/L)

	Aglianico Grape	AGL M1	AGL M2	AGL W1	AGL W2	Aglianico control	Fiano Grape	FIA M1	FIA M2	FIA W1	FIA W2	Fiano control
acetone	54,08	0,33	1,71	1,90	0,87	3,458	18,46	0,18	0,70	0,83	2,43	2,679
2,3-butanedione	0,26	0,05	7,71	2,76	2,22	2,358	0,04	0,06	7,29	0,89	0,42	0,463
2,3-heptanedione	0,00	0,00	0,45	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
2-heptanone	0,32	0,00	0,00	0,00	0,00	0,00	0,07	0,00	0,00	0,00	0,00	0,00
Σ Ketones	**54,66**	**0,38**	**9,87**	**4,66**	**3,09**	**5,186**	**18,57**	**0,24**	**7,99**	**1,72**	**2,85**	**3,142**

Table 11. Concentration of esters (µg/L)

Compounds	Aglianico Grape	AGL M1	AGL M2	AGL W1	AGL W2	Aglianico control	Fiano Grape	FIA M1	FIA M2	FIA W1	FIA W2	Fiano control
ethylformate	0,05	0,11	2,69	4,78	4,37	1,572	0,00	0,00	2,68	2,96	2,00	2,205
ethylpropionate	0,22	0,39	12,15	10,32	6,65	6,471	0,00	0,00	1,93	1,61	1,40	1,543
isoamylformate	0,00	0,00	0,18	0,10	0,00	0,052	0,00	0,00	0,16	0,07	0,07	0,077
propylpropionate	0,00	0,00	0,00	0,00	0,00	0,000	0,00	0,00	0,00	0,26	0,00	0,000
ethyl-2-methylbutyrate	0,00	0,00	0,09	0,11	0,15	0,105	0,00	0,00	0,06	0,02	0,06	0,066
ethylisovalerate	0,00	0,05	0,07	0,10	0,17	0,157	0,00	0,01	0,04	0,09	0,07	0,077
ethylhexanoate	0,59	1,71	1,34	0,43	0,84	0,720	0,23	0,67	0,94	0,22	8,29	9,138
methylacetate	0,71	1,57	10,16	8,96	7,16	7,938	0,04	0,02	1,17	1,40	2,05	2,260
ethylacetate	12,86	48,98	260,30	195,40	207,30	220,975	1,06	3,38	179,30	181,80	236,70	260,914
propylacetate	0,00	0,00	1,65	0,94	0,52	0,511	0,00	0,00	3,46	2,17	2,02	2,227
ethylisobutyrate	0,02	0,00	1,83	2,15	2,49	1,912	0,00	0,00	1,26	1,12	1,42	1,565
isobutylacetate	0,00	0,17	7,72	5,57	3,26	2,594	0,00	0,03	20,19	9,27	7,74	8,532
ethylbutyrate	0,00	0,31	6,21	4,05	3,37	3,432	0,00	0,13	13,17	7,48	9,37	10,329
isoamylacetate	2,12	5,54	36,52	17,36	17,27	18,574	0,56	1,43	60,34	22,34	81,13	89,430
2-methylbutylacetate	0,15	0,48	3,47	2,01	1,99	1,546	0,03	0,07	8,01	1,76	8,07	8,896
hexylacetate	0,10	0,42	0,04	0,00	0,05	0,052	0,04	0,20	0,03	0,00	1,07	1,179
Σ Esters	**16,82**	**59,73**	**344,42**	**252,28**	**255,59**	**266,611**	**1,96**	**5,94**	**292,74**	**232,57**	**361,46**	**398,437**

Table 12. Concentration of aldehydes (µg/L)

Compounds	Aglianico Grape	AGL M1	AGL M2	AGL W1	AGL W2	Aglianico control	Fiano Grape	FIA M1	FIA M2	FIA W1	FIA W2	Fiano control
acetaldehyde	6,18	0,75	8,73	4,67	6,82	5,436	0,39	2,29	6,88	4,63	7,81	8,609
isobutanal	0,00	0,00	1,11	0,23	0,21	0,144	1,29	0,00	0,78	0,03	0,04	0,044
3-methylbutanal	3,96	0,54	6,65	3,13	2,17	1,860	1,27	1,99	6,42	0,98	0,90	0,992
2-methylbutanal	3,89	0,28	1,70	1,28	2,24	1,323	2,38	0,38	1,31	0,39	1,22	1,345
3-methylpentanal	73,76	83,69	78,21	76,49	81,03	99,668	72,59	89,26	76,01	0,00	119,70	131,945
hexanal	42,81	5,48	0,84	0,00	1,59	0,00	8,75	23,98	1,09	0,00	0,00	0,00
2-hexenal	3,37	0,15	0,00	0,00	0,00	0,00	0,90	0,10	0,00	0,00	0,00	0,00
Σ Aldehydes	**133,97**	**90,89**	**97,24**	**85,80**	**94,06**	**108,431**	**87,57**	**118,00**	**92,49**	**6,03**	**129,67**	**142,935**

Table 13. concentration of terpenes (µg/L)

Compounds	Aglianico Grape	AGL M1	AGL M2	AGL W1	AGL W2	Aglianico control	Fiano Grape	FIA M1	FIA M2	FIA W1	FIA W2	Fiano control
β-damascenone	5,42	8,37	8,84	12,14	16,43	2,585	0,99	1,55	1,61	2,87	3,22	1,926
Geraniol	2,65	3,25	3,86	4,63	5,55	4,736	4,65	5,26	5,47	7,72	8,61	3,528
Citronellol	3,11	4,86	5,14	5,74	6,86	4,265	6,22	7,96	11,12	22,87	32,05	3,177
Linalool	6,33	8,27	8,35	11,14	13,60	7,659	6,22	17,37	21,12	53,49	78,16	5,706
α-terpineol	7,48	9,79	9,95	13,28	15,99	4,989	1,38	1,79	2,14	2,89	3,80	3,717
Σ terpenes	**24,99**	**34,54**	**36,14**	**46,93**	**58,43**	**19,24**	**19,46**	**33,93**	**41,46**	**89,84**	**125,84**	**14,338**

Terpenes. Table 13 shows the different content of nor-isoprenoid molecules in samples. It is interesting to observe that there is an important enrichment in the volatile fraction compared to the control wine. In Aglianico with *R.mucilaginosa* AGWLR12, the terpenes increase three time whereas in Fiano they increase almost nine times. In the controls the nor-isoprenoid compounds are most abundant in red wine and the predominant compounds are α-terpineol and linalool, as they are in Fiano. In experimental wines, the most abundant terpenes in Aglianico are β-damascenone and α-terpineol, while in Fiano they are linalool and citronellol. According to Ugliano and Moio (78), the concentration of β-damascenone is very low (2,87 µg/l) in *Fiano* wine, while the linalool and geraniol are the predominant compounds. As can be seen in fig.26-27, the amount of terpenes constantly grows, even far beyond the end of fermentation. This is proof that the β-glucosidase from *Rhodotorula* AGWLR12 is highly stable in wine.

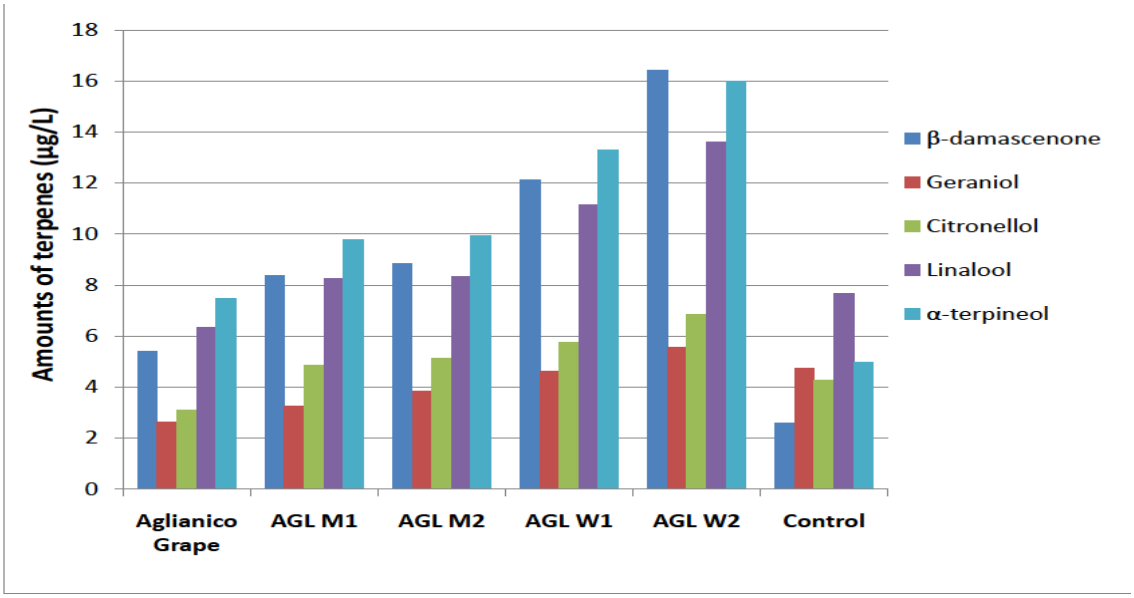


Fig.26:Aglianico samples

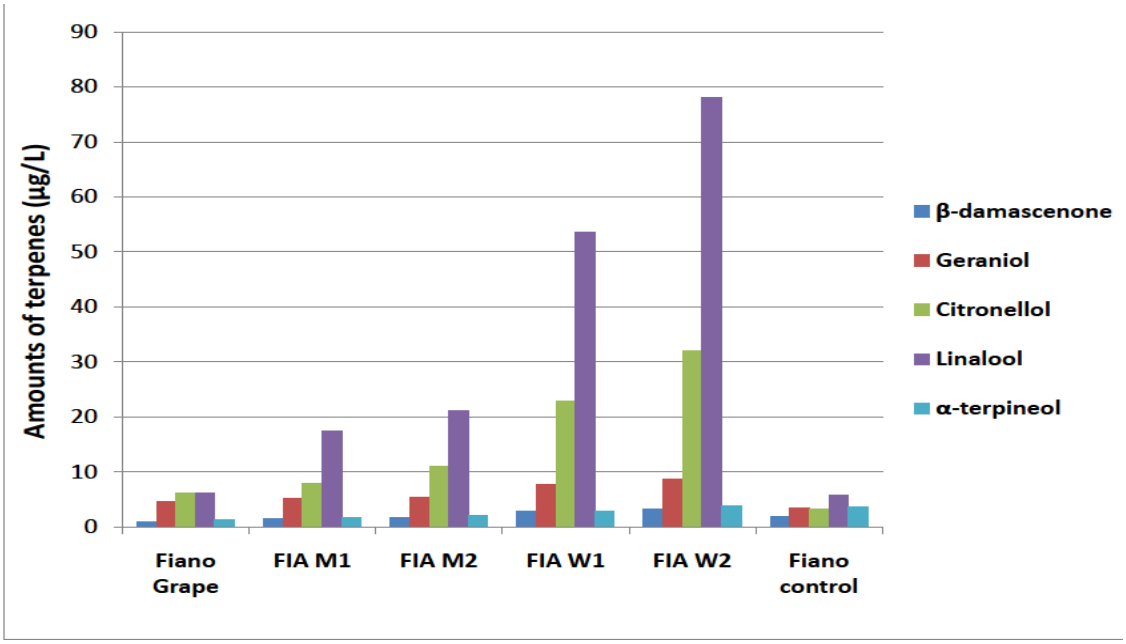


Fig.27: Fiano Samples

7- Sensory analysis

7.1- Sensory analysis of 2007 wines

As can be seen in figure 28 and 29, the wine sensory profiles were similar within each of the wine samples, but interestingly the samples performed with *Rhodotorula spp.* (FIA W2 and AGL W2) scored higher than the control. The general impression, intensity and terpenic flavour attributes improved with *Rhodotorula spp*., the Aglianico wine in particular scored the maximum value on intensity, terpenic and full flavour attributes. It is noteworthy that, despite the higher total acidity, Aglianico AGWLR12 was considered less acidic than the control.

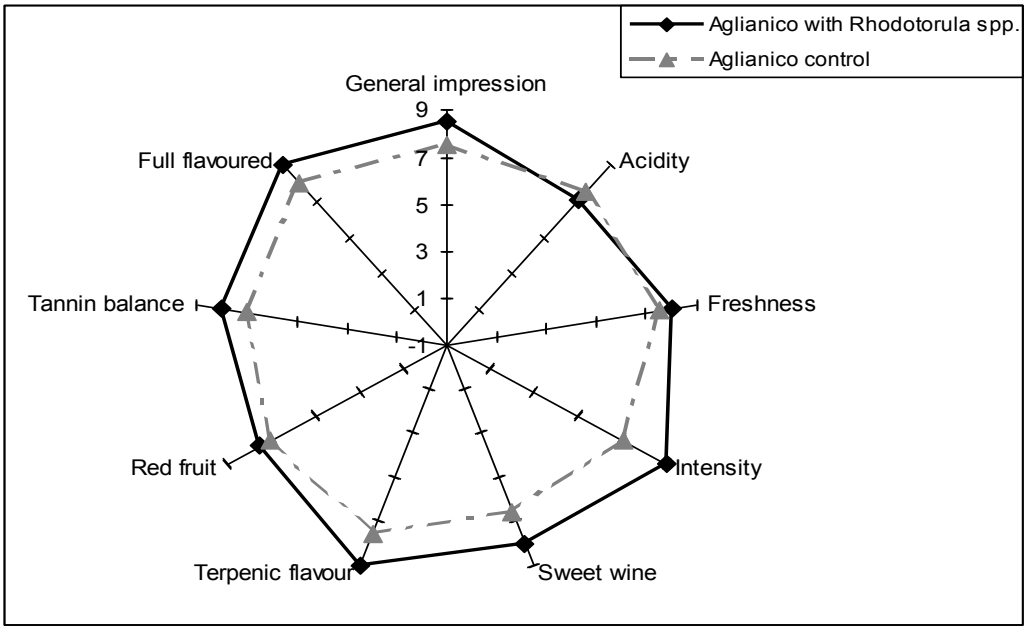


Fig.28

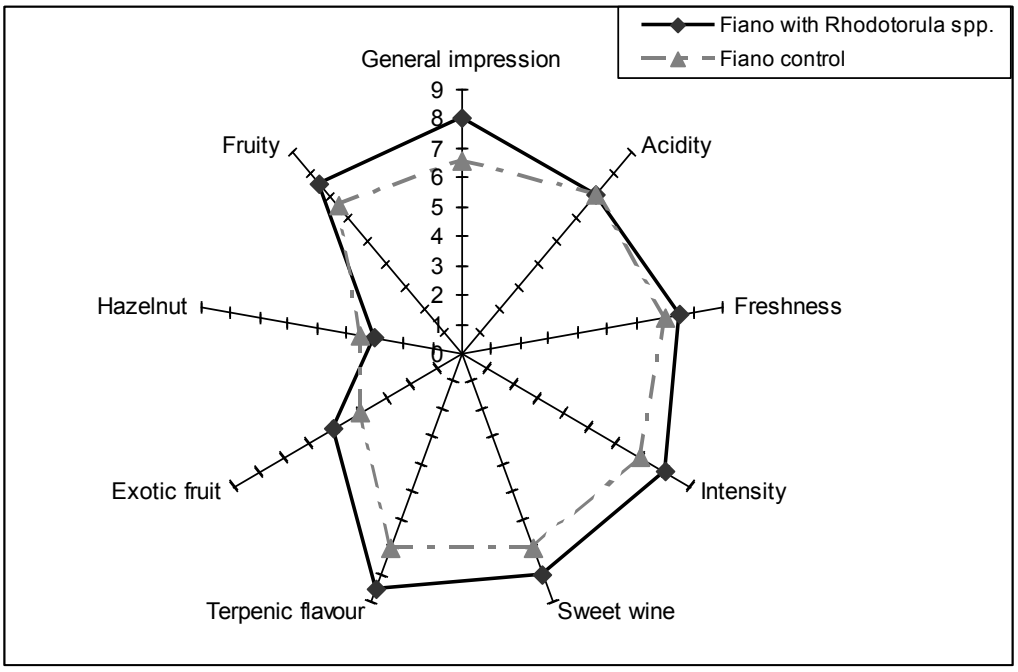


Fig.29

7.2- Sensory profile of 2008 vintage Aglianico samples

The preliminary evaluation of the data obtained by the panel regarding Aglianico showed, for the visual sensations, that the “ruby red intensity” attribute was of the highest intensity, which is usual for a young wine of this variety. It should be noted that the two samples treated with commercial yeast with and without the non-*Saccharomyces* yeast AGYP28 have slightly lower values, this is consistent with the values of anthocyanins as determined by chemical analysis.
With regards to the olfactory sensations, all of the samples scored medium to high for the "olfactory intensity" attribute. In particular, the sample treated with non-*Saccharomyces* AGYP28 and *Saccharomyces* AGYP37 showed a greater intensity of the “fruit odour" and "fruity odour" attributes. The “spicy odour” attribute was also noteworthy.
With regards to the gustatory-tactile sensations, the attributes that characterize the samples are: "astringency", "body" and "acidity". The "bitter" attribute shows medium values. In agreement with the chemical analysis, the sample treated with commercial yeast is less astringent than all other samples. It should also be noted that the sample treated only with the Saccharomyces Aglianico yeast shows higher "Bitter" values than all other samples. This is also apparent after swallowing sensation for the attribute "bitter aftertaste", in agreement with the presence of chlorogenic acid, which is absent in other samples.
After swallowing sensations show that the " fruity odour", "fruit odour" and "spice odour" attributes have medium values. All samples show quite high values for the attribute "positive aromatic intensity," which is also felt after swallowing with the "persistent aroma” attribute.

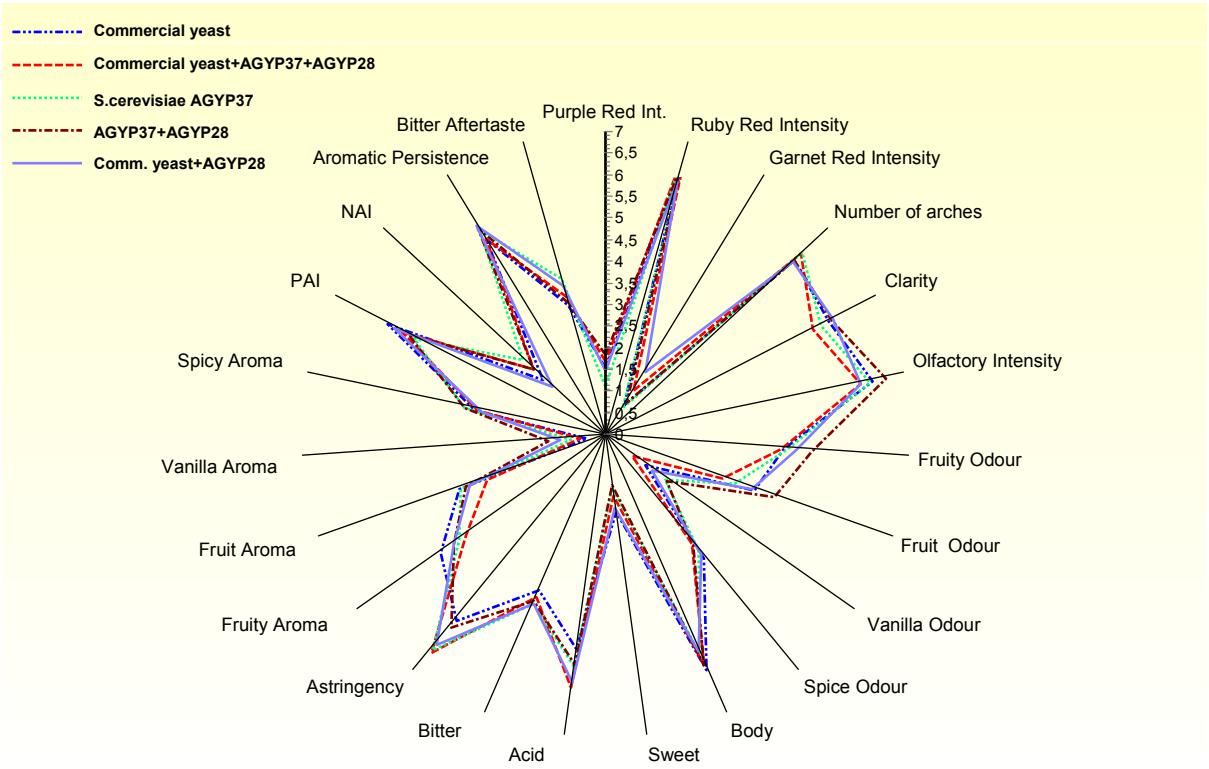


Fig.30

7.3- Sensory profile of 2008 vintage Fiano samples

The overall analysis of the sensory profiles of different Fiano wine samples treated with the five formulations of yeast showed for the visual sensations that the intensity of all attributes except the "intensity of green reflections'" attribute are quite higher. In particular, with regards to the "yellow intensity" attribute, the sample made by the commercial yeast and *H.guilliermondii* FWL66 has a greater intensity than all other samples. With regards to the "green reflections" attribute, the sample made with *S.cerevisiae* FYP69 shows lower values than the other samples. For the "transparency" attribute, sample with commercial yeast has a greater intensity.
Analysis relating to olfactory sensations show that the "olfactory intensity", "floral aroma" and "fruity odour" attributes are of the highest intensity. The "floral aroma" attribute shows the lowest value in the sample treated with commercial yeast , while the "fruity odour" attribute is higher in the sample treated with the commercial yeast and *H.guilliermondii* FWL66.
With regards to the gustatory-tactile sensations all the attributes evaluated show a moderate intensity in all samples. The only difference is found for the "burning" attribute, which is higher in the sample made with the commercial yeast and the FWL66 strain compared to the sample made with *Saccharomyces* FYP69.
With regards to the retro-olfactory and after swallowing sensations the "positive aromatic intensity" (PAI) and "aromatic persistence" attributes, respecitvely, are prevalent in all samples.

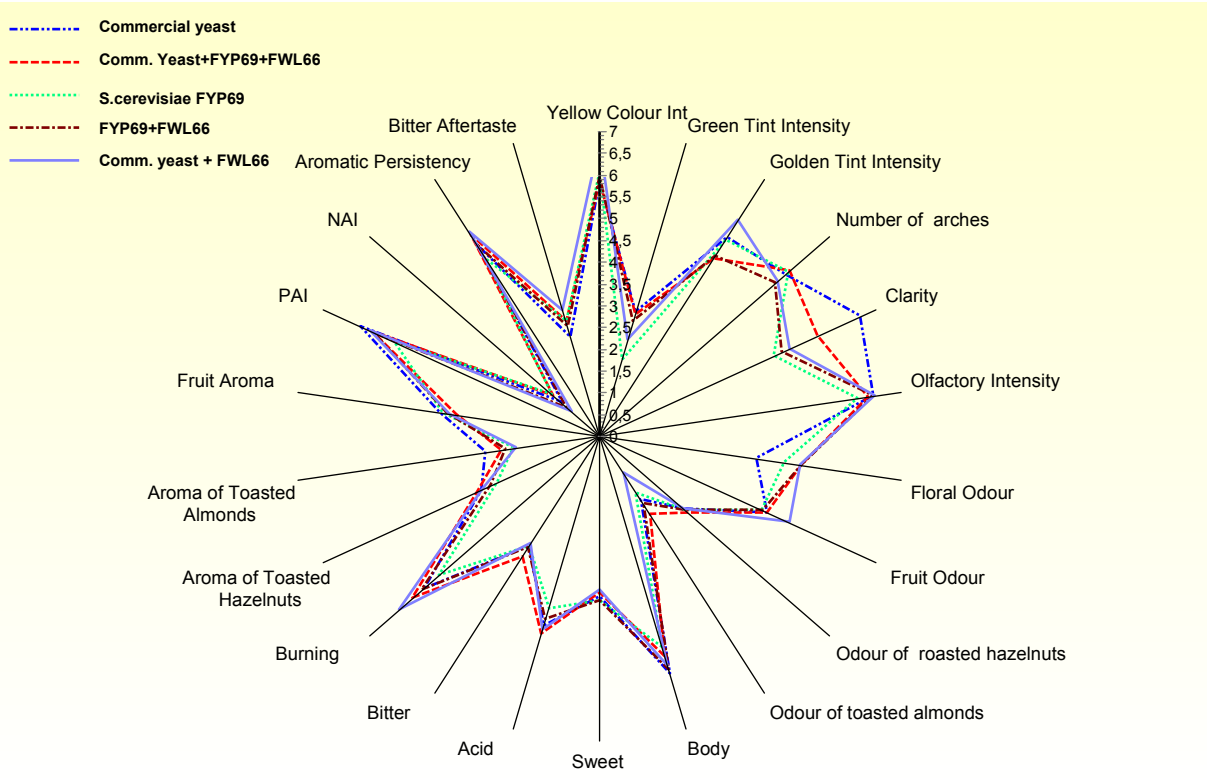


Fig.31

8- Purification and characterization of a β-glucosidase from *R. mucilaginosa* AGWLR12

8.1- Study of the β-glucosidase output in relation to the yeast growth

Before the purification step, a study of the production of β-glucosidase was carried out to assess the maximum amount of this enzyme in the supernatant of a yeast culture (YPD medium, 28°C, 160 rmp) as a function of time (fig.32). For the determination of enzyme units, a standard assay was used and the yeast growth was checked by measuring the absorbance at 600 nm.

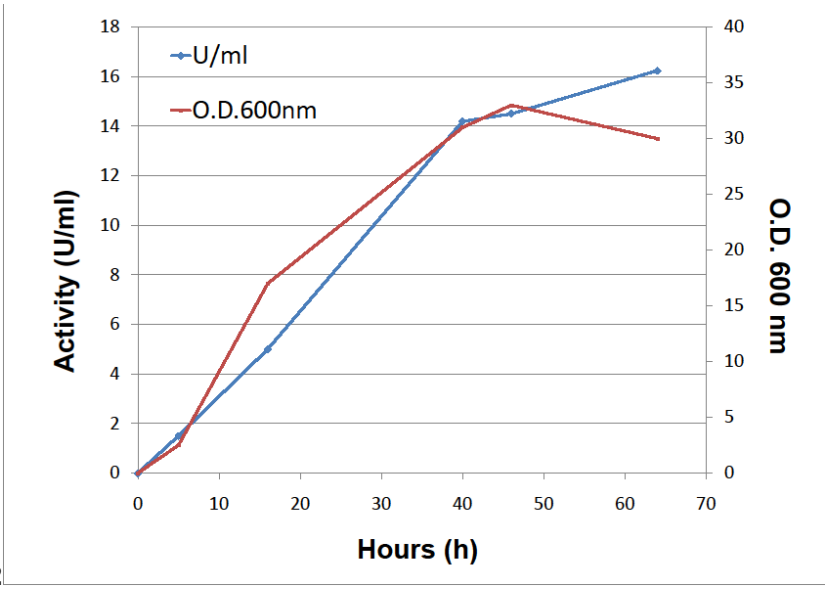


Fig.32

The data show that the production of β-glycosidase is related to growth until the stationary phase, at which point the maximum value is achieved.

8.2- Purification of the β-glucosidase

The enzyme was purified from a 2 litre culture of AGWLR12. Following centrifugation, the supernatant was concentrated to 400 ml by an ultrafiltration cell with a PM 10 membrane (Pellikon – Millipore). It was then further reduced to 60 ml by another ultrafiltration cell (Amicon – mod. 8400) with a YM 10 membrane. The enzyme was purified by ion exchange chromatography on a DEAE SEPHAROSE Fast Flow column and by gel filtration on a preparative SUPERDEX 200 26/60 column. As can be seen in fig. 33, several peaks with β-glycosidase activity were separated, suggesting that *R. mucilaginosa* AGWLR12 can express different β-glycosidase isoforms, as reported in the scientific literature for other oenological yeasts. The peak with the greater activity was eluted at an NaCl concentration of approximately 0,35M and these fractions alone were collected and subjected to the subsequent gel filtration step.

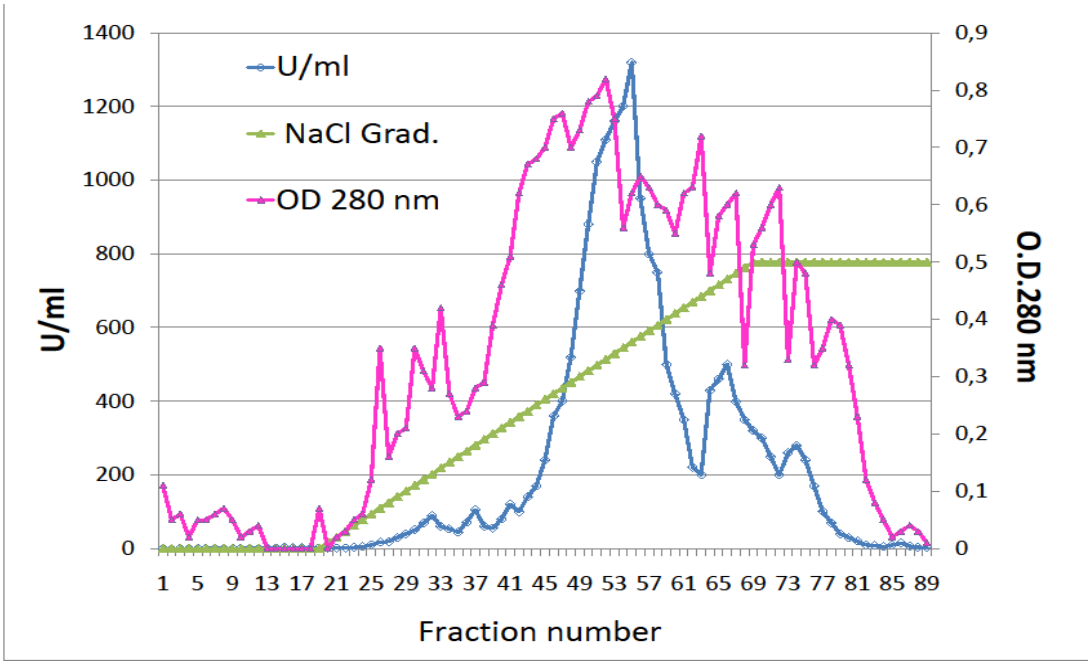


Fig. 33

The gel filtration step was carried out in Tris/HCl buffer 10 mM, pH 8, NaCl 150 mM. The enzyme was eluted in one peak as shown in fig. 34 and an aliquot (1ml) was subjected to another gel filtration step through an analytical Superdex 200 10/30 column that had been previously calibrated. The elution volume shows that the native enzyme has a molecular mass of approximately 190KDa.

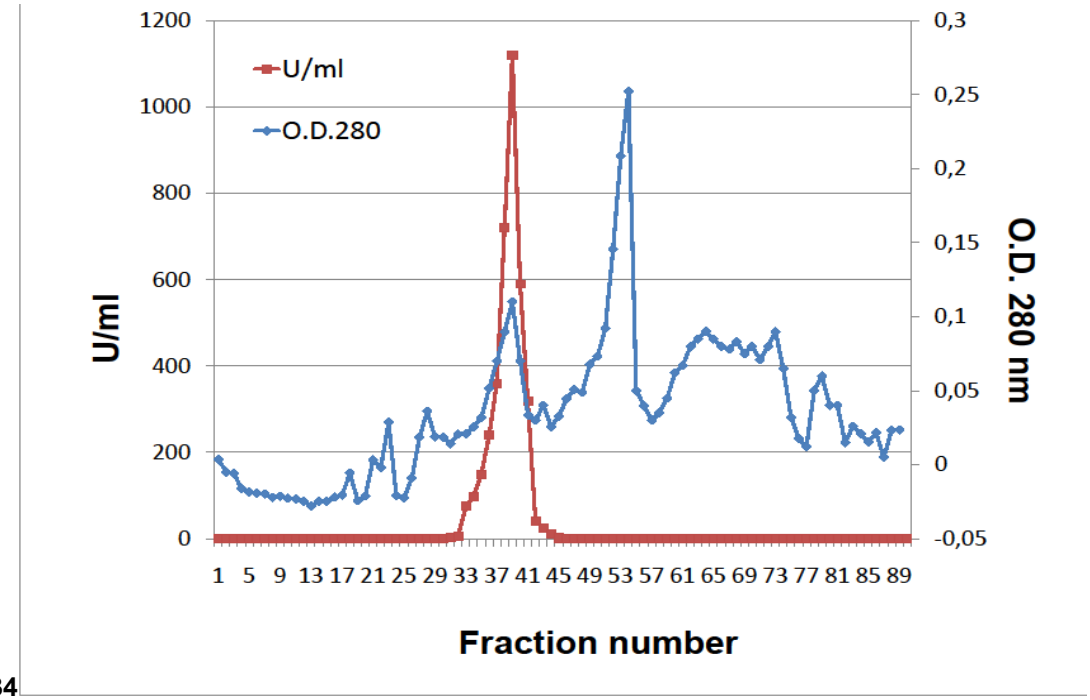


Fig. 34

The fractions eluted from Superdex 200 26/60 column were collected and used for β-glycosidase characterization. The following table summarises the purification step and the relative volumes and yields.

Table 14: Purification steps and yields of β-glycosidase from *R. mucilaginosa* AGWLR12

	Vol. (ml)	U/ml	Tot. U.	Prot. (mg/ml)	A.S. (U/mg)	Tot. Prot.s (mg)	Yield %	Purification
Supernatant	2500	18,3	45.800	0,05	366	125	100	1
Concentration with PM10 membrane	400	111,4	44.560	0,27	412,6	108	97,3	1,13
Concentration with YM10	60	724,2	43.462	1,54	470,2	92,4	94,9	1,28
DEAE Fast Flow	26	1003	26.077	0,33	3039,4	8,58	56,9	8,30
Superdex 200	24	764,6	18.350	0,059	12959	1,41	40,1	35,4

In order to determine the presence of quaternary structures, fractions collected from the Superdex 200 26/60 column were analysed by SDS-PAGE. The gel in fig. 35 shows that the enzyme was homogeneous and that the molecular mass of the single band visualized was 95 kDa, indicating that the native enzyme has a homodimeric structure.

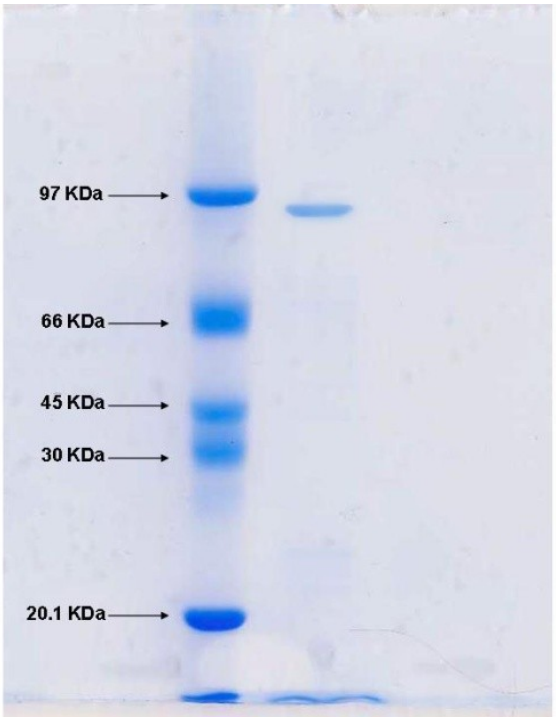


Fig.35

8.3- Dependence of enzyme activity upon pH

The results relating to the pH dependence of β-glucosidase activity are shown in fig. 36. This enzyme was shown to have an optimal pH value between 6.5 and 7.0, a value similar to that of other β-glucosidases reported in the literature for other non-*Saccharomyces* yeasts (15). Furthermore it showed a broad pH range activity and it was still quite active at the acidic conditions of must.

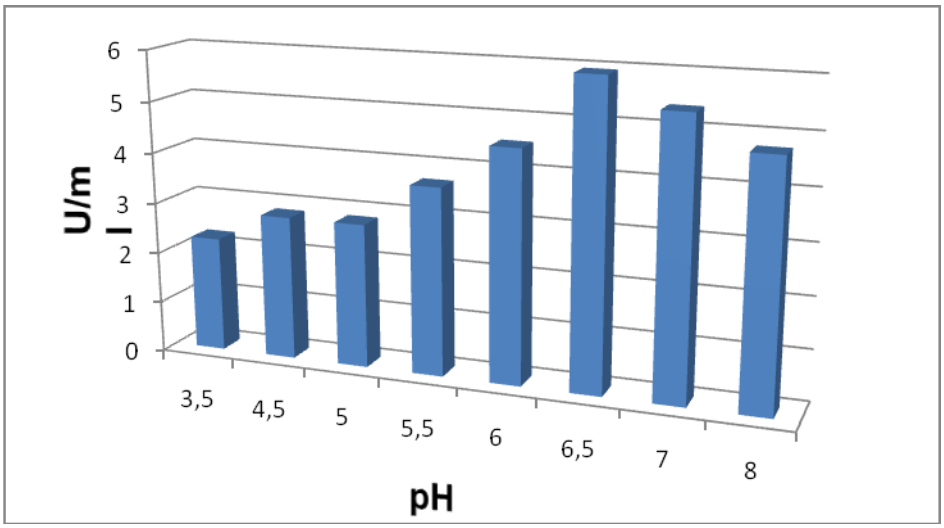


Fig.36

8.4- Temperature dependence of enzyme activity

The effect of temperature on enzyme activity is shown in fig. 37. This enzyme was shown to have an optimal temperature around 60°C, but retained 90% and 80% of its activity at 70°C and 80°C. This is quite interesting as other β-glucosidases from similar microorganisms (like *Debaryomyces hansenii*) has a lower optimal temperature (82).

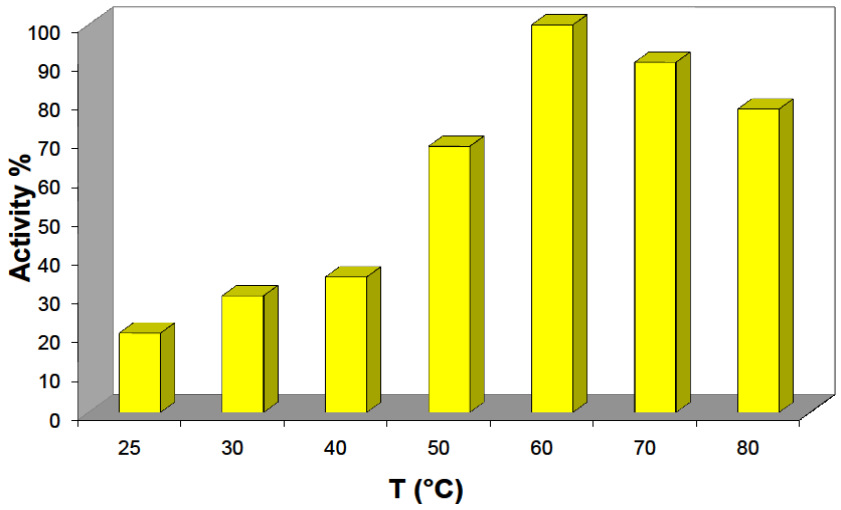


Fig.37

Although this β-glycosidase has a high optimal temperature, it is not thermostable (fig.38). Analysis of its thermostability indicates that its activity does not change at 25°C for 24 hours (90% of residual activity) whereas it decreases to 30% at its optimal temperature, and to 50% at 40°C after just 1 hour.

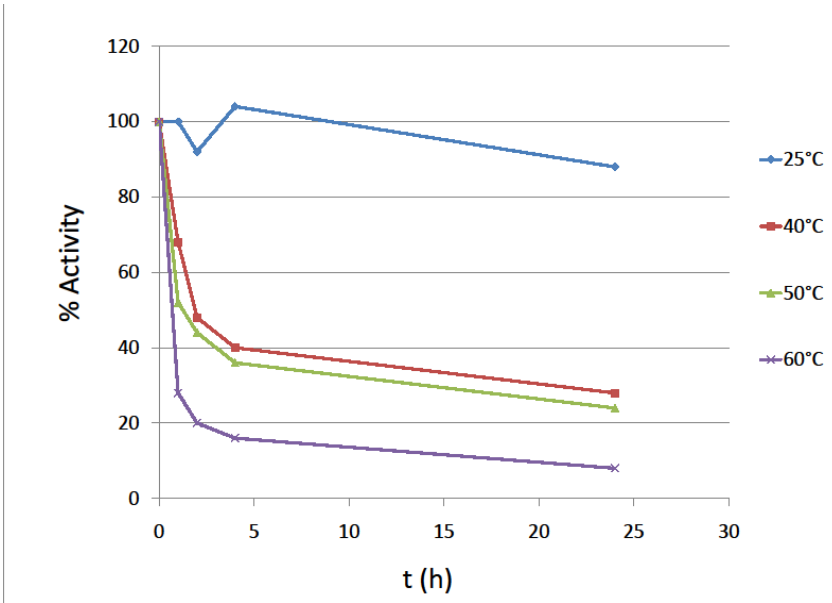


Fig.38

8.5- Study of kinetic constants

The kinetic parameters were calculated by the Michaelis-Menten equation (fig.39) and by way of a relative double reciprocal plot (fig.40) (see Materials and Methods).

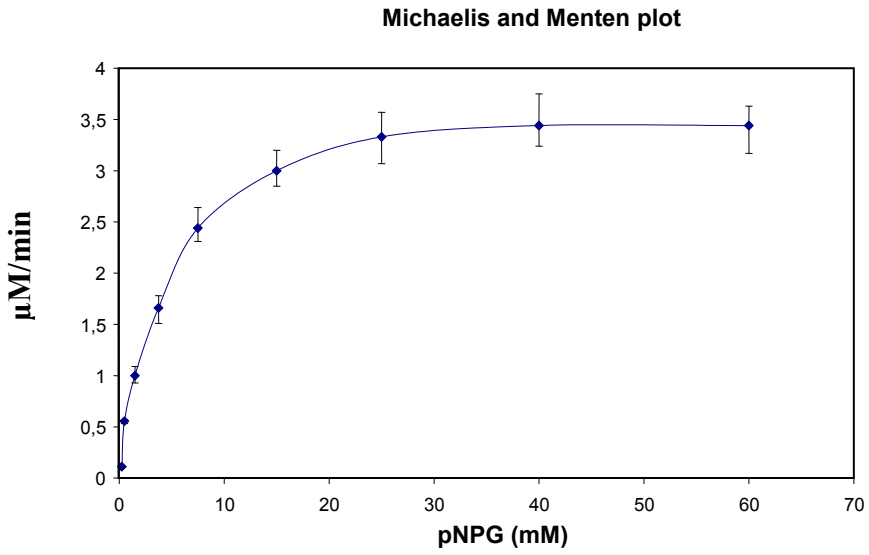


Fig.39

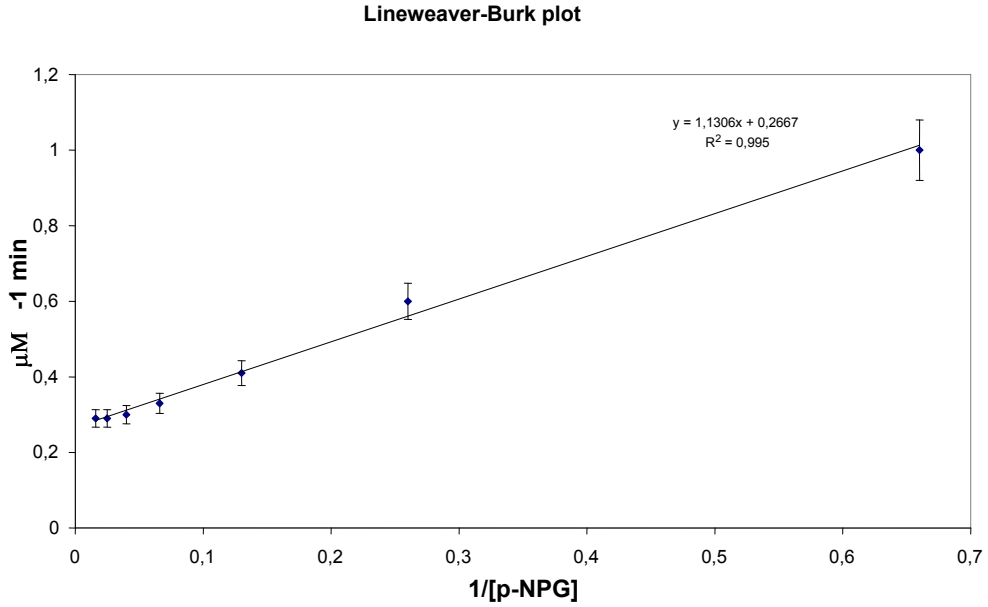


Fig.40

The K_m of AGWLR12 β-glycosidases was calculated as 4,23 mM and the V_{max} 3,75 µM min^{-1}. The K_m value is similar to that of β-glycosidases from *Debaryomyces hansenii* (60). This value is comparable to that obtained from the Michaelis-Menten curve. The K_{cat} value is 118,7 min^{-1}, so the ratio Kcat/Km is 28.06 $M^{-1}min^{-1}$

8.6- Specificity of the β-glucosidase toward synthetic substrates and disaccharides.

The specificity of the β-glucosidase toward different substrates was assessed using synthetic para-nitrophenyl-glycosides and natural disaccharides. The synthetic glycosides were used in a standard colorimetric assay whereas the enzyme hydrolysis of natural disaccharides was assessed by measuring the glucose released through a coupled enzyme reaction (see Materials and Methods). The results are summarized in table 15.

Tab.15

Synthetic Substrates	Activity %	Disaccharides	Activity %
p-nitrophenyl β-D-glucopyranoside	100	Cellobiose	100
p-nitrophenyl α-D-glucopyranoside	14.1±0.1	Maltose	19.0±0.1
p-nitrophenyl α-L-arabinopyranoside	12.5±0.1	Lactose	8.0±0.1
p-nitrophenyl β-D-galactopyranoside	10.2±0.1	Trehalose	18.7±0.1
p-nitrophenyl α-Xilopyranoside	5.4±0.1	Gentibiose	0

The data indicate that the β-glycosidases displays a great specificity towards synthetic substrates with a β 1-4 bond, then for substrates with α 1-4 and α 1-1 bonds, but it has poor or no activity toward β 1-6 and β 1-4 gal-glu bond of lactose.

8.7- Dependence of the β-glucosidase activity upon ethanol and sugar concentration

The study of the β-glucosidase activity shows that the activity of this enzyme is enhanced by ethanol, up to 200% at an ethanol concentration of 15% (Fig.41).

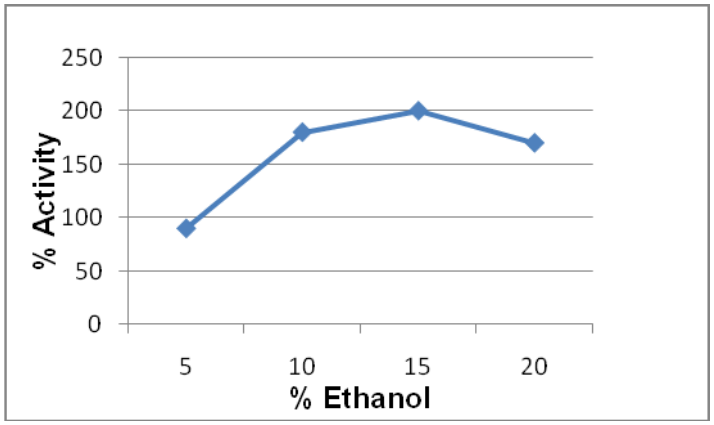


Fig.41

The analysis of activity inhibition by sugars revealed that the β-glycosidase is not inhibited by fructose whereas glucose inhibits 50% of its activity at high concentrations alone, over 15% (Fig.42). The inhibition by sugars is similar to that observed with other β-glycosidase from fungi and yeasts reported in the literature (49,68) as well as enzyme activation by ethanol. Some authors hypothesise that this activation could be due to ethanol acting as an acceptor of a key glycosidic intermediate (43).

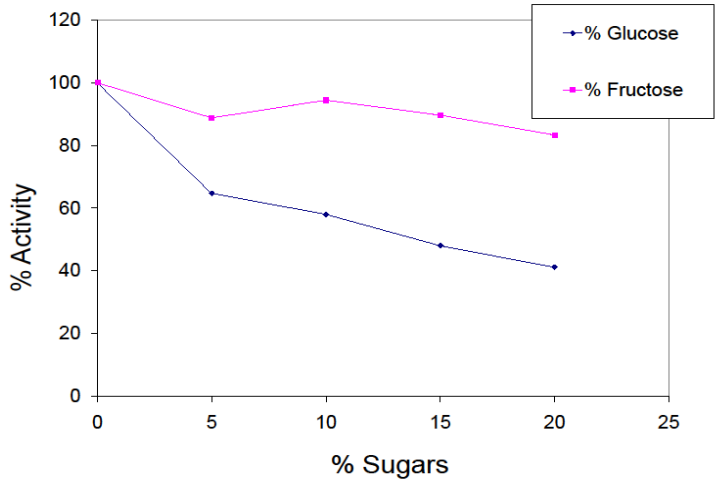


Fig.42

8.8- Effect of the β-glucosidase activity upon hydrolysis of piceid

HPLC analysis shows that β-glucosidase is very efficient in the hydrolysis of piceid and after 72 h at 45°C pH7, 80% of this compound is hydrolysed. This is a very important reaction because piceid has a much lower bioavability than the resveratrol, even if it is more abundant. The ability to hydrolyze piceid by a β-glucosidase has been reported for three other microorganisms only: *Aspergillus niger*, *Aspergillus orizae* and *Candida molischiana* (24,28).

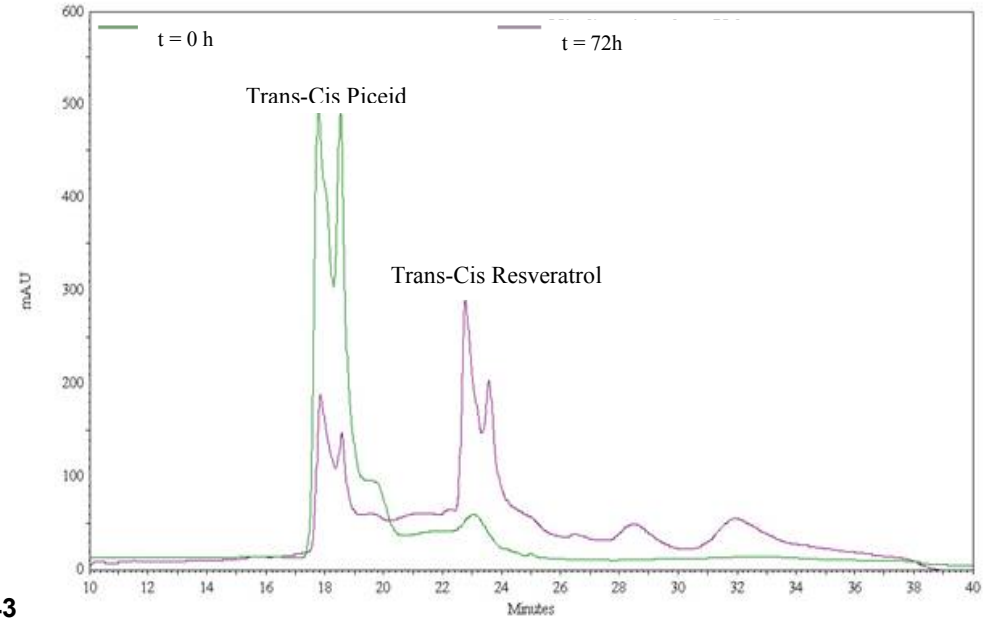


Fig.43

Conclusions

The continued use of sulphur as a plant protection agent in the vineyards and metabisulfite in the winery may have influenced the microbial biodiversity of indigenous yeasts of Aglianico and Fiano to generate more resistant strains. This statement is supported primarily from the morphological and functional analysis of the collection of yeasts isolated from grapes and musts that has lead to the isolation of only four genera that is *Saccharomyces spp., Candida spp., Metschnikowia spp. and Kloeckera spp.* plus a single member of the genus *Rhodotorula spp.* from Aglianico, whereas the literature shows that many more genera are naturally present on grapes.
Secondly, the results obtained from analysis of resistance to SO_2 shows that the majority of strains are resistant to high concentrations of SO_2, even higher than those allowed in the cellar. Resistance is also noticeable in most non-*Saccharomyces* yeasts, which are generally considered very sensitive in the literature. This fact, together with the analysis of the genera isolated during the fermentation of yeast, shows that most of the wild microflora of these two vines multiplies freely in the must in the early stages of fermentation without being impacted by metabisulfite added in the cellar. An inoculum consisting of *Saccharomyces* and non-*Saccharomyces* yeasts selected appropriately could help to dampen the influence of microorganisms in the juice, through competition for nutrients without adding additional SO_2.
Fermentations carried out with the yeast *Rhodotorula mucilaginosa* AGWLR12 on both types of musts can allow the observation of the performance of this organism in different environments. The fact that, compared with their controls, in Aglianico alone there has been a major reduction in fermentation time, demonstrates that AGWLR12 is better placed to grow in the must obtained from its vineyard of origin.
In general, native yeasts have proved to have excellent oenological potential and are worthy competitors of commercial yeast on classic oenological parameters such as efficiency of fermentation, alcohol-power and low production of acetic acid. Furthermore, with regard to those isolated from Aglianico, these improve the extraction of polyphenols, flavonoids and pigments from the skins and seeds, and the amounts of gallic acid and catechin compare to the control, thus increasing the reducing power of wines.
It is worth noting that these yeasts, although they are all selected by us only for the resistance to sulphur dioxide and some even for their ability to secrete a β-glucosidase, also possesses many other features not usually required in a yeast in the field of wine but which make them suitable for a particular type of fermented wort.
All these data seem to support the idea, mentioned in the introduction, that the most suitable yeast strains for wine fermentation should be sought on the grapes themselves. In particular, the spontaneous microflora present on the Aglianico grapes suggests that a selection has taken place in the vineyard.
Sensory analysis of 2007 wines produced with the *Rhodotorula* AGWLR12 strain shows a profile, both in Fiano and Aglianico, which is very similar to the control tones albeit more severe. The wines made therefore possess the same footprint, the same character as the controls but a higher intensity, and this has had a positive impact on their overall assessment by the panel test.
A preliminary sensory analysis on 2008 wines has shown some positive differences among samples as well, with regard the colour, the aroma and taste-tactile sensation. In Aglianico the use of native yeasts has also produced a higher colour intensity and a more intense fruity odour than the control. The increase in colour intensity is a major achievement when you consider that colour is a factor of great influence on the consumer.

In Fiano, the use of *H.guilliermondii* FWL66 has produced a higher value of “fruity odour” and “scorching” attributes.
This study also allowed the exploration, albeit minimally, of the enzyme activities of non-*Saccharomyces* yeasts, confirming their strength and versatility. In the case of AGWLR12 strain β-glucosidase, this allows the wine terpenes to be liberated more efficiently but also to enhance the levels of wine resveratrol, and thus increasing its nutritional value.
Finally the results obtained with the strain of *Rhodotorula mucilaginosa* has lead to the hypothetical use of this yeast in processes not strictly linked to flavor development, such as enhanced clarification efficiency by means of the polysaccharides secreted by the strain itself.

Bibliography

1. **Arevalo Villena M, Diez Perez J, Ubeda JF, Navascues E, Briones Al** (2006) A rapid method for quantifying aroma precursors: Application to grape extract, musts and wines made from several varieties. *Food Chemistry* 99: 183-190

2. **Arevalo Villena M, Ubeda Iranzo JF, Gundllapalli SB, Cordero Otero RR, Briones Perez Al** (2006) Characterization of an exocellular β-glucosidase from *Debaryomyces pseudopolymorphus. Enzyme and Microbial Technology* 39: 229-234

3. **Arnous A, Makris D, Kefalas P** (2002) Correlation of pigment and flavanol content with antioxidant properties in selected aged regional wines from Greece. *Journal of Food Composition and Analysis* 15: 655-665

4. **Arroyo-Lòpez FN, Duràn-Quintana MC, Ruiz-Barba JL, Querol A, Garrido-Fernàndez A** (2006) Use of molecular methods for the identification of yeast associated with table olives. *Food Microbiology* 23: 791-796

5. **Arthur CL and Pawliszyn J** (1990) Solid Phase Microextraction with Thermal Desorption *Analytical chemistry* 62: 2145-2148

6. **Barbagallo RN, Palmeri R, Fabiano S, Rapisarda P, Spagna G** (2007) Characteristic of β-glucosidase from Sicilian bloodoranges in relation to anthocyanin degradation. *Enzyme and Microbial Technology* 40: 570-575

7. **Barbagallo RN, Spagna G, Palmeri R, Restuccia C, Giudici P** (2004) Selection, characterization and comparison of β-glucosidase from mould and yeasts employable for enological applications. *Enzyme and Microbial Technology* 35: 58-66

8. **Beltran G, Torija MJ, Novo M, Ferrer N, Montserrat P, Guillamòn JM, Rozès N, Mas A** (2002) Analysis of yeast populations during alcoholic fermentation: A six year follow-up study. *Systematic and Applied Microbiology* 25: 287-293

9. **Blondin B, Ratomahenina R; Arnaud, A. and Galzy, P.** (1983) Purification and properties of the β-glucosidase of a yeast capable of fermenting cellobiose to ethanol: *Dekkera intermedia* Van der Walt. *European journal of Applied Microbiology and Biotechnology* 17:1-6.

10. **Bradford MM** (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248-254.

11. **Cabredo-Pinillos S, Cedròn-Fernàndez T, Parra-Manzanares A and Sàenz-Barrio C.** (2004). Determination of volatile compounds in wine automated Solid-Phase Microextraction and gas chromatography. *Chromatographia*, 59, 733-738.

12. **Cavazza A** (2004) Lieviti in enologia: origine, proprietà, funzioni. *VigneVini* 7/8: 29-31

13. **Ciani M, Beco L, Comitini F** (2006) Fermentation behaviour and metabolic interactions of multi starter wine yeast fermentations. *International Journal of Food Microbiology* 108: 239-245

14. **Constanti M, Reguant C, Poblet M, Zamora F, Mas A, Guillamòn JM** (1998) Molecular analysis of yeast population dynamics: Effect of sulphur dioxide and inoculums on must fermentation. *International Journal of Food Microbiology* 41: 169-175

15. **Cordero Otero RR, Ubeda Iranzo JF, Briones-Perez AI, Potgieter N, Villena MA, Pretorius IS, Van Rensburg P** (2003) Characterization of the β-glucosidase activity produced by enological strains on non-*Saccharomyces* yeasts. *Journal of Food Science* 68: 2564-2569

16. **Delfini C** (1995) Scienza e tecnica di microbiologia enologica (Ed. Il Lievito)

17. **Esteve-Zarzoso B, Gostincar A, Bobet R, Uruburu F, Querol A** (2000) Selection and molecular characterization of wine yeasts isolated from the 'El Penedès' area (Spain). *Food Microbiology* 17: 553-562

18. **Esteve-Zarzoso B, Manzanares Paloma, Ramòn D, Querol A** (1998) The role of non-*Saccharomyces* yeasts in industrial winemaking. *International Microbiology* 1:143-148

19. **Farris GA, Budroni M, Vodret T, Deiana P**(1990) Sull'origine dei lieviti vinari. I lieviti dei terreni, delle foglie e degli acini di alcuni vigneti sardi. *L'Enotecnico* 26:99-108

20. **Fernàndez M, Ubeda JF, Briones AI** (2000) Typing of non-*Saccharomyces* yeasts with enzymatic activities of interest in wine-making. *International Journal of Food Microbiology* 59: 29-36

21. **Fleet GH** (1999) Microorganisms in food ecosystems. *International Journal of Food Microbiology* 50: 101-117.

22. **Fleet GH** (2003) Yeast interactions and wine flavour *International Journal of Food Microbiology* 86: 11-22

23. **Gazzetta Ufficiale della Repubblica Italiana**, Serie generale n. 301del 27Novembre 2008
Disposizioni di attuazione dei regolamenti (CE) n.479/2008 del Consiglio e (CE) n.555/2008 della Commissione per quanto riguarda l'applicazione della misura della distillazione dei sottoprodotti della vinificazione.

24. **Genovés S, Gil IV, Manzanares P, Aleixandre JL, Vallés S** (2003) *Candida molischiana* β-glucosidase production by *Saccharomyces cerevisiae* and its application in winemaking. *Journal of Food Science* 68: 2096-2100

25. **Genovés S, Gil JV, Vallés S, Casas JA, Manzanares P** (2005) Assessment of the aromatic potential of Palomino Fino grape must using glycosidases. *American Journal of Enology and Viticulture* 56: 188-191

26. **Genovese A, Gambuti A, Piombino P, Moio L** (2007) Sensory properties and aroma compounds of sweet Fiano wine. *Food Chemistry* 103: 1228-1236

27. **Goffeau A, Barrell BG, Bussey H, Davis RW, Dujon B, Feldmann H, Galibert F, Hoheisel JD, Jacq C, Johnston M, Louis EJ, Mewes HW, Murakami Y, Philippsen P, Tettelin H, Oliver SG** (1996). "Life with 6000 genes". *Science* 274: 563–567

28. **González-Candelas L, Gil JV, Lamuela-Raventós RM, Ramón D.**(2000) The use of transgenic yeasts expressing a gene encoding a glycosyl-hydrolase as a tool to increase resveratrol in wine. *International Journal of Food Microbiology* 59: 179-183

29. **Hayashi S , Matsumoto K, Wada Y, Takasaki Y, Imada IK** (1993) Stable β-glucosidase from *Aureobasidium. Letters in Applied .Microbiology* 17**:** 75- 77

30. **Hemingway KM, Alston MJ, Chappell CG, Taylor AJ** (1999) Carbohydrate-flavour conjugates in wine. *Carbohydrate Polymers* 38: 283-286

31. **Hierro N, Gonzàlez A, Mas A, Guillamòn JM** (2006) Diversity and evolution of non-*Saccharomyces* yeast population during wine fermentation: effect of grape ripeness and cold maceration. *FEMS Yeast Research* 6: 102-111

32. **König H, Unden G, Fröhlich J** (2009) Biology of Microorganismson Grapes, in Must and in Wine (Springer eds)

33. **Kourkoutas Y, Kanellaki M, Koutinas AA, Tzia C** (2006) Effect of storage of immobilized cells at ambient temperature on volatile by-products during wine-making. *Journal of Food Engineering* 74: 217-223

34. **Jaillon O, et al**. (2007) The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. *Nature* 449: 463-467

35. **Jolly NP, Augustyn OPH, Pretorius IS** (2003) The effect of non-*Saccharomyces* yeasts on fermentation and wine quality. *South African Journal of Enology and Viticulture* 24: 55-62

36. **Johnson, Hugh** (1989). *Vintage: The Story of Wine*. Simon and Schuster.

37. **Maicas S, Mateo JJ**(2005) Hydrolysis of terpenyl glycosides in grape juice and other fruit juices: a review *Applied Microbiology and Biotechnology* 67:322-335

38. **Makris DP, Buskou G, Andrikopoulos N** (2007) Polyphenolic content and in vitro antioxidant characteristics of wine industry and other agri-food solid waste extracts. *Journal of Food Composition and Analysis* 20: 125-132

39. **Malacrinò P, Zapparoli G, Tosi E, Dellaglio F** (2005) Selezione di lieviti autoctoni *Saccharomyces* per la produzione di vino Amarone. *VigneVini* 4: 131-135

40. **Maldonade IR, Rodriguez-Amaya DB, Scamparini ARP** (2008) Carotenoids of yeasts isolated from the Brazilian eco system *Food Chemistry* 107 :145-150

41. **Manzanares P, Rojas V, Genovés S, Vallés S** (2000) A preliminary search for anthocyanin-β-glucosidase activity in non-*Saccharomyces* wine yeasts. *International Journal of Food Science and Technology* 35: 95-103

42. **Marinova D, Ribarova F, Atanassova M** (2005) Total phenolic and total flavonoids in Bulgarian fruits and vegetables. *Journal of the University of Chemical Technology and Metallurgy* 40: 255-260

43. **Mateo JJ, Di Stefano R.** (1997) Description of the β-glucosidase activity of wine yeast. *Food Microbiology* 14: 583-91.

44. **Mateo JJ, Jiménez M** (2000) Monoterpenes in grape juice and wine. *Journal of Chromatography A* 881: 557-567

45. **McGovern PE**, et al. (2003)Fermented beverages of pre- and proto-historic China. *Proceedings of the National Academy of Sciences* (The National Academy of Sciences).

46. **Moreira N, Mendes F, Guedes de Pinho P, Hogg T, Vasconcelos I** (2008) Heavy sulphur compounds, higher alcohols and esters production profile of *Hanseniaspora uvarum* and *Hanseniaspora guilliermondii* grown as pure and mixed cultures in grape must. *International Journal of Food Microbiology* 124: 231-238

47. **Moreno-Arribas MV, Polo MC** (2009) Wine chemistry and Biochemistry (Springer)

48. **Pellicano MP, Cammarota G, Laurino C, Graziani MP** (2009) Applicazione dell'analisi descrittiva quantitative a tre vini bianchi tipici della Campania. *Ingredienti Alimentari* – VIII: 21-25

49. **Pemberton MS,Brown RD, Emert GH.** (1980) The role of β-glucosidase in the bioconversion of cellulose. *The Canadian Journal of Chemical Engineering* 58: 723-9.

50. **Pérez-Nevado F, Albergaria H, Hogg T, Girio F** (2006) Cellular death of two non-*Saccharomyces* wine-related yeast during mixed fermentation with *Saccharomyces cerevisiae*. *International Journal of Food Microbiology* 108: 336-345

51. **Perrier V, Dubreucq R, Galzy P** (1995) Fatty acid and carotenoid composition of *Rhodotorula* strains *Archieves of Microbiology* 164: 173-179

52. **Pretorius IS** (1997) Utilization of polysaccharides by *Saccharomyces cerevisiae*. In *Yeast Sugar Metabolism* (Zimmermann, F.K. and Entian, K.D., eds) pp 459-501. Technomic Publishing Co

53. **Pretorius IS** (1999) Engeneering designer genes for wine yeast. *Australian and New Zealand Wine Industry Journal* 14: 42-47

54. **Pretorius IS** (2000) Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* 16: 675-729

55. **Pretorius IS** (2001) Gene technology in winemaking: new approaches to the ancient art. *Agri. Consp. Sci.* 66: 1-20

56. **Pretorius IS** (2002) The genetic analysis and improvement of wine yeasts. In *Fungal Biotechnology* (Arora D., ed) Marcel Decker

57. **Pretorius IS, Bauer FF** (2002) Meeting the consumer challenge through genetically customized wine yeast strains. *Trends in Biotechnology* 20: 426-32

58. **Pretorius IS, Van der Westhuizen TJ** (1991) The impact of yeast genetics and recombinant DNA technology. *South African Journal of Enology and Viticulture* 12: 3-31

59. **Pretorius IS et al** (1999) Yeast biodiversity in vineyards and wineries and its importance to the South African wine industry. *South African Journal of Enology and Viticulture* 20: 61-74

60. **Riccio P, Rossano R, Vinella M, Domizio P, Zito F, Sansevrino, D'Elia A and Rosi I** (1999) Extraction and immobilization in one step of two β-glucosidase released from a yeast strain of *Debaryomyces hansenii. Enzyme and Microbial Technology* 24: 123-129.

61. **Rojas V, Gil JV, Pinaga F, Manzanares P** (2003) Acetate ester formation in wine by mixed cultures in laboratory fermentations. *International Journal of Food Microbiology* 86: 181-188

62. **Regodon JA, Perez F, Valdes ME, De Miguel C, Ramirez M** (1997) A simple and effective procedure for selection of wine yeast strains. *Food Microbiology* 14: 247-254

63. **Renaud S**(1992) From French paradox to Cretan miracle. *The Lancet* 355 :48

64. **Riccio P, Rossano R, Vinella M, Domizio P, Zito F, Sansevrino F, D'Elia A, Rosi I** (1999) Extraction and immobilization in one step of two β-glucosidases released from a yeast strain of *Debaryomyces hansenii*. *Enzyme and Microbial Technology* 24: 123-129

65. **Romano P, Fiore C, Paraggio M, Caruso M, Capece A** (2003) Function of yeast species and strains in wine flavor. *International Journal of Food Microbiology* 86: 169-180

66. **Romano P, Suzzi G, Comi G, Zironi R (2008)** Higher alcohol and acetic acid production by apiculate wine yeast *Journal of Applied Microbiology* 73: 123-130

67. **Rojas V, Gil JV, Pinaga F, Manzanares P** (2001) Studies on acetate ester production by non-*Saccharomyces* wine yeast. *International Journal of Food Microbiology* 70: 283-289

68. **Rosi I, Vinella M, Domizio P** (1994) Characterization of β-glucosidase activity in yeasts of oenological origin. *Journal of Applied Bacteriology* 77: 000-000

69. **Sanchez Palomo E, Diaz-Maroto Hidalgo MC, Gonzalez-Vinas MA, Perez-Coello MS** (2005) Aroma enhancement in wines from different grape varieties using exogenous glycosidases. *Food Chemistry* 92: 627-635

70. **Sanford JC, Johnson SA** (1985) The consept of parasite-derived resistance - deriving resistance genes from the parasite's own genome. Journal of Theoretical Biology 115: 395-405

71. **Sansone G, Rezza I, Calvente V, Benuzzi D, Sanz de Tossetti MI** (2005) Control of *Botritis cinerea* strains resistant to iprodione in apple with rhodotorulic acid and yeast *Postharvest Biology and Technology* 35 : 245-251

72. **Santamaria P, Garijo P, Lopez R, Tenorio C, Gutierrez R** (2005) Analysis of yeast population during spontaneous alcoholic fermentation: Effect of the age of the cellar and the practice of inoculation. *International Journal of Food Microbiology* 103: 49-56

73. **Shinohara T, Kubodera S, Yanagida F** (2000) Distribution of phenolic yeasts and production of phenolic off-flavors in wine fermentation. *Journal of Bioscience and Bioengineering*. 90: 90-97

74. **Somers TC and Evans ME** (1977). Spectral evaluation of young red wines: Anthocyanin equilibria, total phenolics, free and molecular SO_2. *Journal of Scientific Food Agriculture* 28: 279-287.

75. **Takita J, Katohda S, Sugiyama H**(2001) Structural determination of an exocellular mannan from *Rhodotorula mucilaginosa* YR-2 using ab initio assignment of proton and carbon NMR spectra *Carbohydrate Research* 335:133-139

76. **Tosi E, Lombardi A, Zilio F** (1998) Lieviti autoctoni per I vini veronesi. Supplemento a *L'Informatore Agrario* 35: 3-5

77. **Tosi E, Zilio F, Lombardi A** (2005) Isolamento e caratterizzazione di lieviti ecotipici per la valorizzazione dei vini Soave e Valpolicalla DOC. *Infowine* 7: 1-5

78. **Ugliano M, Moio L** (2008) Free and hydrolytically released volatile compounds of Vitis vinifera L. cv. Fiano grapes as odour-active constituents of Fiano wine *Analitica Chimica Acta* 621:79-85

79. **Usseglio-Tommaset L** (1978) Chimica enologica (Ed. AEB Brescia)

80. **Vasserot Y, Christiaens H, Chemardin P, Arnaud A, Galzy P** (1989) Purification and properties of a β-glucosidase of *Hanseniaspora vineae* Van der Walt and Tscheuschner with the view to its utilization in fruit aroma liberation. *Journal of Applied Bacteriology* 66: 271-279

81. **Vivier MA and Pretorius IS** (2000). Genetic improvement of grapevine: tailoring grape varieties for the third millennium. *South African Journal of Enology and Viticulture* 21: 52-73

82. **Vivier MA and Pretorius IS**. (2002). Genetically tailored grapevines for the wine industry. *Trends in Biotechnology* 20:472-478.

83. **Yanai T., Sato M.** (1999) Isolation of β-Glucosidase produced by *Debaryiomyces hansenii* and its application in winemaking. *American Journal of Enology and Viticulture* 50(3): 231-236.

84. **Zilio F, Tosi E, Lombardi A, Delfini C** (2004) Contributo alla valorizzazione del vino Valpolicella DOC mediante l'isolamento, la caratterizzazione ed il successivo impiego di lieviti specifici. *VigneVini* 7/8: 1-5

85. **Zoecklein BW, Marcy JE, Williams JM, Jasinski Y** (1997) Effect of native yeasts and selected strains of *Saccharomyces cerevisiae* on glycosyl glucose, potential volatile terpenes, and selected aglycones of white Riesling (*Vitis vinifera* L.) wines. *Journal of Food Composition and Analysis* 10: 55-65

Appendix

Congress communications

1. "**Purification and characterization of a β-glucosidase by a *Hanseniaspora guilliermondii* strain isolated from Fiano grape"**
   By Santomauro F. et al.
   XXVIII Annual Congress of SIMGBM (Italian Society of General Microbiology and Microbial Biotechnology), Spoleto, Italy, Jun 09

2. **"Study of the epiphyte microflora on grapes of Irpinian autochthonous vines"**
   By Santomauro F. et al.
   X Annual Congress of FISV (Italian Federation of Life Sciences), Riva del Garda, Italy, Sep 08

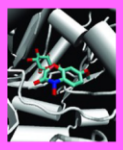
3. **"A novel β-glucosidase from *Rhodotorula spp*. of oenological origin"**
   By Sorrentino A., Esposito G., Santomauro F. et al.
   X Annual Congress of FISV (Italian Federation of Life Sciences), Riva del Garda, Italy, Sep 08

4. "**Effect of polysaccharide based pellets on fatty acids in muscle tissue of Juvenile Cherax spp"**
   By Volpe M.G., Santomauro F. et al.
   XVII International Symposium of the Association of Astacology in Kuopio, Finland , Aug 08

Publications

"Volatile composition of *Aglianico* and *Fiano* wine obtained with an autochthonous selected yeast"
By Calabretti A, La Cara F, Sorrentino A, Di Stasio M, Santomauro F, Rastrelli L, Gabrielli L, Limone F, Volpe MG
This article has been submitted for publication to LWT – Food Science and Technology

Purification and characterization of a β-glucosidase by a *Hanseniaspora guillermondii* strain isolated from Fiano grape



F. Santomauro[1], E. Ionata[1], A. Sorrentino[2], M.G. Volpe[2], F. La Cara[1]
[1]Istituto di Biochimica delle Proteine (IBP), C.N.R. – Napoli, Italy;
[2]Istituto di Scienze dell'Alimentazione (ISA), C.N.R. – Avellino, Italy;

Industrial starter cultures used in wine fermentation are made almost exclusively by strains of *Saccharomyces cerevisiae*. These strains are selected to be tolerant to high concentrations of SO_2 and ethanol, produce low level of foam and for other technological features in order to standardize the wine production. The other species of yeast, usually present during a traditional fermentation (without inoculation) are excluded. These yeasts belong to various species of *Candida, Debaryomyces, Hanseniaspora, Hansenula, Kloeckera, Metschnikowia, Pichia, Schizosaccharomyces, Torulaspora* and *Zygosaccharomyces*. They are called low-fermentative because are alcohol untolerant and their growth is limited to the first two or three days of fermantation. In the last ten years many studies have stressed that they are not only good acetic acid producers but also a possible rich source of metabolites and enzymes which could improve the final quality of wine. Among the enzymes of interest in wine production, β-glycosidases are very important because they can improve wine aroma hydrolizing the present terpenyl-glycosides. The β-glucosidases usually used in industrial wine production are from *Aspergillus niger* but their activity is quite inhibited at the conditions of must fermentaion. The aim of this work has been to purify and characterize a β-glucosidase from *H. guilliermondii* that seems to have better features for a possible use on industrial scale.

Materials and methods

The enzyme was purified from a 2 liters culture of *H. guilliermondii* grown in YPD for 18 h at 20°C under a gentle agitation (40 rpm). The culture was centrifuged and the supernatant was concentrated in an Amicon cell with a PM10 membrane. After precipitation by ammonium sulfate, proteins were resuspended in Tris-HCl 20mM pH 8, dialysed, and loaded in two successive steps on a Sepharose DEAE-FF XK10 and a Sephadex 200 XK 26/10 gel filtration column to obtain the pure enzyme.
The β-glucosidase activity was characterized at different conditions of pH (from 5.0 to 1.6 utilizing citrate-phosphate buffer), temperature (from 10 to 50°C), ethanol concentration (from 0 to 20 %), glucose or fructose concentration (from 0 to 20%). The thermostability of the enzyme was assessed at pH 8.0 at three different temperatures (25, 45 and 65°C) for 24 hours.

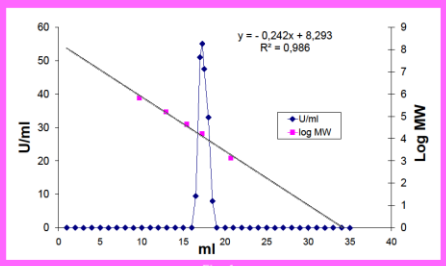
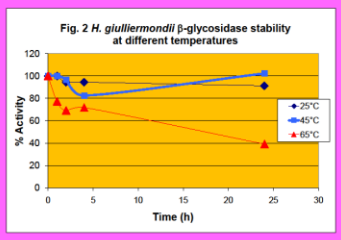
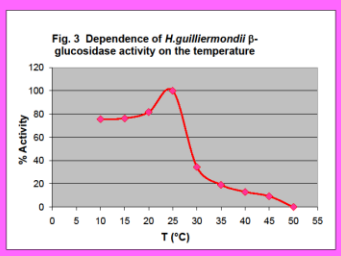


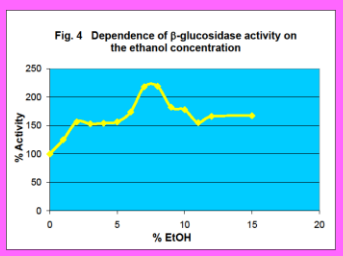
Fig. 1

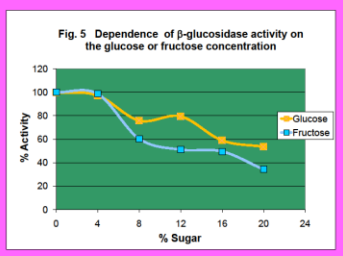
Results

The elution volume of β-glucosidase from the gel filtration column showed that the molecular mass of the enzyme is around 20 Kda (fig. 1). The analysis of enzyme features revealed that *H. guilliermondii* β-glucosidase is quite thermostable: its activity decreases under 50 % only after a incubation of 24 hours at 65°C, while it is stable at lower temperatures (fig. 2). Moreover the β-glucosidase shows an optimum of temperature around 25°C and retains the 80% of activity at lower temperatures. However we observed a rapid decrease of activity already at 30°C (60% of initial value) and no activity at 50°C (fig. 3). The enzyme is not inhibited by high concentration of ethanol and slightly by glucose or fructose (fig. 4 - 5). In particular its activity is enhanced more than twice by 7-8 % ethanol concentration and it retained more than 50% of its initial value even at 15% concentration of glucose or fructose. This glucosidase works in a very acidic environment because it is active only at pH below 4. It displays a broad range activity towards glycosides with different sugars (glucose, galactose, arabinose) and linkages (alpha and beta).









Discussion

The final analysis of data reveals that the β-glucosidase from *Hanseniaspora guilliermondii* presents some peculiar features. Its molecular weight is the smallest among all the β-glucosidases so far characterized, that have a mass usually around 100-150 KDa. Moreover its optimum working conditions are very close to those present in must during fermentation: high sugars and ethanol concentration, low temperature and pH around 3.0. These characteristics are unusual for this class of yeast enzymes which often have a pH optimum in the range of 5-7, a higher optimal temperature and a low resistance to high concentration of glucose and ethanol. Its broad range of substrate specificity allows to hydrolyze the most of glycosilated terpenes in wine. On the basis of these results *H. guilliermondii* β-glucosidase has a great potential in the improvement of wine aroma during fermentation as an alternative to *Aspergillus spp* β-glucosidases usually utilized on industrial scale in the wine-making process.

1. Cordero Otero RR et al. *Characterization of the β-glucosidase activity produced by enological strains of non-Saccharomyces yeast*, JFS Food Microbiology and Safety pp 2564-2569 (2003)
2. Fleet, G.H., Heard, G.M.,. *Yeasts: growth during fermentation*. In: Fleet, G.M. (Ed.), Wine Microbiology and Biotechnology. Harwood Academic Publishers, Chur, Switzerland, pp. 27– 54, (1993).
3. R. Mortimer, M. Polsinelli. *On the origins of wine yeast*. Res. Microbiol. 150, pp. 199-204, (1999).
4. K. Zott, C. Miot-Sertier, O. Claisse, A. Lonvaud-Funel, I. Masneuf-Pomarede. *Dynamics and diversity of non-Saccharomyces yeasts during the early stages in winemaking*. Int. J. of Food Microbiology (2008).
5. P. Romano et al. *Function of yeast species and strains in wine flavour* , Int. J. of Food Microbiology pp. 169-180 (2008).

Study of epiphytic microflora of "Aglianico" and "Fiano" Irpinian grape-vines

F. Santomauro[1], A. Sorrentino[2], F. La Cara[1], F. Limone[3], E. Ionata[1], F. Petitto[2], R. Pellecchia[1], M.G. Volpe[2]

[1]Istituto di Biochimica delle Proteine (IBP), C.N.R. – Naples, Italy;

[2]Istituto di Scienze dell'Alimentazione (ISA), C.N.R. – Avellino, Italy; [3]Istituto Tecnico Statale Agrario De Sanctis – Avellino, Italy

Autochthonous yeasts thriving on vine grapes determine the sensorial and qualitative characteristics of wines. During the spontaneous must fermentation there is a succession of non-*Saccharomyces* and *S. cerevisiae* "sensu stricto" strains. Recently the role of non-*Saccharomyces* has been re-evaluated for their ability to produce enzymes and metabolites which act on the sensorial characteristics of wine. Objective of this work was to assess the evolution of yeasts populations during the ripening and wine-making processes. Our results showed an increase of yeasts count on the grapes up to the harvesting. The epiphytic microflora of studied Irpinian grapes is composed of oxidative and fermentative non-*Saccharomyces* yeasts belonging to the genera *Kloeckera, Hanseniaspora, Torulaspora, Candida, Metschnikowia* and *Rhodotorula*. These microorganisms, with low fermentation activity and slight alcohol tolerance, contribute only to the early phase of the wine fermentation. The *Saccharomyces* species, more competitive and tolerant to ethanol and SO2, successively become the dominant. This species sequence enhances the aromatic properties of the wines giving them complex and novel flavour profiles.

Materials and methods

The grapes were taken from the vineyards of the Istituto Statale Tecnico Agrario "De Sanctis", the grapes were cut, placed in sterile bags and transported in containers at low temperature in the laboratory where microbiological tests were made. 10g of sample were taken in sterile condition, added to 90 ml of sterile Ringer solution and homogenized with a Stomaker Lablender 400 (PBI, Milan). Serial dilutions were spread on YPD plates (yeast extract 10 g/l; peptone 20 g/l; dextrose 20 g/l; agar 20 g/l) of WL agar (Oxoid) and Lysine Medium (Oxoid) to test the population of yeast. The plates were incubated at 28 °C for 5 days. Five colonies per plate were isolated randomly, grown on YPDA and kept at 4 °C. The isolated cultures were tested using the API 20 AUX kit (BioMérieux, France) to define genera and species they belong to. They were tested also for industrial parameters such as sporulation and resistance to sulphur dioxide (100 and 250 ppm). Fermentation tests have been carried out on Aglianico and Fiano musts in the winery of the Istituto Tecnico Statale Agrario "De Sanctis" with a strain of *Rhodotorula spp.* isolated from Aglianico grapes.

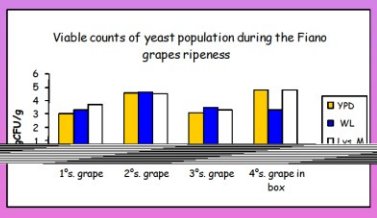
Vine of the Istituto Tecnico Statale Agrario "De Sanctis"

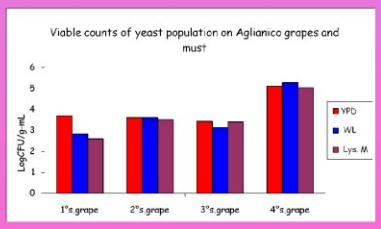
Fermenting vats in the winery of the Istituto Tecnico Statale Agrario "De Sanctis"

Results

The results obtained by testing the epiphytic population on the Fiano and Aglianico grapes during the ripening showed that yeasts number increase from 10^3 cfu/g on green bunches to 10^4 cfu/g at the time of véraison (change of colour) and up to 10^5 cfu/g during the vintage. The data obtained by physiological and biochemical tests show that the most of epiphytic microorganism on Fiano grapes belong to the genus *Kloeckera sp.*, followed by genres *Torulaspora sp., Candida sp.* and *Rhodotorula sp.* Yeasts isolated from grapes of "Aglianico" belong mainly to the genus *Kloeckera sp.* and at a lesser percentage to genus *Hanseniaspora*

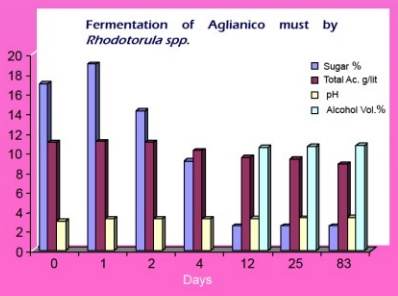
cultures of indigenous yeasts were also tested for the ability of sporulation and it was found out that only a small percentage can form spores. Tests for resistance to sulphur dioxide show that the 60 % of yeasts isolated from grapes and must of Fiano and Aglianico is resistant to a concentration of 100 ppm and the 40% is resistant to 250 ppm. These tests were carried out also on yeasts isolated during fermentation achieved with *S. cerevisiae* starter cultures. The fermentation with *Rhodotorula spp.* and a *S. cerevisiae* starter is faster than a traditional process and it produces an intense and characteristic fruity flavour.





Discussion

The aim of this study was to isolate yeasts from Irpinian autochthonous vines "Fiano and Aglianico" belong to the group of non-*Saccharomyces*; preliminary data showed that the most of these yeasts have an oxidative metabolism, while only a small percentage displays fermentative activity. These are not alcohol-tolerant yeasts and they usually grow in the early stages of fermentation, due to their prevalence on the grape peel. Our results agree with what is reported in the literature. Therefore we can assume that many of the sensory characteristics that distinguish these wines derive from the fermentation of indigenous yeasts, which start the fermentation of must. Their metabolites are very important to compose the bouquet of these wines.



References

1. Zambonelli C. - *Microbiologia dei vini.* Edagricole, Bologna (1988).
2. Fleet, G.H., Heard, G.M.,. *Yeasts: growth during fermentation.* In: Fleet, G.M. (Ed.), Wine Microbiology and Biotechnology. Harwood Academic Publishers, Chur, Switzerland, pp. 27– 54, (1993).
3. R. Mortimer, M. Polsinelli. *On the origins of wine yeast.* Res. Microbiol. 150, pp. 199-204, (1999).
4. Domizio P, Lencioni L, Ciani M, Di Blasi S, Pontremolesi C, Sabatelli MP. *Spontaneous and inoculated yeast populations dynamics and their effect on organoleptic characters of Vinsanto wine under different process conditions.* Int J Food Microbiol. Apr 20;115(3):281-9, (2007).
5. K. Zott, C. Miot-Sertier, O. Claisse, A. Lonvaud-Funel, I. Masneuf-Pomarede. *Dynamics and diversity of non-Saccharomyces yeasts during the early stages in winemaking.* Int. J. of Food Microbiology (2008).

A novel β-glucosidase from *Rhodotorula spp.* of oenological origin

A. Sorrentino[2], G. Esposito[1], F. Santomauro[1], M.G. Volpe[2], E. Ionata[1], R. Pellecchia[1], I. Castellano[1], F. La Cara[1]

[1]Istituto di Biochimica delle Proteine, CNR Napoli; [2]Istituto di Scienze dell'Alimentazione, CNR Avellino

Alcoholic fermentation of grape is a complex process characterized by the presence of a large number of different yeast genera and species that also contribute to the flavour of wines. The chemical composition of these aromas is mainly due to volatile compounds such as monoterpenes which are linked to β-D-glycosides or diglycosides. These volatile molecules are liberated in free form by the sequential hydrolysis operated by several enzymes closely followed by the action of a β-D-glucosidase (1). Non-*Saccharomyces* yeasts are recognized to play an important role in determining the aromatic profile of wines. Among several non-*Saccharomyces* yeasts, isolated from grapes and musts of Aglianico and Fiano cultivars, we have identified a strain of *Rhodotorula spp.* that produce a peculiar extracellular β-glucosidase.
Here we report the characterization of an extracellular β-glucosidase purified from this non-*Saccharomyces* yeast useful for oenological application.

The extracellular β-glucosidase of *Rhodotorula spp.* (strain WRL12) was obtained by fermenting the yeast in YPD medium. The enzyme activity was monitored during the growth assaying the cell free supernatant. As shown in Fig. 1 the enzyme production was growth related and reached the maximum value in the stationary phase.

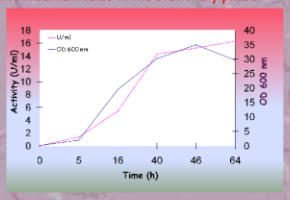


Fig.1 Growth rate and β-glucosidase activity

The enzyme purification was performed from the cell free culture supernatant, after two sequential steps of concentration on PM10 membranes, by a combination of two chromatographic step on DEAE Sepharose Fast-Flow XK16 and Superdex 200 XK 26/60 columns. The enzyme was purified 35,4 fold with a final yield of 40,1%. The overall purification scheme is summarized in Table 1 The β-glucosidase obtained was judged to be homogeneous by SDS-PAGE analysis. The enzyme molecular mass was determined both in native and denaturing conditions by gel filtration chromatography on a previously calibrated Sephadex 200 HR10/30 column and by SDS-PAGE respectively. Gel filtration results revealed a molecular mass of 190 kDa while SDS-PAGE showed a single band in correspondence of 95 kDa (Fig. 2). From these data a quaternary structure of two identical subunits has been deduced for the native enzyme.

Purification steps	Activity * (U)	Proteins (mg)	Specific Activity (U/mg)	Purification (fold)	Yield (%)
Supernatant	45800	125	366	1	-
Ultrafiltration 10 kDa	43462	92.4	470.2	1.28	94.9
DEAE Fast Flow	26077	8.58	3039.4	8.30	56.9
Superdex 200	18350	1.41	12959	35.4	40.1

Tab. 1 Purification of *Rhodotorula spp.* β-glucosidase.
* One unit is defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol of *p*-nitrophenyl glucopyranoside per minute.

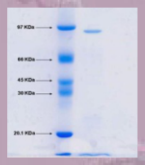


Fig. 2 SDS-PAGE of purified β-glucosidase

The effect of pH on enzymatic activity was determined using *p*-nitrophenil-β-D-glucopyranoside (*p*-NPG) as substrate in phosphate-citrate buffer at different pH from 3.5 to 8.0. The optimal activity was found at pH between 6.5 and 7.0. Moreover at pH around 3.5, characteristic of must fermentation, the enzyme retain 50% about of the maximum activity (Fig. 3a). The optimal temperature was at 60 °C but also at 70 and 80 °C the activity was high (Fig. 3b). This is an interesting finding because for similar yeast species such as *Debaryomyces hansenii* the optimal temperature is lower (2,3). β-glucosidase thermostability is not elevated; in fact at 60°C it retain only 30% of the initial activity. However the enzyme is stable for long time at room temperature (90% of initial activity after 24 h) but with an halflife of only one hour at 40 °C (Fig. 3c).

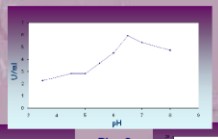


Fig. 3a

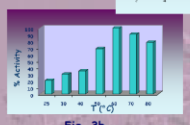


Fig. 3b

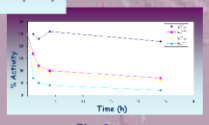


Fig. 3c

Fig. 3 a) Effect of pH, b) of temperature on activity c) Effect of temperature on enzymatic stability

The ability of β-glucosidase to hydrolyze glycosidic linkages with α and β configuration was assessed using natural disaccharides and various synthetic substrates, composed of *p*-nitrophenyl (*p*-NP) linked to a sugar mojety. *Rhodotorula spp.* enzyme is very specific in hydrolyzing inter-sugar bonds between two glucose molecules and, for this reason, it can be defined a true glucosidase. Moreover the enzyme is more specific for β1-4 than α1-4 and α1-1 glucosidic bonds while its activity has not been detected for β1-6. The enzyme presents also other side activities such as arabinosidase, galactosidase and xylosidase which, as displayed in Tab. 2 are only the 10% respect to the highest value obtained for the *p*-NP-β-D-glucopyranoside

Synthetic substrates	Activity (%)	Disaccharides	Activity (%)
*p*NP-β-D-Glu *p*-nitrophenyl β-D-gluco pyranoside	100	Cellobiose	100
*p*NP-α-D-Glu *p*-nitrophenyl α-D-gluco pyranoside	14.1	Maltose	19
*p*NP-α-L-Ara *p*-nitrophenyl α-L-Arabino pyranoside	12.5	Lactose	8
*p*NP-β-D-Gal *p*-nitrophenyl β-D-galacto pyranoside	10.2	Trehalose	18.7
*p*NP-α-Xylo *p*-nitrophenyl α-Xylo pyranoside	5.42	Gentibiose	0

Tab. 2 Substrate specificity of β-glucosidase from *Rhodotorula spp.*

The enzyme kinetic was monitored for 10 minutes at different *p*-NPG concentrations in 100 mM citrate-phosphate buffer at pH 7.0 at 30°C. Kinetic parameters (averaged from three values) are summarized in Tab. 3

Kinetic Constants	
K_m	4.23 mM
V_{max}	3.75 μmoli min $^{-1}$
K_{cat}	118.7 min^{-1}
K_{cat}/K_m	28.06 mM^{-1} min^{-1}

Tab. 3 Kinetic constants of β-glucosidase from *Rhodotorula spp.*

According to literature data on yeast β-glucosidases (1) the enzyme from *Rhodothorula spp.* is not inhibited by sugars. In fact, as reported in Fig. 4, the activity is slightly influenced by fructose and is halved only at 20 g/l glucose while at typical must concentration (about 10 g/l) it retain 60% of the maximum value. Moreover the enzyme showed no reduction of activity in presence of ethanol up to 10% and an increase at higher concentration Fig 5.

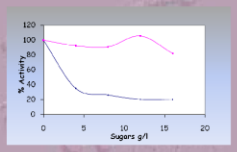


Fig. 4a

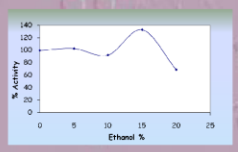


Fig. 4b

Fig. 4 a) Effect of sugars and b) ethanol concentrations on enzymatic activity

In conclusion the extracellular β-glucosidase, purified from *Rhodotorula ssp.*, showed a good enzymatic activity at the characteristic condition of must fermentation mainly regarding pH, sugars and ethanol concentration. For these reasons studies are in progress for future winemaking application of the enzyme during all stages of fermentation and in young wines which still retain a great part of glycosidically bound aromatic molecules.

References

1 Palmeri R, Spagna G. β-glucosidase in cellular and acellular form for winemaking application. Enzyme Microb. Technol. 2007; 40:382-389
2 Riccio P, Rossano R, Vinella M, Domizio P, Zito F, Sanseverino F, D' Elia A, Rosi I. Extraction and immobilization in one step of two β-glucosidases released from a yeast strain of *Debaryomyces hansenii* . Enzyme Microb. Technol. 1999; 24:123-129
3 Takaaki Y, Michikatsu S. Isolation and Properties of β-glucosidase produced by *Debaryomyces hansenii* and its application in winemaking. Am. J. Enol. Vitic. 1999; 50:231-235

POSTER

EFFECT OF POLYSACCHARIDE BASED PELLETS ON FATTY ACIDS IN THE MUSCLE TISSUE OF JUVENILE *Cherax spp.*

Volpe M.G.[1], Santomauro F[1]., Coccia E. [2], Monetta M.[2] , Di Stasio M. [1], Paolucci M. [2]

[1] ISA - CNR Via Roma 52, 83100 Avellino, Italy mgvolpe@isa.cnr.it

[2] Faculty of Sciences, Department of Biological and Environmental Sciences, University of Sannio, Via Port'Arsa, 11, 82100 Benevento, Italy paolucci@unisannio.it

ABSTRACT

Juvenile *Chrax spp*. were fed formulated diets in form of pellets containing the natural polysaccharides alginate, agar, chitosan and pectin as binders. Formulated diets contained the same amount of nutrients (proteins, lipids and carbohydrates) mixed with each one of the different polysaccharides Here we present data about the polysaccharides effect on the fatty acid composition of the juvenile crayfish body. Feeding trials lasted twelve weeks. At the end of this period, crayfish were sacrificed and from each animal muscle tissue from the tail was analyzed by gas chromatography. The presence of polysaccharides affected the fatty acid composition of the crayfish muscle tissue. In particular, the percentages of stearic acid, EPA and DHA were higher in the animals fed formulated diet with respect to the control animals fed fresh diet. On the other hand, he percentages of myristic acid, oleic acid, linoleic acid and palmitic acid did not change in the animals fed formulated diet with respect to the control animals fed fresh diet. It is interesting to note that the animals fed formulated diets with added polysaccharides showed a higher percentage of unsaturated fatty acids in the muscle tissue. This result may have important consequences for human health.

KEYWORDS: Cherax spp., formulated diet, polysaccharides, fatty acids, muscle tissue.

Ringraziamenti

L'attività sperimentale, i cui risultati sono descritti in questa tesi, è stata condotta presso l'Istituto di Scienze dell'Alimentazione (ISA) del CNR di Avellino e presso l'Istituto di Biochimica delle Proteine (IBP) del CNR di Napoli, in collaborazione con l'Istituto Tecnico Agrario "F. De Sanctis" di Avellino.
Vorrei ringraziare per primi il prof. Mosè Rossi (IBP-CNR) che in questi anni ha seguito con pazienza lo sviluppo del mio lavoro, la dott.ssa Maria Grazia Volpe (ISA-CNR) che per prima ha creduto nelle mie capacità offrendomi questo progetto di ricerca, e il dott. Francesco La Cara (IBP-CNR) per la sua costante e attenta guida scientifica. Vorrei inoltre ringraziare la dott.ssa Alida Sorrentino (ISA-CNR), la dott.ssa Elena Ionata (IBP-CNR) , il dott. Roberto Pellecchia (IBP-CNR) , il dott. Mario Paolo Pellicano (ISA-CNR), il dott. Giancarlo Cammarota (ISA-CNR) e la dott.ssa Antonella Calabretti (Università degli Studi di Trieste) per il loro supporto scientifico.