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PhD COURSE IN

FOOD BIOTECHNOLOGY - XXIV CYCLE

YEAST LYSIS DURING BOTTLE-FERMENTED SPARKLING WINES AGEING IN PRESENCE OF A β-GLUCANASE OENOLOGICAL PREPARATION

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To my family

ABSTRACT

This PhD thesis dealt with the study of yeast lysis during bottle-fermented sparkling wines ageing in presence of a β-glucanase oenological preparation. A four-step experimental procedure was set up by performing the following tests; 1) Preliminary study of Lallzyme MMX® oenological preparation (Lallemand Inc., Canada), commonly used to improve the short maturing of wines on lees, both in presence and in absence of two secondary fermentation yeast strains: *Saccharomyces cerevisiae bayanus* BCS103® (Fermentis, division of S.I. Lesaffre, France) and *Saccharomyces cerevisiae bayanus* Lalvin EC1118® (Lallemand Inc., Canada) in synthetic buffers, in dependence of pH and ethanol concentrations, kinetic parameters determination; 2) Sparkling wines production by the traditional method. Base wine inoculum was performed using the same selected yeast strains, both in absence and in presence of Lallzyme MMX®; 3) Base wine and sparkling wines sampling at 4, 8 and 12 months after *tirage* and study of: yeasts morphological changes by TEM (Transmission Electron Microscopy) and SEM (Scanning Electron Microscopy) observations; foaming properties; free amino acids composition; total proteins content. A fourth step was focused on perform a sensory analysis 12 months after *tirage* by a trained panel group aimed at assess the differences between sparkling wines produced in presence and in absence of Lallzyme MMX®. Moreover, a PCA bi-plot was carried out considering total proteins and free amino acids 12 months after *tirage*.

The preliminary charaterization of Lallzyme MMX® showed that even if the optimum pH for β-glucanase was 5.00, at wine pH (3.20), it exhibited a satisfactory activity, useful for oenological purposes. Moreover, a lytic activity on yeast cell wall glucans was pointed out, in particular for EC1118 strain. Ethanol inhibition did not compromise the lytic activity of β-glucanase, that resulted enhanced in presence of yeast cells, in particular with EC1118. TEM and SEM observations were useful to describe yeast lysis during ageing. Free amino acids

content showed the effect of enzymatic treatment on yeast lysis, particularly manifested for some amino acids, such as Gaba (γ-aminobutyric acid), Hystidine, Threonine and Isoleucine 12 months after *tirage*. Total proteins content showed that autolysis occurred already 8 months after *tirage*, even if this parameter seemed to have no positive influences on foaming properties, which presented a decreasing trend during sparkling wines ageing. Sensory analysis showed no significant differences between samples even if all sparkling wines were considered as good. Future perspectives may look at increase knowledge about enzyme inhibitors both in synthetic buffers and in wine, aimed to better understand enzyme behaviour in real wine.

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RIASSUNTO

Questa tesi di dottorato ha riguardato lo studio della lisi cellulare durante la maturazione di spumante rifermentato in bottiglia in presenza di una preparazione enologica a base di β-glucanasi. Il progetto di tesi ha previsto una sperimentazione suddivisa in quattro fasi: 1) Studio preliminare del preparato enzimatico ad uso enologico Lallzyme MMX® (Lallemand Inc., Canada), utilizzato per migliorare ed accelerare la maturazione dei vini sulle fecce. Lo studio è stato condotto sia in presenza che in assenza di due ceppi di lievito per rifermentazione: *Saccharomyces cerevisiae bayanu*s BCS103® (Fermentis, division of S.I. Lesaffre, France) e *Saccharomyces cerevisiae bayanus* Lalvin EC1118® (Lallemand Inc., Canada) in tamponi sintetici, sono stati valutati la dipendenza dal pH, da due concentrazioni di etanolo e i parametri cinetici dell'enzima; 2) Produzione di spumante metodo classico utilizzando i due ceppi selezionati, sia in assenza che in presenza del preparato enzimatico; 3)

Campionamento del vino base e degli spumanti ottenuti a 4, 8 e 12 mesi dopo il *tirage* e studio di: cambiamenti morfologici dei lieviti tramite TEM (Transmission Electron Microscopy) e SEM (Scanning Electron Microscopy), caratteristiche della spuma, amminoacidi liberi, proteine totali. La quarta fase ha previsto l'analisi sensoriale sui campioni 12 mesi dopo il *tirage*, allo scopo di valutare le differenze tra gli spumanti prodotti in presenza e in assenza del preparato Lallzyme MMX®. Inoltre, è stata condotta una PCA (Principal Component Analysis), considerando come variabili proteine totali e amminoacidi liberi 12 mesi dopo il *tirage*.

Dallo studio preliminare del preparato Lallzyme MMX® è emerso quanto segue: il pH ottimale per la β-glucanasi è 5.00 tuttavia, al pH del vino (3.20), l'enzima ha manifestato un'attività soddisfacente, utile agli scopi enologici prefissati. Inoltre, è stata rilevata un'attività litica sui glucani della parete cellulare del lievito, in particolare per il ceppo EC1118. L'inibizione esercitata dall'etanolo non ha compromesso l'attività litica della β-glucanasi, che è risultata più elevata in presenza delle cellule di lievito, in particolare del ceppo EC1118. Le osservazioni al TEM e al SEM si sono rivelate utili per descrivere la lisi cellulare durante la maturazione dello spumante. Il contenuto in amminoacidi liberi invece ha mostrato l'effetto della preparazione enzimatica sulla lisi cellulare, particolarmente evidente per alcuni amminoacidi come Gaba (acido γ-amminobutirrico), Istidina, Treonina e Isoleucina 12 mesi dopo il *tirage*. Le proteine totali hanno evidenziato l'avvenuta lisi cellulare 8 mesi dopo l'inizio della presa di spuma, anche se questo parametro non ha avuto influenze positive sulle caratteristiche della spuma, che hanno mostrato un andamento decrescente durante la maturazione degli spumanti. All'analisi sensoriale tutti gli spumanti sono stati giudicati di buona qualità. Le prospettive future potrebbero riguardare l'acquisizione di ulteriori conoscenze riguardo gli inibitori della β-glucanasi sia in tamponi sintetici che in vino, per comprendere meglio il comportamento dell'enzima in condizioni reali.

SUMMARY

1 INTRODUCTION

A sparkling wine is obtained by a secondary fermentation of a still wine, called base wine. Depending on the production technology, they can be classified into sparkling wines produced by traditional in-bottle fermentation and sparkling wines produced by secondary fermentation in hermetically-sealed tanks.

The production of sparkling wines is a long process that follows many different steps, and most of them require long time and expensive skilled labor. In the traditional method technology, secondary fermentation takes place directly into the same bottle that will be marketed. Base wine, yeasts and saccharose in the correct amunt to produce the desired CO_2 pressure are introduced into the bottle and secondary fermentation occurs. Refermentation is followed by a prolonged ageing in contact with lees and during this time, yeasts autolysis takes place. This process is characterized by the hydrolysis of intracellular biopolymers by endogenous yeast enzymes, resulting in the release of several intracellular compounds like amino acids, peptides, proteins, polysaccharides, nucleic acid derivatives and lipids that have a positive effect on sparkling wine quality (Alexandre and Guilloux-Benatier, 2006). However, yeasts autolysis is a slow process lasting from a few months to years and depending on various factors such as temperature, nutrients availability, grape variety, or the yeast strain used (Nunez et al., 2005).

Over the years many attempts to simplify the production process have been made, trying, at the same time, to keep the quality and the characteristics of sparkling wines unaltered. This revolution is still currently a matter of debate within the winemaking industry. Some new approaches in this area include the use of modern biotechnologies, in order to obtain a quality end product with reduced production times and costs (Tiţa, et al., 2003). In addition to the

improvement of the yeasts used for secondary fermentation, also procedures to go faster the natural process of autolysis have been studied (Pozo-Bayón et al., 2009).

For years on end, two methods have been available to accelerate this process during sparkling wine production: by adding yeast autolysates to the wine and by increasing the ageing temperature (Charpentier and Feuillat 1992). However, both techniques cause organoleptic defects in the final product, often described as toasty. Many researchers addressed their studies towards the selection of autolytic yeast strains for secondary in-bottle fermentations (Gonzalez et al. 2003, Martínez-Rodríguez et al. 2001a, Todd et al. 2000).

Over the years, the winemaking industry has developed specific commercial enzyme preparations with the aim of accelerating autolysis in wines aged over lees. These products are a mix of several enzymes such as β-glucanases and pectinases that considerably increase the polysaccharide concentration of both white and red wines (Pellerin and Tessarolo 2001, Trione and Martínez 2001). Many studies are available about the use of these enzymatic preparations for yeast autolysis enhancement in red and white wines (Masino et al. 2008, Palomero et al. 2009, Palomero et al. 2007). On the other hand, to our knowledge, there are no research works about the use of these enzymatic preparations for the production of sparkling wines by the traditional method. Moreover, any preliminary study of these commercial enzymatic preparations is still available.

The present work focuses on the preliminary study of Lallzyme MMX® activity, an enzymatic preparation containing β-glucanase activity, both in presence and in absence of two secondary fermentation yeast strains: *S. cerevisiae bayanus* BCS103® and *S. cerevisiae bayanus* Lalvin EC1118®, and its further application in sparkling wines production by the traditional method.

Enzyme activity was studied in different buffers and in a model wine solution. pH and ethanol content effects, i.e the main wine characteristics possibly affecting enzyme activity, were also considered. In addition, the interactions between the enzyme and two different yeast strains

was verified. After sparkling wines production, direct and indirect evaluation of EC1118 and BCS103 strain lysis was performed. The analysis of enzyme effects on yeasts morphology, foaming properties, chemical composition changing and sensory quality implications on the obtained sparkling wines was conducted.

2 STATE OF THE ART

2.1 Sparkling wines production by the traditional method

A sparkling wine is a special wine obtained by a secondary fermentation of a still wine, called base wine. Depending on the production technology, they can be classified into sparkling wines produced by traditional in-bottle secondary fermentation and sparkling wines produced by secondary fermentation in hermetically-sealed tanks.

The traditional method is used in Spain to produce "*Cava"* sparkling wine, in Italy for "*Talento"* production and also in the French *Champagne* region to obtain the "*Champagne"* sparkling wine. In this case, the production technology is strictly regulated by the principles of the "*Appellation d'Origine Contrôlée*" (*AOC*).

Even if the winemaking techniques adopted over the world may be very similar among them, because of the exceptional *terroir*, the European Union authorized the use of the expression "*méthode champenoise*" only for sparkling wines produced in the *Champagne* region (EU regulation n°3309/85). As a consequence, sparkling wines produced elsewhere cannot use this expression but "traditional method", "classic method" or similar terms.

In general, the traditional method requires a second in-bottle alcoholic fermentation of a base wine and a prolonged ageing in contact with lees. During this process, the characteristic endogenous CO_2 is produced with the consequent release of carbon dioxide when the bottle is uncorked (Torresi et al., 2011).

Although the production of sparkling wines is lower compared to that of still wines, the economical impact of this product is very important because of its high added value. Today, the orientation of customer preferences toward the quality of fermented beverages is a

certainty and sparkling wines made through the traditional method present distinct and unique characteristics compared to the whole range of sparkling wines produced by other techniques (Torresi et al., 2011). The production of sparkling wines requires some devices suitable to sustain the internal bottle pressure typical of these special wines. This led in the past to the introduction of special cork closures and the development of new technologies in glass manufacture. It was fundamental to produce resistant glass for bottles able to withstand the high pressures that develop during base wine refermentation. During the reign of King James I (1603–1625), in England, glassmakers started to use coal to fire the glass furnaces, this expedient allowed to obtain bottles resistant to pressure. The introduction of corks, at the end of 1500s, permitted to hold carbon dioxide inside the bottle, contributing to the evolution of sparkling wines production technology (Jackson, 2008).

2.1.1 Elaboration of base wines

The elaboration of a sparkling wine involves filling the bottle with the base wine, derived from the fermentation of grape musts produced by the traditional white wine technology.

Some of the many cultivars used in various growing regions for the traditional sparkling wine's production are *Chardonnay*, *Pinot Noir*, *Pinot Meunier* and *Pinot Blanc*. The use of autochthonous cultivars has become a common practice, even if their use is forbidden for the production of *Champagne*.

The base wine should always presents typical characteristics like moderate alcohol content (about 11% v/v), because a subsequent amount of ethanol is produced during secondary fermentation (up to a final concentration of about 13% v/v); pale colour; fruity aroma; low residual sugar content; low volatile acidity and should usually have been subjected to tartaric stabilization. Moreover, is convenient for base wines to show quite higher total acidity values

than traditional white wines. In fact, during the production process, total acidity decreases, because of potassium bitartrate precipitations, malic acid degradation operated by yeasts and also dilution due to dosage material addition (*liqueur d'expedition*) (Ribéreau-Gayon et al., 2004). For these reasons, harvesting is generally slightly anticipated respect to traditional white wine grapes. As a consequence, must extraction must be very accurate, in order to avoid skins macerations, leading to the formation of vegetal and bitter defects. Also colour extraction should be prevented, especially if red grapes are used for the so called "*blanc de noirs*" sparkling wines (Zoecklein, 2002). Harvesting is a very delicate process, grapes should be hand-harvested into small containers, berries breakage should be avoided, defective grapes should be discarded and fruit should be transported to the cellar as soon as possible to prevent undesired fermentations.

In order to limit oxidations and macerations, grapes are generally directly pressed, without crushing. This phase should be very fast to obtain a good quality must. Immediately after juice extraction, sulphur dioxide is added to prevent oxidations and then solids are removed to minimize phenols oxidations that may occur during fermentation. This is generally made through the use of pectolytic enzymes. The primary fermentation is usually carried out in stainless steel tanks were temperature is maintained under 20°C after selected yeasts addition.

Once the primary fermentation is completed, base wine is clarified, decanted, filtered and bentonite is added to prevent proteins precipitations. Cold stabilization to avoid potassium bitartrate precipitation is also performed. Immediately before bottling, base wine is generally filtered again, to completely eliminate solid particles.

2.1.2 Secondary fermentation

Even if base wines contain a quite low amount of ethanol, in order to facilitate the beginning of secondary fermentation, an active selected yeasts inoculum is usually prepared, and wine is

gradually added in order to accustom yeasts to alcohol. After base wine introduction into the bottle, a *tirage* solution, composed of saccharose, yeasts, grape must or wine, in the correct proportion to produce the desired CO_2 pressure, is added. Moreover, a small amount of bentonite is usually employed (Martínez-Rodríguez and Pueyo, 2009) in order to simplify the procedure of lees removal (*dégorgement*).

After the base wine (*cuvée*) has been bottled, the *bidule* is also inserted. This is a hollow polyethylene cup usually 17mm Ø and 14 mm high. *Bidules* help to prevent leakage and metal contact from the crown; furthermore, they give a better seal and help the disgorging process. Further to the hand or machine insertion of the *bidule*, a closure is placed on the bottle usually consisting of a crown cap. These corks are especially designed for sparkling wines and are generally made in stainless steel, coated mild steel or aluminium, although in some countries plastic lined crown caps are also used (Zoecklein, 2002).

The bottles are then stored horizontally in special places furnished for the settling of the sparkle. The bottle storage area should be cool and have minimum temperature fluctuations and minimum lighting. There are several bottle storage systems: by stacking bottles on the floor, but this is a very labour-intensive one; by using wood or caged bins, where 380-504 bottles may be stacked, this is a very space-saving system; a third method of bottle storage is to place bottles into cartons (the same cartons that will go to market). This is a system designed and patented by California's Korbel (Zoecklein, 2002).

During this time, secondary fermentation takes place, the CO_2 is formed and stabilized and the ageing with the yeasts occurs (Martínez-Rodríguez and Pueyo, 2009).

The rate of the secondary fermentation depends on the yeasts, temperature and *cuvée* chemistry, however, a secondary fermentation at 12-15°C occurs in no less than 0.5-1.5 months. The fermentation progress is monitored by reducing sugars analysis and internal bottle pressure control. At the end of secondary fermentation, when sugars have been

depleted, a prolonged ageing in contact with lees starts. During this time, sparkling wines mature and yeasts release their compounds into the wine, modifying its characteristics.

2.1.3 Ageing over lees

During the ageing period, sugars are almost exhausted, yeasts sediment accumulates and its volume decrease. The horizontal position of the bottle allow an efficient sparkling wine-yeast sediment contact so a slow release of yeast compounds (mainly amino acids) takes place. These compounds originate both from yeasts and wine, during fermentation, in starvation conditions, yeasts accumulate them as a reserve and once the fermentation has finished, yeasts release these compounds into the wine, modifying its characteristics. This is still not autolysis, but simply a free exchange back to the wine (Zoecklein, 2002). These phenomena play an important role in sparkling wines quality, along base wine composition and ageing times. The length of the ageing period is a key parameter in sparkling wines production, in fact, the reducing conditions inside the bottle ensure wine protection from oxidations and an adequate ageing over lees (*sur lie*). During this time, several months after secondary fermentation, the autolysis process takes place (Chapter 3). This is an enzymatic self-degradation of cells constituents thus causing the release of many parietal and cytoplasmic compounds with proved foaming and sensory properties (Alexandre and Guilloux-Benatier, 2006; Nunez et al., 2005; Moreno-Arribas et al., 2000). This process allows to obtain an end product with typical characteristics that make it a worldwide prestigious wine. During ageing roundness, flavour and complexity develop, a typical characteristic is the so called “yeasty character”. This is a reduction scent due to thiol groups formation because of sulphur amino acids photodegradation. This reaction depends on riboflavin (vitamin B_2) photosensitivity, and the reaction products, methanethiol and dimethyl disulfide are responsible of “light struck” or (*Goût de Lumière*) flavour (Ribéreau-Gayon et al., 2004). It has been observed that

Champagne quality diminished if bottles were exposed in supermarkets instead of traditional liquor stores and the "light struck" flavour appeared. It was due to the intense fluorescent lighting typically used in large retail stores and triggered by photochemical transformations involving sulphur components, such as methionine and cysteine (Maujean and Seguin, 1983; D'Auria et al., 2003). In order to protect sparkling wine from "light struck" appearance, the use of glass bottles impermeable to wavelengths below 450 nm has become a common practice. Moreover, the addition of small amounts of ascorbic acid and SO_2 before the final corking, allow to prevent this unpleasant drawback (Ribéreau-Gayon et al., 2004).

2.1.4 Riddling, disgorging and dosage

When the winemaker considers that wine has matured for a sufficient time over lees, the procedure of riddling, also called *rémuage* is carried out. This technique allows to convey yeasts sediment into the *bidule*, facilitating lees removal during the subsequent disgorging process. During riddling, gravity slowly conveys the sediment to the neck of the bottle. This process was originally carried out with the bottles on desks, by manually turning them 1/8 of a turn for about 15 days and gradually increasing inclination until they were almost perpendicular to the floor. Now this method has been replaced by automated systems that can move all the bottles simultaneously (Martínez-Rodríguez and Pueyo, 2009). When the sediment has been completely conveyed into the *bidule*, bottles are ready for the final step, the disgorging process, or *dégorgement*. This procedure is performed by inserting the neck of the bottle in a solution which freezes the sediment trapped in the *bidule*. The *bidule* helps to ensure that the yeast plug will be ejected uniformly and that no yeast residue will be left. Bottles are then placed neck-up, uncorked and the pressure within the bottle ejects both *bidule* and ice plug. The bottle is then filled with the *liqueur d'expédition*, or dosage solution, composed of wine, sugar, brandy, SO_2, citric acid, with a different formula for each firm. The

amount of sugar (g l^{-1}) added with the dosage solution differentiates sparkling wines by sweetness, thus they are classified as follows: brut, 0–15 g $l^{-1}$of sugar; extra-dry, 12–20 g l^{-1}; sec, 17–35 g l^{-1}; demi-sec, 33–50 g l^{-1}; doux, >50 g l^{-1} (Zoecklein, 2002).

2.1.5 Sparkling wines characteristics

Once the production process has ended, a special wine typified by carbon dioxide development (*perlage*), foam appearance and some representative sensory characteristics is obtained. The main factor distinguishing sparkling wines from still wines is effervescence, this is due to the carbon dioxide bubbles that continuously go up through the liquid. Effervescence represents a pivotal quality criterion, it also behaves as an aromas carrier because bubbles contain both CO_2 and some odorous compounds. When the bottle is uncorked, the CO_2 is rapidly released as a consequence of the difference between bottle and room pressure. When the sparkling wine is poured into the glass, carbon dioxide gives rise to the *perlage* (CO_2 bubble chains that go up through the sparkling wine) and foam appearance. Bubbles originate from nucleation sites formed by tiny solid particles localised into the wine or on glass walls. CO_2 bubbles keep enlarging because their own internal pressure is lower than that of the wine. When bubbles diameter reaches a critical value, they get up to the wine free surface. Some wine components such as polysaccharides, proteins and lipids influence bubbles size, persistence and also foam, since they form a rigid coat around each bubble, reducing liquids' surface tension.

Although there is no scientific correlation between foam quality and effervescence finesse, consumers often believe that small bubbles are a sign of a lasting and elegant effervescence (Gallart *et al.,* 2004). A good quality foam is characterized by a slow CO_2 release from the bottom of the glass, bubbles should be small and form a foam crown once they reach the

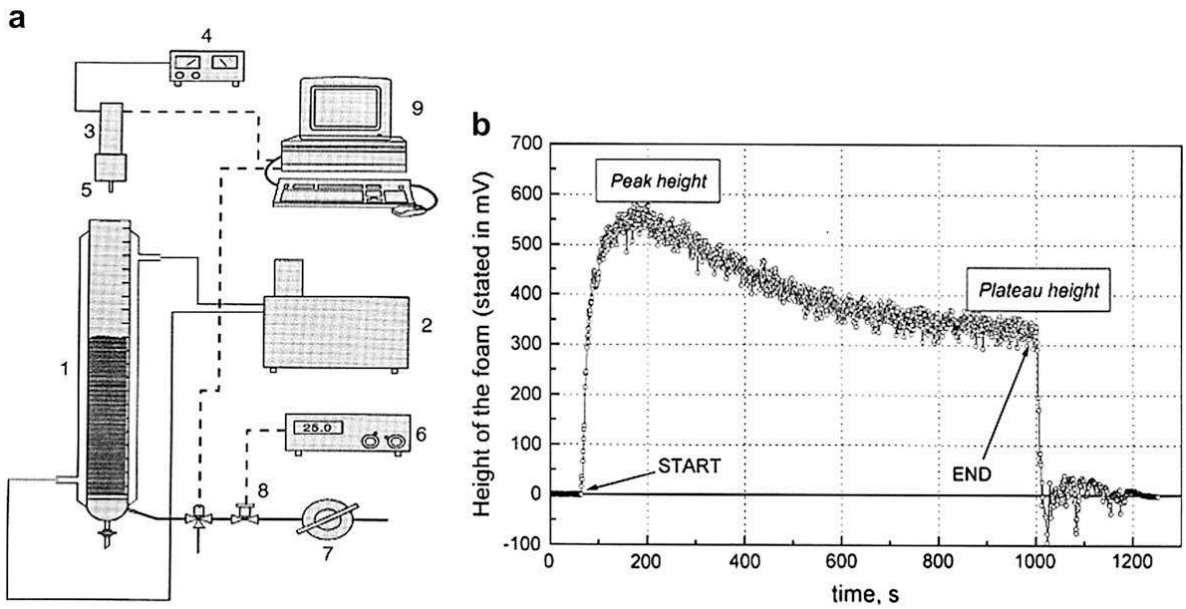
liquid surface. Foam persistence depends on the balance between the rate of bubbles formation and disappearance (Martínez-Rodríguez and Pueyo, 2009).

Since consumers consider foam and effervescence a fundamental criterion for sparkling wines quality, the study of the mechanisms involved in foam formation and its chemistry is essential for winemakers (Robillard et al., 1993).

Many studies are available about the influence of different factors on foam appearance, such as grape variety, time of harvesting, winemaking process, ageing time, wine composition (Hardy, 1990; Moreno-Arribas et al., 2000). Numerous research groups, addressed their studies toward the development of instrumental techniques able to evaluate foam characteristics and match different sparkling wine samples. The methods available to measure foam are classified as follows: methods based on CO_2 release kinetic study; gas sparging methods and image analysis methods (Martínez-Rodríguez and Pueyo, 2009). Methods based on CO_2 release kinetic, involve the study of free CO_2 spontaneously released from the bottle. Once all the CO_2 is come out, bottle is shaken to quantify the further released CO_2. Gas sparging methods are widely used and are based on the Bikerman's procedure (1938). This method involves the estimation of Σ (Bikerman's coefficient), the ratio between foam volume (in stationary state) and gas volumetric flux injected into the sample. This parameter is independent from both instrument and gas flux injected (Gallart et al., 1997; Senée et al., 1998). Bikerman's method consists of injecting a controlled gas flux through a known volume of wine to produce foam. After gas injection, it is possible to measure the maximum height reached by foam column and also the time elapsed before all the bubbles disappear (after gas flux interruption) (Pueyo at al.,1995). Maujean et al. (1990), developed an automatic apparatus for foam measurement, based on the Bikerman's method and called "Mosalux" (Figure 1). This apparatus is widely used both in research laboratory and cellars producing

sparkling wines. It uses a photoelectric cell to register foam parameters, moreover, it is equipped with a processor for data acquisition.

Figure 1 *Mosalux apparatus scheme for foam measurements (a) (1) Graduated glass cylinder, (2) Thermostated bath, (3) Ultrasound transmitter/receiver (4) 24 Volts power supply, (5) Wavelenghts selector, (6) Flow controller, (7) Pressure reducing valve, (8) Control valve, (9) Personal computer; (b) Base wine foam profile, example (Pozo-Bayón et al., 2009).*



Mosalux apparatus measures three parameters: HM (foamability), expressed in millimiters and representing the maximum height reached by foam 1 or 2 minutes after CO_2 injection; HS (Height stability), expressed in millimiters, is obtained maintaining the same conditions of pressure and flow of CO_2, in this circumstance, foam collapses to stabilize at a level HS; TS (Foam stability time), expressed in seconds, this is the time elapsed before all the bubbles disappear after gas flux interruption (Gallart et al., 1997; Poinsaut, 1991). It has been demonstrated that HM and HS are independent among them, in fact, a wine with attitude to form much foam, not necessarily is also able to provide a stable foam (Maujean, 1990). Generally, during sparkling wines ageing on lees, HS decreases, while TS tends to increase.

The validity of the results obtained with the Mosalux apparatus has been repeatedly tested and a good correlation between the measured parameters and the Bikerman's coefficient has been found. Foam quality has also been evaluated by mathematical equations established between foam parameters (dependent variables of the equations) and wine physicochemical characteristics, such as ethanol content, proteins, pH, titratable acidity and organic acids (independent variables of the equations) (López-Barajas et al.,1999). Many studies have tried, over the years, to suggest the introduction of new parameters for foam measurements, even if Gallart et al. (1997) retains that HM, Σ e TS are the most appropriate. Also image analysis is considered as a valid method to study foam characteristics directly into the glass (Machet et al., 1993), this technique evaluates automatically foam quality in real condition using video cameras. However, this method does not apply to base wines to predict sparkling wine's foam quality (Gallart et al., 2004).

A correlation between sparkling wines foam and proteins content has been pointed out (Ribéreau-Gayon et al., 2004). Solubility greatly influences effervescence; because of their hydrophobicity, proteins are adsorbed on the external surface of the gas bubble. The gas bubble is so stabilized, because of surface tension decrease. The adsorption is favored by a low proteins molecular weight. Winemaking techniques may negatively influence foam quality, among them, filtration and clarification are the most hazardous, since they cause proteins and solid particles decrease. These compounds forms nucleation sites allowing bubbles to grow, so their removal from wine may allow a reduction of foam stability.

The use of bentonite may be responsible of foam reduction, Martínez-Rodríguez and Polo (2003), found that bentonite addition (0,03 g l^{-1}) to the *liqueur de tirage* modifies proteins and peptides composition of sparkling wines, while the amino acidic content remains unaltered.

Also grape variety may greatly influence sparkling wines' foam characteristics (Girbau-Sola et al., 2002a; Girbau-Sola et al., 2002b). Because of the importance of foam appearance and

perlage characteristics, Obiols et al. (1998) developed a valuable protocol including scorecard to evaluate the foaming and effervescence properties of sparkling wines.

When a sparkling wine is poured into the glass, foam appearance precedes the liquid and it's considered as a fundamental quality criterion during tasting. Sparkling wines visual aspect is then essential, an unpleasant visual perception in fact can affect the whole sensory anlaysis.

A good quality foam has small CO_2 bubbles, independent from each other and able to retain their spherical shape. Furthermore, effervescence behaves like an aromas detector, since bubbles contain, in addition to CO_2, also odorous compounds (Ribéreau-Gayon et al., 2004).

In sparkling wines tasting, glass shape plays an important role, it should be a *flûte* with typical characteristics. This is a tapered glass, with a slight shrinking aperture to favor both liquid rotation and aromas conveyance to the taster's nose. The *flute* should contains about 100 ml of sparkling wine, spread over a 75 mm height. This is considered as an adequate wine quantity to give a good effervescence and a proper tasting.

About sparkling wines tasting, Hardy (1986, 1987a, 1987b, 1988), wrote a complete guide, therefore visual aspects should be considered as important as olfactory and gustative characteristics (Torresi et al., 2011). The first part is about foam visual aspect examination, the second part concerns wine visual aspect and the third part analyzes both olfactory defects and aromas. The last section of the guide is for gustative evaluation, CO_2 perception, sparkling wines acidity and sweetness are part of it. Hardy's scorecard for sparkling wines tasting subdivides flavorings into four categories: primary, prefermentatives, secondary and tertiary. Primary aromas derive from the grape, mainly present in young products. Pre-fermentative aromas, that may have a positive influence on wine quality (such as cold maceration aromas), or negative effects due to an erroneous grape processing after harvesting. Secondary aromas, instead, originate from both first fermentation and secondary fermentation in the bottle, they are mainly affected by must and base wine quality, fermentation temperature and yeast strain.

Finally, tertiary aromas arise from wine maturing on lees and develop also by yeast cells lysis, after long ageing as a consequence of low molecular weight intracellular compounds release (Ubigli, 2004; Parodi, 2002).

2.1.6 Improvements in sparkling wines production

The modern technologies for the production of sparkling wine are very different from the one developed by Abbot Dom Pierre Pérignon in the 17th century. The wine history in fact, encompasses several millennia of craftsmanship and innovation. In ancient times, wine was considered as a magical, spontaneous gift of nature. For this reason, the application of biotechnologies to grape and wine represents a challenge to tradition and a balance should be found between technological innovations and winemaking traditions (Pretorius and Høj, 2005). In the last few years, there have been considerable developments in wine-making techniques affecting all stages of wine production, but more importantly, the fermentation process (Kourkoutas et al., 2010). Over the years, wine-making technology has experienced significant progresses, that have led to a large set of good quality products. Among the wide range of biotechnological applications available, some of them have been particularly useful for the improvement of wine quality; selected yeasts, immobilised yeasts and enzymatic treatments have been particularly important for winemaking technology and also in sparkling wines production. Even if the principles of oenology have been established over the course of several centuries of rigorous and methodical observation, the production of quality wine has mostly benefited, in the last quarter of a century, from research developments. In this area, biotechnological knowledge may well become more and more important, not only as regards the microorganisms involved in the fermentation process but also because of the use of enzymes in pressing grapes, prefermentation treatments and refinement of the wines (Torresi et al., 2011).

Over the years, researchers addressed their study toward the selection of yeasts strains able to withstand the typical adverse conditions of in-bottle fermentations. Even if the history of wine yeast starts with the first civilisations, the fermentation of must was not understood until 1863. In that year Louis Pasteur showed that a living microorganism, the yeast, was responsible for the biotransformation of the sugar present in the must into ethanol and CO_2.

Although many genera and species of yeasts are found in the musts, the genus *Saccharomyces*, and mainly the species *S. cerevisiae*, is responsible for this biotransformation. For this reason, *S. cerevisiae* is the preferred strain for wines alcoholic fermentation (Kirtadze and Nutsubidze, 2009) and is referred to as “the wine yeast” (Pretorius, 2000; Pérez-Ortín et al., 2002).

However, in the past, must fermentation happened by pure chance, it was very difficult to control the factors involved in yeasts growth because there was scarce knowledge about them. Over the years, a deeper understanding of yeast strains was reached and a selection strategy started to be adopted in order to obtain a wide range of suitable strains for different winemaking technologies and products, capable of withstanding the adverse conditions that may occur during fermentation. This is especially true for sparkling wine production, for which the secondary fermentation occurs in very particular conditions for yeasts. Base wine presents a high alcohol content (about 10% v/v), under these conditions, only *S. cerevisiae* (but not all strains) can grow and ferment. Fermentation temperatures are quite low (12–18 °C) and slow down yeast activity, even if this is useful for sparkling wines quality improvement. A slow fermentation rate allows to obtain a better foam and a good bouquet. SO_2 amount is generally quite low, moreover, most of it is in the combined form and can’t exhibit an inhibitory effect. Carbon dioxide, which is produced during secondary fermentation, has no negative effects on fermentation. In fact, yeasts can tolerate more than a 4 atm pressure, even if their activity is quite slowed down, mainly at low pH and high alcohol

content conditions (Zambonelli, 2006c; Ribéreau-Gayon et al., 2004). However, even if in-bottle conditions are not excellent for yeasts, a good quality base wine, a careful strain selection and a controlled temperature are requirements good enough to guarantee a correct refermentation process. In order to avoid unpleasant drawbacks during fermentation, in recent years, the use of selected autochthonous yeasts during secondary fermentation has become a common practice (Martínez- Rodríguez et al., 2001a). It has been stated that the yeast strain used for refermentation plays an important role in the ageing process and it affects the final composition of sparkling wines. This is also due to the fact that refermentation occurs in sealed bottles with no compounds dissipation. Many *S. cerevisiae* strains produce good quality sparkling wines but with different characteristics, the strain could affect sparkling wine quality such as the grape variety, in fact, some yeast strain are so flavouring that it is possible to obtain a good quality sparkling wine from non-aromatic base wines. Likewise, a sparkling wine produced using a flavouring yeast and an aromatic base wine might be too fragrant (Zambonelli, 2006c). Yeasts that carry out secondary fermentation must satisfy a series of additional characteristics compared to those used in the first fermentation of base wine production. Besides being resistant to ethanol, they must have high flocculation capacity, in order to facilitate their elimination from the bottle (Bidan et al., 1986; Bartrà, 1995). Flocculation is a distinctive yeast feature consisting in cells aptitude to form compact lumps which form a deposit in the bottle. This characteristic is a frequently occurring trait in *S. cerevisiae* (Suzzi et al., 1984; Castellari et al., 1994). After shaking, large flocs resuspend but they heavily precipitate and form a sediment. As regards the importance of flocculation during disgorging, many studies have been carried out over the years in order to understand its mechanism and effects (Bayly et al., 2005; Bester et al., 2006; Zambonelli, 2006a). In food and biotechnological industries, where fermentation occurs and cell reactors are employed, suspended cells must normally be separated from the media prior to further processing.

Beverage alcohol production is a typical example of this process (Jin and Speers, 1999). In flocculent yeast strains, cells separate from each other after budding and subsequently form large flocs that rapidly sediment. Flocculation is a very important characteristic, especially in sparkling wine production by the traditional method. Yeast flocs, indeed, do not cause turbidity and form a deposit that rapidly accumulates in the *bidule* thus facilitating disgorging (Zambonelli, 2006a). Coloretti et al. (2006), obtained interspecific hybrids from flocculent *S. cerevisiae* and nonflocculent *S. uvarum* (*S. bayanus var. uvarum*), these yeasts are flocculent to a higher degree and have the capacity to reach a good fermentation rate at both low and high temperatures. If employed for the production of sparkling wines by bottle re-fermentation, they form a deposit that fragments into large flocs which rapidly sediment without causing turbidity. Certain genes involved in flocculation are extremely variable and cause frequent changes in the flocculation profile of some strains. In the future, genetic modification may offer a valuable method to improve imperfect flocculation profile of some sparkling wine strains (Pozo-Bayón et al., 2009).

Because of autolysis importance during sparkling wines ageing, many studies have been directed to select autolytic strains for secondary fermentations, this aspect will be best described below (Paragraph 3.3).

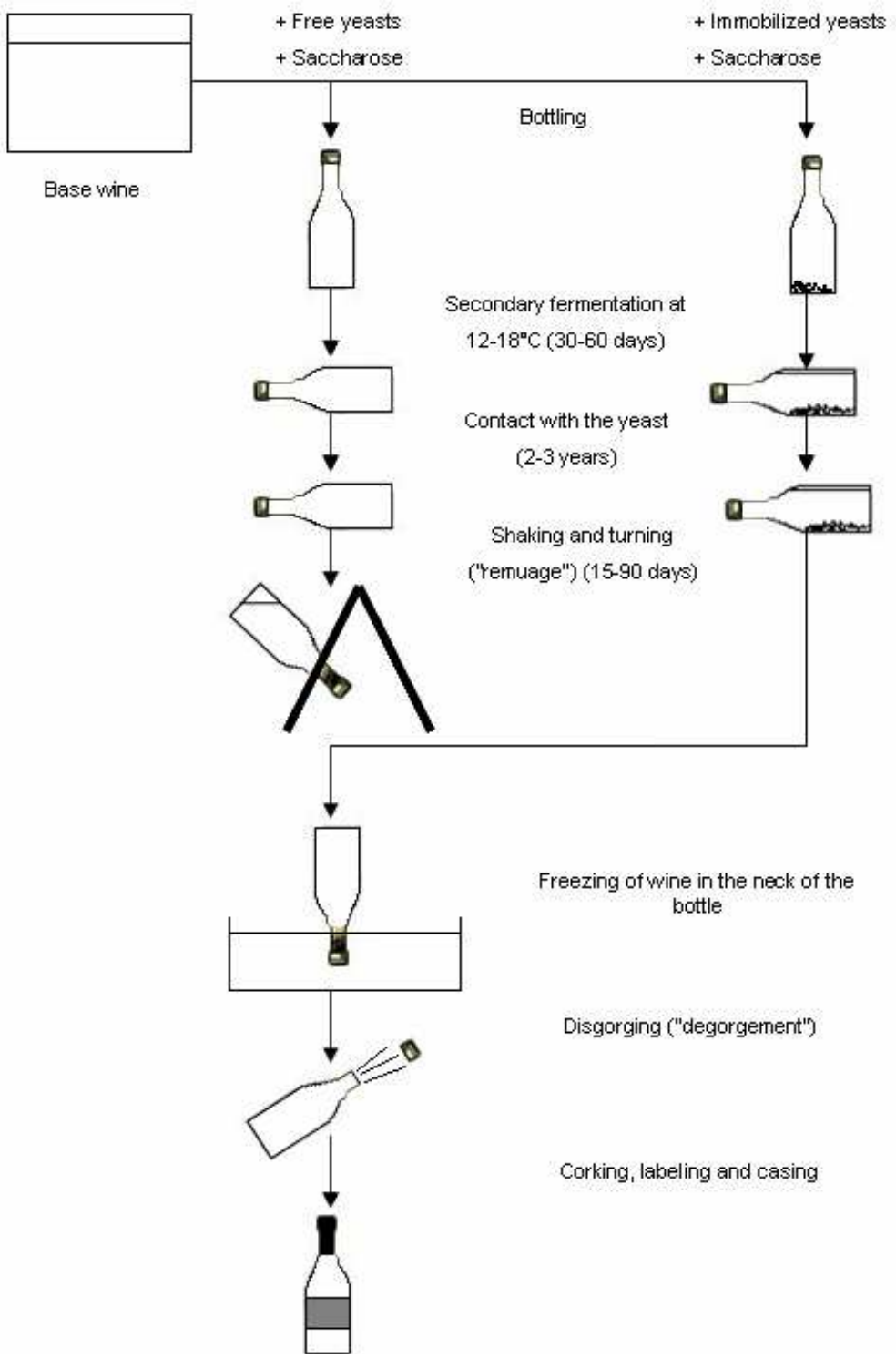
Moreover, in order to obtain efficient biocatalysts for sparkling wines produced by in-bottle fermentation, the use of immobilised yeasts has been introduced. In the traditional sparkling wines production, lees removal is a very labor-intensive and time-consuming process and the use of immobilised yeasts has been investigated in order to reduce and simplify the riddling and disgorging procedures. Yeasts immobilisation consists in the introduction into the bottle of a certain amount of beads containing yeast cells (Figure 2). While the cells grow, they remain trapped into the beads and wine clarity is preserved (Zambonelli, 2006b). The first applications of immobilised yeasts for industrial purposes have been performed in k-

carrageenine by Wada et al. (1979) and in sodium alginate by Veliky and Williams (1981). Bidan et al. (1980), in France, have been the first to obtain a patent for the production of sparkling wines using immobilised yeasts (Martynenko and Gracheva, 2003). Over the years, immobilised yeasts have been frequently used in sparkling wines production, the first applications have been reported by Fumi et al. (1987), Fumi et al. (1988) and Fumi et al. (1994). Immobilisation supports suitable for the wine industry must have additional prerequisites such as food-grade purity, low cost, abundance, non-degradable nature and suitability for low-temperature fermentation. Although many immobilisation supports have been suggested for winemaking applications, the industrial use of this technology is still uncertain (Kourkoutas et al., 2004). Among the immobilisation techniques available, occlusion in polysaccharide gels such as agar, alginate and carrageenan is the most important and widely used. Yokotsuka et al. (1997), used yeasts immobilised in double-layer gel beads for bottle-fermentation, beads were easily inserted into the bottles and simply removed with the ice plug during disgorging.

When a bottle containing immobilised yeasts is inverted, the beads quickly settle into the neck and they can be easily removed. Moreover, Yokotsuka et al. (1997) found that this technique allows to obtain sparkling wines similar, in taste and bouquet, to those made by using free yeasts. Diviès et al. (1994), showed that the use of immobilised yeast cells for the production of sparkling wine could greatly simplify the procedure of "*remuage*". The compared metabolism of entrapped and free cells during the bottle fermentation shows some differences, but the final products do not reveal significant sensory disparity. Efremenko et al. (2005), determined cell growth conditions in order to ensure yeast biomass accumulation for the further development of an efficient biocatalyst for sparkling wines production, poly(vinyl alcohol) cryogel (PVA C) was used in order to entrap yeast cells. The cultivation conditions used for biomass accumulation before its immobilisation could affect the biocatalyst features

and, thereby, the characteristics of sparkling wine production. Even if the use of immobilised yeasts in the classic technology of sparkling wines production my allows to reduce and simplify the procedure of *remuage*, some drawbacks occur. Indeed, sparkling wine frequently loses transparency because of the release of yeast cells from the carrier matrix. Gòdia et al., (1991) used both yeast cells immobilised in simple calcium alginate beads and supports with an external layer free of cells around them, in order to reduce cells release from the carrier matrix. They found that externally coated beads allow to obtain, at the end of the process, a wine free of cells. Organoleptic characteristics were in both cases improved compared to the traditional method. Martynenko et al. (2004), studied a process consisting in the use of *Champagne* yeasts immobilised by inclusion into cryogels of polyvinyl alcohol. Cells were also treated with the auto regulatory factor d_1, thus preventing cells release from the carrier matrix. Furthermore, in order to avoid cells discharge from the support, the market also provides selected yeasts confined by micro-filtration membrane like ‘‘Millispark’’ cartridge, which was developed by Millipore (Billerica, Massachusetts, USA**)** for the production of bottle-fermented sparkling wines (Ramon-Portugal et al., 2003). This technique presents, nonetheless, different disadvantages like mass transfer limitation (Lebeau et al., 1998) and possible membrane biofouling caused by cells growth (Gryta, 2002; Kourkoutas et al., 2005). Even if, in experimental conditions, immobilised yeasts have been widely used for sparkling wines production, their application in oenological practices is still unfrequent.

Figure 2 *Scheme of the process for preparing bottled sparkling wine by traditional method and by immobilised yeast (Colagrande et al., 1994)*



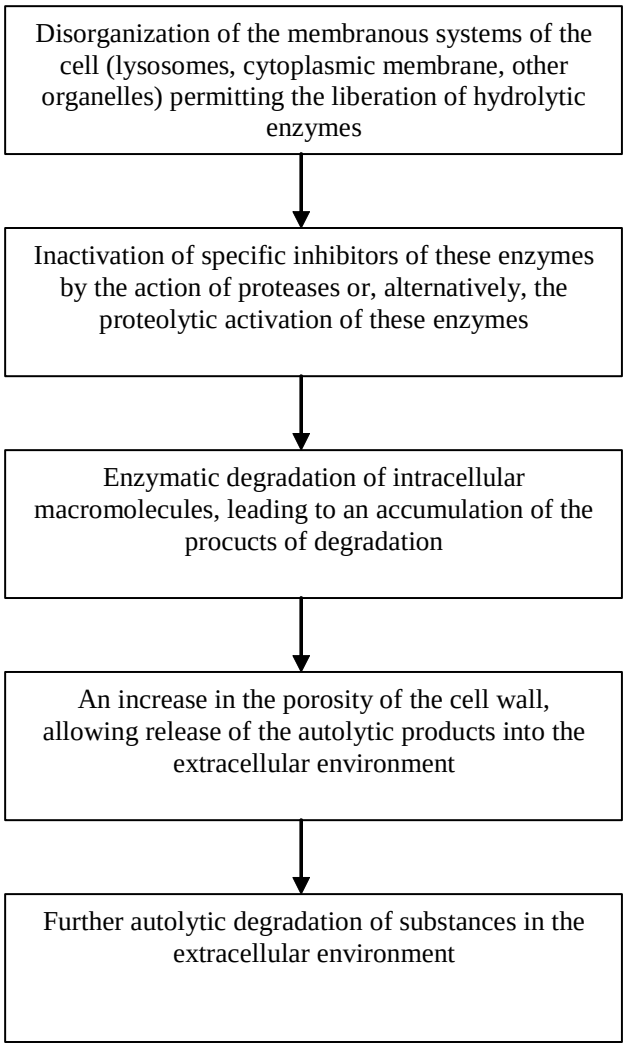
2.2 Yeast autolysis in sparkling wines

2.2.1 Yeast autolysis

The term autolysis was introduced by Salkowsky in 1875 (Farrer, 1956). It can be defined as the hydrolysis of biopolymers under the action of hydrolytic enzymes which release cytoplasmic (peptides, amino acids, fatty acids and nucleotides) and cell wall (glucans, mannoproteins) compounds into the wine (Alexandre and Guilloux-Benatier, 2006). Generally autolysis occurs when sugars and other nutrients are exhausted, in this starvation conditions yeasts use their own nutrients reserve, consuming glycogen and other compounds. When also these internal store is depleted, autolysis begins (Connew, 1998).

The autolysis process consists of five stages, as reported in Figure 3. In the first phase, yeast cell membranous systems break up and hydrolytic enzyme are released. After enzymes activation, the enzymatic degradation of cells constituents leads to the accumulation of degradations product. Yeast cell wall becomes more porous and the autolytic products come out of the cell (Charpentier and Feuillat, 1993). The compounds released in the medium have a low molecular weight and are biologically inactive (Zambonelli et al., 2000). This process has several implications in winemaking. It occurs during storage of wine on lees and is associated with cell death releasing into the wine yeast constituents that strongly influence wine sensory properties. Moreover, it has a unique significance during sparkling wine production by the classic method (Charpentier and Feuillat, 1993). During a prolonged ageing in contact with lees, a typical step in the sparkling wine production by the traditional mehod, yeast autolysis occurs and organoleptic and foaming properties are modified, reflecting changes in the wine composition (Alexandre and Guilloux-Benatier, 2006).

Figure 3 *Stages of the autolysis process (Charpentier and Feuillat, 1993)*



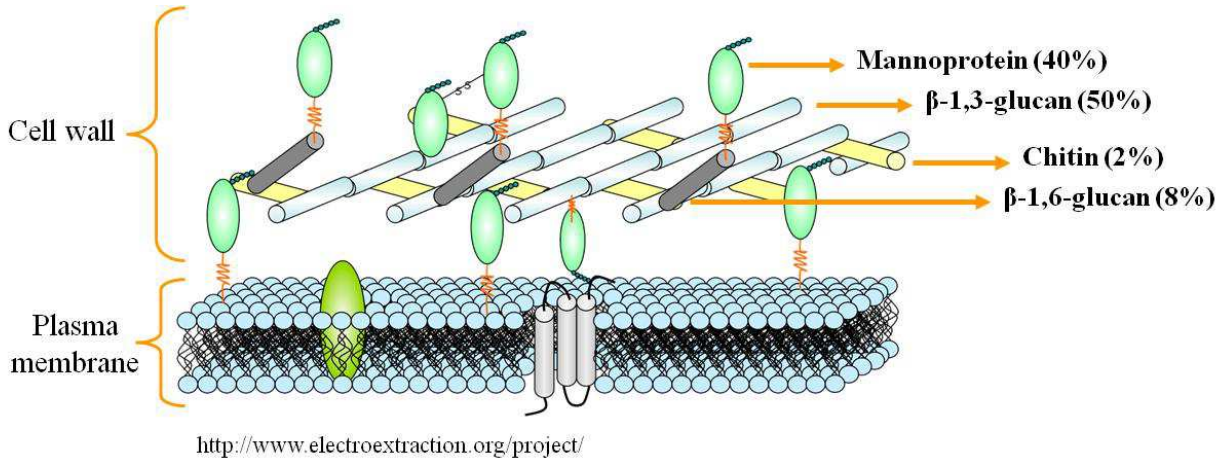
However, yeasts autolysis is a slow process lasting from a few months to years and depending on various factors such as temperature, nutrient availability, grape variety, or the yeast strain used (Nunez et al., 2005).

The cell wall of *S. cerevisiae* is the main cell structure involved in the autolysis process (Figure 4). It is made of mannoproteins crossed by fibres of glucan and chitin (Pretorius, 2000). Glucans from *S. cerevisiae* are mainly β-D-1,3-linked glucose units with β-D-1,6-linked lateral glucose chains. Some branched β-1,6-glucans with some β-1,3-links are also present (Manners et al., 1973a; Manners et al., 1973b). β-Glucanases, classified as endo- and

exo-glucanases, hydrolyse the β-O-glycosidic linkage of β-glucan chains, leading to the release of glucose and oligosaccharides (Dubourdieu et al., 1981).

During autolysis, the cell wall is so gradually degraded due to the breakage of these glucan and chitin fibres caused by endogenous enzymes including glucanases (which are present in the cell wall for up to 4 months after the yeasts' death) and mannosidases (Charpentier and Freyssinet, 1989).

Figure 4 *Scheme of yeast cell wall*

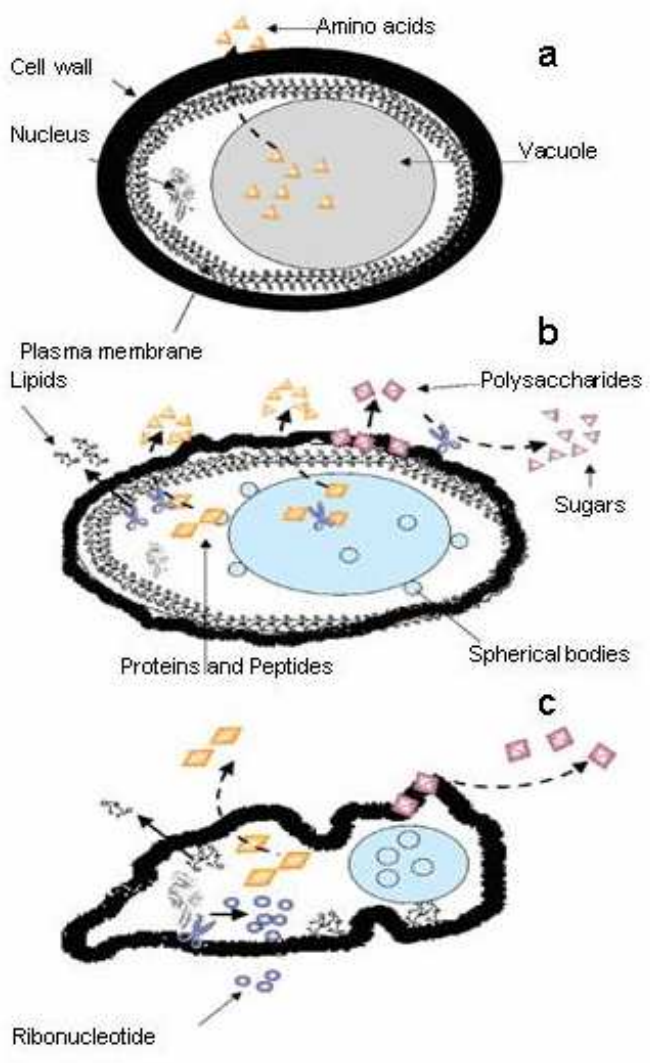


The autolysis process involves yeast cells during sparkling wines maturation on lees and it results in the release of several autolytic products, these compounds are both cytoplasmic (peptides, amino acids, fatty acids and nucleotides) and parietal (glucans, mannoproteins) and they have a positive effect on the quality of the aroma, flavor, and foam of the wine (Charpentier and Feuillat, 1993; Alexandre and Guilloux-Benatier, 2006; Martínez-Rodríguez and Polo, 2000).

In Figure 5, cells morphological changes are displayed. At the end of secondary fermentation, cells show an elliptical shape and the cell wall appears thick and smooth, inside the cell, a big vacuole is surrounded by numerous spherical bodies (Figure 5a). Between three and six

months after the beginning of the autolysis process, cell and vacuole are smaller in size and spherical bodies are uniformly distributed inside the vacuole, on the cell wall surface, ridges and folds appear (Figure 5b). Between nine and twelve months instead, the cell collapses and is smaller in size. The cell wall remains unbroken, but many wrinkles appear on the external surface, moreover, cell lost most of its cytoplasm (Figure 5c).

Figure 5 *Schematic representation of biochemical and morphological changes during yeast cell autolysis in sparkling wines. At the end of the refementation process (a), between three and six months of ageing (b), between nine and twelve months of ageing (c). Alexandre and Guilloux-Benatier (2006).*



During the autolysis process, also biochemical changes occurs. At the beginning of the process, a passive diffusion of amino acids occurs. Afterwards, sparkling wine enriches of amino acids from proteins and peptides hydrolysis and a polysaccharides increase derived from yeast cell wall is generally detected. Plasma membrane is degraded and also lipids are released in the sparkling wine. Between nine and twelve months after the beginning of the autolysis process, amino acids concentration decrease and proteins and peptides are released. Cell wall polysaccharides, lipids and ribonucleotides increase too (Alexandre and Guilloux-Benatier, 2006).

Several research groups addressed their study toward the relationships between yeast autolysis and autophagy (Andrés-Lacueva et al., 1997; Cebollero et al., 2005; Cebollero and Gonzalez, 2006). Autophagy is a catabolic process omnipresent in eukaryotic cells that involves cytoplasm degradation in the vacuole or lysosome lumen. This degrading process seems to be essential for cells adaptation to external and internal stress factors (Cebollero and Gonzalez, 2007). For the yeast *Saccharomyces cerevisiae*, in starvation conditions, the mechanism of autophagy is activated and generates an internal pool of nutrients in order to allow survival during prolonged nutrition deficient conditions. In eukaryotic cells, like yeasts, the beginning of autophagy induces the transport of cytoplasm into the vacuole, mediated by autophagosomes: double-membrane bounded vesicles. Autophagosome, enclosing a portion of cytosol, fuses its outer membrane with the vacuole, releasing the internal vesicle named the autophagic body into the lumen. This autophagic body is finally degraded by resident hydrolases (Takeshige et al., 1992). This process is essential for yeasts to withstand to adverse stressful conditions such as sparkling wines ageing. During this starvation situation, the dissolution of intracellular organelles occurs, leading to the release of hydrolytic enzymes from the vacuole into the cytoplasm. Many studies have been made in order to establish autophagy implications in the autolysis process and many advances have been made (Paragraph 3.3). The selection of secondary fermentation strains defective or over expressing autophagy may be an interesting tool to enhance sparkling wines quality.

2.2.2 Autolytic products and their effects on sparkling wines quality

Yeast autolysis in sparkling wine production has been the subject of many studies. In the research work carried out over the years, two approaches have been followed: the study of structural and ultrastructural changes of yeast cells during autolysis (Martínez-Rodríguez et al., 2001b) and the analysis of the different products released into the medium during the

process, following the change in nitrogen compounds, polysaccharides, glycoproteins, nucleic acids, lipids and other macromolecules (Martínez-Rodríguez and Polo, 2000; Martínez-Rodríguez et al., 2002; Nunez et al., 2005; Martínez-Rodríguez and Pueyo, 2009) (Table 1).

Table 1 *Yeast autolysis compounds affecting sparkling wines quality*

Autolysis compounds	**Origin**	**Main characteristics affected by autolysis compounds**	**Average amount (mg l^{-1})**
Nitrogen compounds: Proteins, Peptides, Amino acids	Yeast cell content	Organoleptic characteristics, Foam quality (Martínez-Rodriguez et al.,2003)	Proteins: 5-10 (Liger-Belair, 2005) Peptides (Ile–Arg, Arg–Ile, Ile–Val, Tyr–Lys) : < 10 (De Person et al., 2004) Amino acids: 0,8-2 (Liger-Belair, 2005)
Polysaccharides: Mannoproteins	Yeast cell wall, grapes	Mouthfeel, Yeast flocculation, Foam quality, Wine stability (Alexandre,and Guilloux-Benatier, 2006, Caridi, 2006)	≈ 200 (Liger-Belair, 2005)
Lipids and their derivatives: Fatty acids, Esters, Ketones, aldehydes	Yeast cell content	Foam quality, Flavour (Alexandre and Guilloux-Benatier, 2006)	≈ 10 (Liger-Belair, 2005)
Nucleic acids: 5'-UMP 5'-AMP 5'-GMP	Yeast cell content	Mouthfeel, Flavour (Charpentier et al., 2005)	5'-UMP ≈ 1.5 5'-AMP ≈ 0,2 5'-GMP ≈ 0,06 (Charpentier et al., 2005)
Volatile compounds: Esters, Terpenic alcohols, Higher alcohols, Aldehydes	Yeast Cell	Aromatic quality	Volatile organic compounds (VOC) ≈ 700 (Liger-Belair, 2005)

Among them, nitrogen compounds have been considered to be the best markers of yeast proteolytic activity (Fornairon-Bonnefond et al., 2002; Martínez-Rodríguez and Pueyo, 2009). Even if proteins are minor constituents of wine, they greatly contribute to the quality of the product, in fact, they are responsible for the ''body'' sensation, they can bind volatile compounds retaining wine aroma and also have a positive effect on foam stability (Brissonnet

and Maujean, 1991; Luguera et al., 1998). Proteins seem to be foam active substances because of their surface properties, they act as tensoactive compounds and enhance foam stability (García et al., 2009). During wine ageing on lees, proteins decrease is observed, because proteases activity and the resulting hydrolysis to lower molecular weight compounds. For this reason, sparkling wines present a lower proteins content then base wines from which they originate. Among the different types of proteases involved, protease A is the main enzyme responsible for this process. Recently, Alexandre et al. (2001), found that this enzyme was responsible for 60% of the nitrogen released during autolysis in wine, but they also found that the correlation between protease A, cell death and autolysis was unclear. As a consequence of proteins degradation, peptides concentration increase and then they are hydrolyzed to free amino acids.

During the ageing of sparkling wine over lees, Martínez-Rodríguez et al. (2002), established that the yeast strain used for refermentation influences the content of free amino acids and peptides. They also detected four main stages in the ageing of wines with yeasts: in the first one, amino acids and proteins diminish and peptides are released; in the second stage, there is a release of nitrogen compounds used as nutrients for the viable cells which in this stage coexist with dead cells. An intracellular protease activity is also detected and proteins are degraded to peptides which then are hydrolysed to amino acids. In the third stage, no viable cells are present and proteins and peptides are released due to the enzymatic activity still present in wine. The last phase occurs after about 270 days after *tirage* and authors found a decrease of amino acids in some of the wines studied.

Even if peptides and amino acids are generally considered as the most important compounds released during autolysis, yeasts can liberate amino acids into the wine even before the process begins. This is due to a cell response to the stress caused by starvation conditions, however, this phenomenon shouldn’t be mistaken for autolysis, which starts later (after three-

nine months). The beginning of autolysis mainly depends on both wine composition and yeast strain. It has been stated that during sparkling wines ageing, total amino acids increase before free amino acids content. This demonstrates that peptides are first released and then hydrolyzed to lower molecular weight compounds. Moreno-Arribas *et al.* (1996), studied nitrogen fractions evolution during ageing of a sparkling wine made through the traditional method. Between three and nine months after *tirage*, authors did not found differences in free amino acids concentration. After nine months of ageing instead, this concentration was increased, demonstrating the beginning of the autolysis process. By studying peptides amino acidic composition of sparkling wines, serine and threonine are the most abundant. This demonstrates that sparkling wines peptides are mainly derived from yeast autolysis, since these amino acids are involved in glycosidic linkages between cell wall proteins and mannans (Klis et al., 2002). Peptides derived from the autolysis process mainly originate from yeasts and their molecular weight tends to decrease during the ageing time. Sparkling wines enrichment in peptides may have a positive influence on its organoleptic characterstics. Peptides in fact are precursors of some aromatic molecules deriving from deamination and decarboxylation reactions (Feuillat and Charpentier, 1982). Even if peptides are considered to account for the majority of the compounds released during the autolysis process, they are less investigated than other nitrogen compounds, mainly because of the problems related to their isolation and characterisation in wines (Moreno-Arribas et al., 2002).

Moreno-Arribas et al. (2002) found that the final concentration of peptides in sparkling wines can be influenced by different factors such as temperature, ageing time, yeast strain etc. Pozo-Bayón et al. (2009), also reported that during the first stages of the autolysis process, hydrophobic high molecular weight peptides are released, these compounds are then hydrolysed thus producing hydrophobic low molecular weight compounds and free amino acids. Low molecular weight peptides have important functional properties in wines, such as

tensoactive, bioactive, antioxidant, antimicrobial and antihypertensive properties they are also a good source of nutrients for yeasts and bacteria, play a role in foam stability and are involved in sensorial properties (Pozo-Bayón et al., 2009; Desportes et al., 2000). Alcaide-Hidalgo et al. (2007), established that peptides released by *S. cerevisiae* EC1118 during autolysis in model wine could present a multifunctional activity.

As previously mentioned, during autolysis in sparkling wines, glucanases and proteases allow the release of polysaccharides, macromolecules mainly containing mannose (43%) and glucose (31%). During autolysis in fact, enzymatic activities make the cell wall less rigid and cause the breakdown of glucans and the release of cell wall mannoproteins (Babayan and Bezrukov, 1985; Feuillat, 1998; Feuillat, 2003; Leroy et al., 1990), which have been particularly studied in recent years for their interesting properties (Martínez-Rodríguez and Pueyo, 2009). Mannoproteins are glycoproteins located in the outermost layer of the yeast cell wall, where they are connected to a matrix of amorphous β-1,3-glucan by covalent bonds (Klis et al., 2002). When found in wine, they exist as polysaccharide and protein moieties. Polysaccharides are among the most studied autolytic compounds derived from yeasts. These compounds differenziate from grape polysaccharides because they are mainly composed by mannose and glucose, instead of arabinose (Martínez-Rodríguez and Polo, 2000). The mannose/glucose ratio increase during autolysis, as a consequence of mannoproteins derived from glucans degradation. The activity of proteases and glucanases during yeast lysis in fact, lead to the hydrolysis of glucans with the consequent increase of parietal mannoproteins in sparkling wines. Among the various wine glycoproteins, yeast mannoproteins have been widely investigated, because of their interesting properties. These compounds may have utility in bottle fermentation of sparkling wines (Feuillat, 2003), because they contribute to the flocculation of yeast strains (Caridi, 2006; Klis et al., 2002). Moreover, Nunez et al. (2006), found a relationship between mannoproteins and foaming properties of sparkling

wines. Mannoproteins also play an important role in controlling cell wall's porosity, thereby regulating leakage of proteins from the periplasmic space and entrance of macromolecules from the environment (Caridi, 2006). The increased porosity of the cell wall facilitates the release of autolytic products towards the extracellular environment (Charpentier and Feuillat, 1993). However, the production and release of mannoproteins into the wine depends both on the yeast strain (Pérez-Serradilla and Luque de Castro, 2008) and the autolysis process which is very slow and may take several months. It has been also observed that the concentration of polysaccharides containing mannose and glucose increases three to four times during sparkling wines ageing on lees. Charpentier (2000), detected an increase in polysaccharides concentration from 366 g l^{-1} in base wine to 602 g l^{-1} in the obtained sparkling wine, after nine months of contact with lees.

Also lipids are among the components released by yeasts during autolysis. Even if they are present in small amount, they can have an important role in the sensorial character of sparkling wines. In fact, the release of fatty acids could produce volatile components with low sensory thresholds, either directly or through derivatives such as esters, ketones and aldehydes (Charpentier and Feuillat, 1993; Martínez-Rodríguez and Pueyo, 2009). During ageing in contact with lees, indeed, lipids content increases and qualitative changes occur, depending on the ageing time. However, the results of studies on the interaction between lipids and foaming properties are contradictory. Gallart et al. (2002), reported a negative influence of fatty acids C8, C18 and C10 on foamability, whereas the ethyl esters of hexanoic, octanoic and decanoic acids were positively related to this parameter. Maujean et al. (1990), reported a negative effect on foam stability after adding octanoic and decanoic acid, instead, no effects were found on this parameter by Dussaud et al. (1994).

The concentration of nucleic acids is also modified during autolysis. In general, their content tend to decrease and the rate of this depletion depends on the yeast strain used during

secondary fermentation (Hernawan and Fleet, 1995). This probably explains results variability among different studies. In fact, some authors found a complete DNA degradation (Houg and Maddox, 1970), while Trevelyan (1978) and Zhao and Fleet (2003), detected no DNA reductions or just a partial decrease. Since the tendency to form DNA-proteins complexes is known, this may protect the nucleic acid from DNAase activity during autolysis (Hernawan and Fleet, 1995). However, DNA degradation depends on several enzymatic activities and lead to oligonucleotide, nucleotide and nucleoside degradation products.

Also RNA is degraded during autolysis, even if low fermentation temperatures and high ethanol concentrations (typical of sparkling wines), could slow down the process. Zhao and Fleet (2005) reported that RNA degradation plays a key role in yeast autolysis, this reaction leads to the release of 2', 3' and 5' ribonucleotides. Recently, Charpentier et al. (2005) recognised 5'-UMP (uridine-5'-monophosphate), 5'-GMP (guanosine 5′-monophosphate) and 5'-IMP (inosine-5'-monophosphate) in *Champagne* aged over lees. Even if these compounds are well recognised as flavor compounds, further studies are required to establish their impact on the sensorial properties of sparkling wines (Martínez- Rodríguez and Pueyo, 2009).

Finally, many volatile compounds are released or formed during autolysis, some of them with low sensory threshold. The most represented family is the esters one; when autolysis starts, short-chain (C_3-C_4) and medium-chain (C_6-C_{12}) acyl esters, with typical fruity aromas appear and subsequently diminish. Also long-chain acyl esters have been detected in sparkling wines. Many authors related the quality of sparkling wine to the concentration of these substances such as isoamyl-caproate, octyl-acetate, phenylethyl-acetate, ethyl-linoleate (Pueyo et al., 1995; Pozo-Bayón et al., 2003). Terpenic alcohols and higher alcohols are also formed during yeast autolysis, among these, geraniol, α-terpineol, citronellol and farnesol have been identified, their sensory threshold is between 100 e 300 μg l^{-1}. Moreover, a rapid formation of

isoamyl alcohol and 2-phenylethanol (rose scent) has been observed in model wine (Chung, 1986; Alexandre and Guilloux-Benatier, 2006).

Moreover, aldehydes have been detected, among them, 3-methylbutanal is the most abundant (40% of total), this compound derives from the oxidation of isoamyl alcohol. Some aldehydes have a negative influence on sparkling wine quality because of their grassy odour, but most of them fortunately disappear during ageing (Chung, 1986). Francioli et al., (2003), characterised some volatile compounds released during the autolysis process and which should be potentially useful as age markers. Vitispirane, TDN (1,1,6-trimethyl-1,2-dihydronaphthalene) and diethylsuccinate may help to discriminate between old and young sparkling wines.

2.2.3 Yeast lysis enhancement

During a prolonged ageing in contact with lees, yeasts autolysis takes place. This process is characterized by the hydrolysis of intracellular biopolymers by endogenous yeast enzymes, resulting in the release of several intracellular compounds like amino acids, peptides, proteins, polysaccharides, nucleic acid derivatives and lipids that have a positive effect on sparkling wine characteristics and affect its sensory quality (Martínez-Rodríguez et al., 2001b; Martínez-Rodríguez et al., 2001c; Alexandre and Guilloux-Benatier, 2006). However, yeasts autolysis is a slow process lasting from a few months to years, depending on various factors such as temperature, nutrient availability, grape variety, or the yeast strain used (Nunez et al., 2005). For years on end, two methods have been available to accelerate the autolytic process during sparkling wine production: by adding yeast autolysates to the wine and by increasing the ageing temperature (Charpentier and Feuillat 1993). However, both techniques caused organoleptic defects in the final product, often described as toasty. Over the years, many studies aimed at obtaining yeast autolysis improvement have been carried out in order to

avoid these drawbacks. Many researchers addressed their studies toward the selection of autolytic yeast strains for secondary in-bottle fermentations (Gonzalez et al. 2003, Martínez-Rodríguez et al. 2001a, Todd et al. 2000). With the aim to improve sparkling wines quality, Tini et al., (1995) used autolytic strains derived through meiosis from an industrial second fermentation yeast. Tabera et al. (2006), applied the deletion of the BCY1 gene trying to obtain autolytic yeast strains to accelerate sparkling wines ageing. As a means of speeding up the onset of autolysis, Todd et al. (2000), suggested to use interactions between two killer strains of *S. cerevisiae* and two sensitive strains of *S. cerevisiae*. In their study, authors showed that it is possible to exploit the death of the sensitive strain for the purpose of promoting yeast autolysis. In order to obtain secondary fermentation strains with improved autolytic capacity, Gonzalez et al. (2003), used a selection method based on a temperature-sensitive autolytic phenotype. They gain a second fermentation *S. cerevisiae* yeast strain by UV mutagenesis, the mutations affected cell morphology, growth kinetics, sporulation and the release of nitrogenous compounds in an accelerated autolysis experimental model. UV mutagenesis can also be used to obtain thermosensitive autolytic mutants altered in cell wall integrity that increases the release of cell wall polysaccharides during alcoholic fermentation (Giovani and Rosi, 2007). Martínez-Rodríguez et al. (2004), also used autolytic wine yeasts obtained by UV mutagenesis, and reported the occurrence of morphological changes and the presence of autophagic bodies. They found a relationship between autophagy and autolysis for wine yeasts that may be an important tool to set strategies aimed at the genetic improvement of wine yeasts. Delving into selection of yeast strain defective in autophagy or overexpressing this characteristic may contribute to increase the wide set of selected yeast available for secondary fermentation processes (Torresi et al., 2011).

Moreover, since mannoproteins are among the major compounds released by yeasts during autolysis, and their positive effect on sparkling wines quality has been widely described (Klis

et al., 2002; Feuillat, 2003; Caridi, 2006), searching for strains that could release great quantity of these compounds is today an interesting strategy to improve sparkling wine's quality. For example, *S. cerevisiae* EC1118, a widely used secondary fermentation strain, has been genetically modified in order to obtain a strain which overproduces mannoproteins (Gonzalez-Ramos et al., 2008).

Moreover, in recent years, the use of enzymatic treatments to enhance yeast lysis has been studied in wine (Paragraph 4.2). These enzymatic preparations mainly contain β–glucanases, able to hydrolyze yeast cell wall glucans. Enzymes activities allow to facilitate the release of parietal and cytoplasmic compounds with proved effects on sparkling wines characteristics.

2.3 Yeast lysis enhancement by β–glucanase oenological preparations

2.3.1 Enzymes in winemaking

Winemaking is a biotechnological process where enzymes play a fundamental role and the use of exogenous enzymatic preparations may contribute to overcome problems related to the insufficient activity of endogenous enzymes (Colagrande et al.,1994). From pre-fermentation, to fermentation, post-fermentation and ageing, enzymes catalyze various biotransformation reactions. Most of these biological catalysts originate from the grape indigenous microflora or from yeasts and bacteria present during winemaking (Humbert-Goffard et al., 2004). Many sensorially-active constituents of wine are affected, at different stages of winemaking, by biochemical transformations catalysed by specific enzymes. In addition, their participation in juice clarification, colour extraction and protein stabilization is fundamental for the efficiency of these technological steps during winemaking (Ugliano 2009).

Numerous biochemical transformations, such as grape phenolics oxidation, volatile compounds formation, wine clarification, colour extraction and protein stabilisation are catalysed by different enzymes from different sources. Grape berries and wine yeasts are the major sources of enzymes; however, typical winemaking conditions such as high sugars and ethanol concentration, low pH and high polyphenols content can potentially inhibit their activity. Because of this, endogenous enzymes of grape, yeasts and other microorganisms usually present in must and wine are often unable to sufficiently catalyse the different bio-transformations that occur during winemaking. For these reasons, reactions are often incomplete and a significant portion of substrate remains untransformed. Since many of these reactions are beneficial for wine quality, enzymatic activities may be enhanced by the use of exogenous enzymatic preparations exhibiting higher efficacy under winemaking conditions. Their use has been introduced in order to obtain the desired level of substrate transformation (Ugliano, 2009). In the 70's, the first enzymatic preparation was marketed in order to facilitate the hydrolysis of must pectic substances. At that time, enzymatic treatments were mainly used in order to improve must clarification and grape pressing. Since the 80's, enzymes have been applied also for colour extraction, aromas release and filterability enhancement.

Enzymatic preparations for oenological applications are commonly obtained by fermentation of selected culture of fungi like *Aspergillus niger* or *Trichoderma harzianum*, by extraction from bacterial cultures such as *Lactobacillus fermentum* or from egg white (lysozyme). Microorganisms are cultured on different substrates (potatoes starch, soy flours etc.). After fermentation, recovery and purification are carried out and finally, the preparation is standardized in order to maintain its characteristics unaltered over the years. Enzymatic preparations are generally composed of a mix of different enzymes like pectin-lyases, pectinesterases, polygalacturonases and cellulases. Otherwise, it is possible to obtain a pure

culture, but genetically modified microorganisms are needed. Nonetheless, the product must be as pure as possible, in order to ensure the effectiveness of the treatment.

Enzymatic preparations manufacturers operate within the laws in force in each country in order to avoid risks for the consumers and safeguard their healthcare. All the commercial enzymatic preparations used in winemaking must comply with the specifications recommended by Joint FAO/WHO (Food and Agriculture Organization/World Health Organization) Expert Committee on Food Additives (JECFA) and by Food Chemicals Codex (FCC) for food enzymes. The method applied to produce enzymatic preparations for wines produced in the European Union is also regulated by the *Organization Internationale de la Vigne et du Vin, OIV* (Resolution Oeno 14/2003, amended by Resolution Oeno 365/2009), (Whitehurst and Van Oort 2009). Producers who use these enzymatic preparations during winemaking and who export wines to the EU, are obliged to use enzymes that comply with these prerequisites (Lourens and Pellerin 2004). The EC Regulation 1332/2008 (partially amended by EC Regulation 1130/2011), provides the rules for the use of enzymatic preparations in food industry. For oenological applications, the EC Regulations 1493/1999, 423/2008 and 606/2009 establish rules for the correct use of enzymes in winemaking.

Exogenous enzymes in winemaking are useful to improve clarification and the release of varietal compounds, to reduce the formation of ethyl carbamate and to lower alcohol levels (Van Rensburg and Pretorius 2000). Another important application concerns the improvement of the yeast lysis process by breaking the glucan and chitine fibers of the cell wall, this operation may contributes to enhance the release of intracellular and parietal compounds from yeast cells thus improving the quality of wines and sparkling wines aged on lees. β-glucanases are among the different enzymatic preparation currently available and suitable for sparkling aged over lees (Paragraph 4.2).

2.3.2 β-glucanase oenological preparations

As previously described, ageing over lees is a very important step in sparkling wines production by the traditional method, however, the occurrence of the autolysis process may takes from a few months to years, depending on various factors such as temperature, nutrient availability, grape variety or the yeast strain used (Nunez et al., 2005). Over the years, the winemaking industry has developed specific commercial enzyme preparations with the aim of accelerating autolysis in wines aged over lees. Since exogenous β-glucanases may represent an important tool to accelerate and intensify cell lysis, these products are designed as a mix of several enzymes like β-glucanases and pectinases that considerably increase the polysaccharide concentration of both white and red wines (Pellerin and Tessarolo 2001, Trione and Martínez 2001). Traditionally, β-glucanases have been used to improve the filtration of wines obtained from grapes infected with the fungus *Botrytis cinerea*. Glucans secreted by *Botrytis* into the infected juice can cause blockages during filtration.

The use of β-glucanases to enhance yeast lysis instead, is a more recent application (Resolution oeno 18/2004) and may contributes to enhance the release of intracellular and parietal compounds from yeast cells thus improving the quality of wines and sparkling wines aged on lees.

As previously reported, in *Saccharomyces cerevisiae*, the cell wall makes up 15 to 30% of the dry weight of the cell (Orlean 1997). The yeast cell wall is mainly composed of mannoproteins and fibrous β-1,3 glucan, branched β-1,6 glucan is also present and links other components of the wall. A significant minor component is chitin, which contributes to the insolubility of the fibers. The β-1,3 glucan-chitin complex is the major constituent of the inner wall, while β-1,6 glucan connects the components of the inner and outer layer of the wall. O and N glycosylated mannoproteins are also located on the outer surface of the wall. They are densely packed and regulate the wall permeability to solutes (Lipke and Ovalle 1998).

The commercial β-glucanase enzymes authorised for winemaking are synthesised by species of *Trichoderma* (e.g. *T. harzianum*), Enzyme Commission number 3.2.1.58 (Resolution oeno 27/2004, OIV), growing under conditions that optimise their production (Humbert-Goffard et al., 2004). The activity of these enzymes determines the hydrolysis and solubility of complex compounds, without involving the cell wall directly (Palomero et al., 2007). As a consequence of this degradation, the cell wall becomes less rigid and polysaccharides are released (Feuillat, 1998). Enzymatic activity causes the breakdown of glucans and the release of cell wall mannoproteins (Babayan and Bezrukov, 1985; Leroy, et al., 1990), showing interesting properties such as the contribution to yeast strains flocculation, the control of cell wall porosity, thereby regulating leakage of proteins from the periplasmic space and entrance of macromolecules from the environment (Klis et al., 2002; Feuillat, 2003; Caridi, 2006). As main consequence of this enzymatic activity, the increased cell wall porosity makes easy the release of autolytic products to the extracellular environment (Charpentier et al., 1993). Since the production and release of mannoproteins depends on the yeast strain (Pérez-Serradilla and Luque de Castro, 2008) and also on the autolysis process, the addition of exogenous β-1,3-glucanases to wines stored on lees, may be a valid tool to reduce sparkling wines production times and costs.

In particular, the addition of exogenous β-1,3-glucanases in wine aging over lees has been recently studied by Palomero et al. (2007). They used the enzymatic preparation Lallzyme MMX® (Lallemand Inc., Canada) and studied polysaccharides release in a model wine system. The addition of β-1,3-glucanases to the medium notably accelerated the process of autolysis, thus facilitating the release of polysaccharides characterized by uniform-in-size fragments smaller than those obtained with conventional ageing over lees. In particular, the addition of exogenous β-1,3-glucanases highly reduces the time required for the autolysis process and enhances the release of polysaccharides, mannoproteins and intracellular

compounds that have a positive effect on wine quality. Even if the enzymatic activities have been the subject of several studies in wines, their implications in the autolysis process have been less investigated.

3 AIMS AND SCOPE

Although the production of sparkling wines is lower compared to that of still wines, the economical impact of this product is very important because of its high added value. Because of this, over the years, many attempts to simplify the production process, reducing time and costs and enhance the quality of this special wine have been done. Among the various factors influencing sparkling wine characteristics, autolysis is one of the most studied. Through the years, the use of exogenous enzymatic preparation to accelerate this slow process has been developed. Many studies are available about the use of these enzymatic preparations for yeast autolysis enhancement in red and white wines. However, to our knowledge, there are no research works about their application for sparkling wines production by the traditional method, moreover, any preliminary study of these oenological preparations in synthetic buffers is still available. The present work focuses on the preliminary study of Lallzyme MMX®, an enzymatic preparation containing β-glucanase activity, and its further application in sparkling wines production by the traditional method. Enzyme activity was studied in different buffers and in a model wine solution. pH and ethanol content effects, i.e the main wine characteristics possibly affecting enzyme activity, were also considered. In addition, the interactions between the enzyme and two different yeast strains were verified. Finally, the analysis of enzyme effects on yeasts morphology, foaming properties, chemical composition changing and sensory quality implications on the obtained sparkling wines was conducted.

4 MATERIALS AND METHODS

4.1 Preliminary characterization of Lallzyme MMX® oenological preparation

4.1.1 Yeast stock solutions

Two different yeast strains: *Saccharomyces cerevisiae bayanus* BCS103® (Fermentis, division of S.I. Lesaffre, France) and *Saccharomyces cerevisiae bayanus* Lalvin EC1118® (Lallemand Inc., Canada) were used.

S. cerevisiae bayanus BCS103® was a strain selected in the *Champagne* region on *Chardonnay* for its excellent fermentation characteristics and its great resistance to extreme wine making conditions (information provided by the manufacturer, Technical Data Sheet OE_L_EN_BC S103 Rev: OCT2009). *S. cerevisiae bayanus* EC1118® presents a strong competitive character, the ability to ferment at low temperature, a good flocculation capacity and an excellent alcohol tolerance (information provided by the manufacturer, Technical Data Sheet Lalvin). A fresh stock solution of each yeast at 1 mg ml^{-1} was daily prepared in deionized water.

4.1.2 Enzyme stock solution

The commercial enzymatic preparation used for all experiments was Lallzyme MMX® (Lallemand Inc., Canada). This preparation is commercially used to improve yeast autolysis of wines on lees and contains β-glucanase, poly-galacturonase and pectinase sourced from *Trichoderma spp.* and *Aspergillus niger* in the following amounts: 90 glucanase U g^{-1} (International Unit; μmol min^{-1} of substrate), 1840 poly-galacturonase U g^{-1}, 545 pectin-esterase U g^{-1} and 25 pectin-lyase U g^{-1} (Informations provided by the manufacturer)

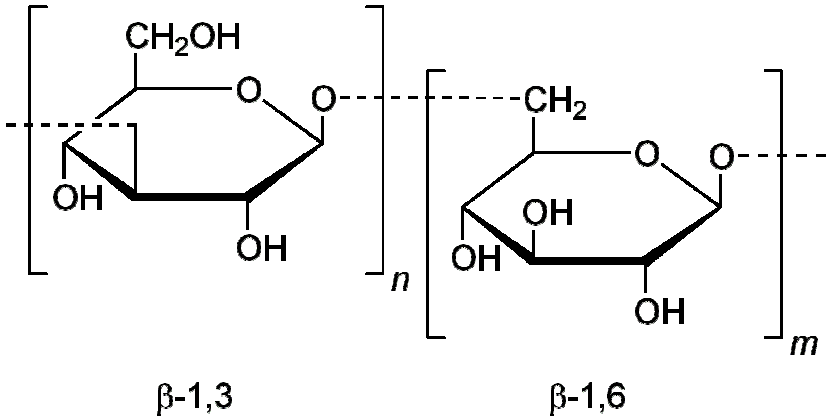
(Palomero et al. 2009). A stock solution of enzyme at 1 mg ml^{-1} was daily prepared in deionized water.

4.1.3 β-glucanase substrate stock solution

The selected substrate for enzymatic activity determinations was Laminarin (Humbert-Goffard et al. 2004, Vázquez-Garcidueñas et al. 1998). It is a linear glucose polysaccharide made up of β(1→3)-glucan with β(1→6)-linkages, with a β(1→3):β(1→6) ratio of 3:1, sourced from the brown alga *Laminaria digitata* (Sigma-Aldrich, Missouri, USA) (Figure 6). A solution of Laminarin at 1 mg ml^{-1} was freshly prepared in deionized water.

All chemicals came from Sigma-Aldrich, Missouri, USA and were reagent grade.

Figure 6 *Laminarin*



4.1.4 Enzymatic activity determination

The enzyme solution for all the experiments was at 0,01 g l^{-1} in water, this concentration is, generally, the same used by winemakers. Since the Laminarin substrate is a polysaccharide having a variable molecular weight, the enzymatic activity was expressed as the amount of

enzyme that release 1 mg l^{-1} min^{-1} of glucose instead of International Unit (μmol min^{-1} of Laminarin) and substrate concentration as mg ml^{-1} instead of mM.

The enzymatic assays were performed incubating at room temperature the substrate Laminarin (1 mg ml^{-1}) and the enzyme at different pH values. Blank assays were performed to take in account the residual glucose content of Laminarin, yeasts and enzyme.

After 45 minutes of incubation, the reaction was stopped by heat inactivation (100°C, 2 minutes), (Humbert-Goffard et al. 2004) and the glucose released was determined after centrifugation (14000 rpm for 3 minutes) through the enzymatic test-combination D glucose/D-fructose kit (R-Biopharm-Germany), following the manufacturer's instructions.

The test is based on the following reactions:

1) D-Glucose + ATP $\xrightarrow{HK}$ G-6-P + ADP

2) G-6-P + $NADP^+$ $\xrightarrow{G\text{-}6\text{-}P\text{-}DH}$ D-gluconate-6-phosphate + NADPH + H^+

The amount of NADPH (Nicotinamide adenine dinucleotide phosphate) formed during the reaction is stoichiometric to the amount of D-glucose. NAPDH is measured by absorbance increase at 340 nm by using a Perkin-Elmer Lambda 25 UV/VIS spectrophotometer (Beaconsfield Buks B).

Enzyme activity (U ml^{-1}) was then determined as described in the manufacturer's instructions adequately modified as follows:

U ml^{-1} = [ΔA / incubation time (min) x 180,16 (g mol^{-1}) x Final volume (ml)] / [6,3 (l $mmol^{-1}$ cm^{-1}) x Sample Volume (ml)]

Were:

- ΔA was obtained by subtracting from each sample the absorbance of the blank assays.
- 180,16 (g mol^{-1}) is the D-glucose molecular weight

- 6,3 (l $mmol^{-1}$ cm^{-1}) is the extinction coefficient of NADPH at 340nm

Standard errors were reported as bars in figures or as SD in tables.

4.1.5 Effect of pH on enzyme activity

The effect of different pH (from 2.60 to 7.00) on the β-glucanase activity of Lallzyme MMX® with and without yeast cells (EC1118 or BCS103 strains) was determined in McIlvaine buffer (McIlvaine, 1921). This buffer solution was employed for all the experiments, because of its capacity to cover a range of pH from 2.2 to 8.0, thus avoiding drawbacks caused by the use of different buffer solutions. Samples were prepared in triplicate in McIlvaine buffer at different pH (2.60, 3.20, 4.00, 5.00, 6.00 and 7.00). Laminarin and enzyme were incubated both in absence (E) and in presence of EC1118 (T^{EC}) or BCS103 (T^{BCS}) strains, as reported in Table 2. Both yeasts were also incubated with substrate, without enzyme, to detect their own possible β-glucanasic activity (L^{EC} and L^{BCS}). Blank assays counting the residual glucose content of Laminarin (S), yeasts (Y^{EC} and Y^{BCS}) and enzyme (β), were also performed (Table 2). Enzyme activity was determined as above described.

Table 2 *Samples outline for the β-glucanase activity determination*

	T^{EC}	T^{BCS}	E	L^{EC}	L^{BCS}	S	Y^{EC}	Y^{BCS}	β
Buffer, ml	5,92	5,92	5,92	5,92	5,92	5,92	5,92	5,92	5,92
EC1118 (1 mg ml^{-1}), ml	1,60	---	---	1,60	---	---	1,60	---	---
BCS103 (1 mg ml^{-1}), ml	---	1,60	---	---	1,60	---	---	1,60	---
Laminarin (1 mg ml^{-1}), ml	0,40	0,40	0,40	0,40	0,40	0,40	---	---	---
LallzymeMMX® (1 mg ml^{-1}), ml	0,08	0,08	0,08	---	---	---	---	---	0,08
Deionized water, ml	---	---	1,60	0,08	0,08	1,68	0,48	0,48	2,00

4.1.6 Effect of ethanol content on enzyme activity

The ethanol influence on enzyme activity was analysed for the enzymatic preparation both in presence and in absence of yeasts (BCS103 and EC1118).

Two different ethanol concentrations were selected for the experiment, 11,3 % v/v and 12,7 % v/v, corresponding to those of a base wine and a sparkling wine respectively, which will be further tested in experimental applications.

Samples were prepared as above described in Table 2, by just replacing the McIlvaine buffer with the Tartaric one (0,1M at wine pH, 3.20) which contained the suitable amounts of ethanol. As control, an experiment without ethanol was also performed. Enzyme activity was determined as above described.

4.1.7 Enzyme kinetics and kinetic parameters determination

Enzyme kinetics were performed in McIlvaine buffer at the optimum pH value, obtained from the activity *vs.* pH curves. Lallzyme MMX® solution (1 mg ml^{-1}, 0,08 ml) and different amounts (from 0 to 1 ml) of Laminarin 1 mg ml^{-1} were employed. Similar experiments were prepared in presence of the two selected yeast strains. All samples were prepared following the same outline reported in Table 2. Enzyme activity was determined as above described.

Kinetic parameters were determined by the non linear regression analysis of the Michaelis-Menten kinetic equation using the software GraphPad Prism 4®. The following kinetic parameters were estimated:

V_{max}: Corresponding to the maximum velocity at which an enzyme catalyzes a reaction, expressed as mg l^{-1} min^{-1} of glucose.

K_M: Representing the amount of substrate corresponding to ½ V_{max}. This parameter reflects the efficiency of the enzyme-substrate complex formation.

A.S. (Specific Activity): Is the enzymatic activity per milligram of total proteins (expressed as $mg_{glucose}\ l^{-1}\ min^{-1}\ mg_{proteins}^{-1}$). Specific activity gives a measurement of the purity of the enzyme.

4.1.8 Total proteins content determination

Total proteins content was determined in the Lallzyme MMX® enzymatic preparation by the Bradford dye-binding assay (Bradford 1976). BSA (Bovine Serum Albumin) 2 mg ml^{-1} was used as standard to make the calibration curve (from 0 to 2 mg ml^{-1}) in Tartaric buffer at pH 3.20. 100 µl of Lallzyme MMX® solution (1 mg ml^{-1}) was introduced into a 4 ml Poly(methyl methacrylate) (PMMA) couvette, then 3 ml of Bradford reagent were added. After 45 minutes of incubation, the absorbance was measured at 595 nm for both calibration curve points and samples. Total proteins content was determined by comparing the absorbances of the calibration curve with those of the samples.

The Lallzyme MMX® preparation used for all experiments contained 1,5% of total proteins and a Specific Activity of 0,107 $mg_{glucose}\ l^{-1}\ min^{-1}\ mg_{proteins}^{-1}$.

4.2 Sparkling wines production by the traditional method

Sparkling wines were obtained with a base wine produced with Roscetto grapes. Roscetto is a Latium autochthonous white grape variety belonging to Verdicchio's family and very widespread in Montefiascone's territory, near Viterbo (Italy). Base wine was produced following the classic white wine technology and sparkling wines were manufactured using the traditional method. Base wine and sparkling wines production process was performed in the Falesco winery in Montecchio (Terni province). Yeast strains EC1118 and BCS103 were used

to prepare a *pied de cuve* and then to inoculate the base wine. 80 bottles were prepared with yeasts alone (40 for each strain), and 80 with Lallzyme MMX® addition (40 for each strain). Bottles were then stacked into caged bins, a very space-saving system (Figure 7a). The bins were stored in a special place furnished for the settling of the sparkle. The bottle storage area was cool and had minimum temperature fluctuations and minimum lighting. Secondary fermentation and ageing with yeasts were carried out at 14°C. CO_2 pressure increase was monitored by aphrometers (Figure 7b), devices suitably designed to measure the pressure within a bottle of sparkling wine. Three bottles for each experiment (both in absence and in presence of Lallzyme MMX®) were fitted by aphrometers. Samples were taken at 0, 4, 8 and 12 months after *tirage* and all the analyses were performed in triplicate on vacuum degassed wine obtained by mixing the content of 2 bottles (Figure 8).

Figure 7 *Sparkling wine bottles placed into caged bins (a), Aphrometer on a sparkling wine bottle (b)*

Figure 8 *Sparkling wine degassing by vacuum pump*

4.3 Direct evaluation of yeast lysis

4.3.1 Scanning Electron Microscopy and Transmission Electron Microscopy analyses

A direct estimation of yeast lysis was obtained on sparkling wine samples by SEM (Scanning Electron Microscopy) and TEM (Transmission Electron Microscopy) observations of yeast lees performed by the C.I.M.E. (Interdepartmental Centre of Electron Microscopy, University of Tuscia, Viterbo. Italy), at time 0 (immediately after the inoculum) and in sparkling wines at 4, 8 and 12 months after *tirage*. Vacuum degassed samples were centrifuged at 10.000 rpm for 10 minutes. The pellet was collected and immediately prepared for SEM and TEM observations. The method of Wright (2000) was used for samples preparation.

4.4 Indirect evaluation of yeast lysis

4.4.1 Free amino acids analysis

Free amino acids analysis was performed on base wine and on sparkling wine samples at 4, 8 and 12 months after *tirage* by the Laboratory of Proteomics and Mass Spectrometry of the University of Tuscia (Viterbo, Italy). Samples preparation was performed using extra sep C18 columns (Lida Manufacturing Corp., 25th Avenue, Kenosha, WI 53143-6615, USA) for solid phase extraction, the obtained samples were dehydrated and then resuspended into solvent A (0.1% formic acid in water). An Ultimate 3000 rapid resolution fast HPLC system (LC Packings, DIONEX, Sunnyvale, USA) was used to perform metabolite separation. The system featured a binary pump and vacuum degasser, well-plate autosampler with a six-port micro-switching valve and a thermostated column compartment. A Dionex Acclaim RSLC 120 C18 column 2.1mm×150mm, 2.2 μm was used to separate the extracted metabolites.

LC parameters: injection volume, 20 µl; column temperature, 30 °C; flow rate, 0.2 ml min^{-1}. The LC solvent gradient and timetable were identical during the whole period of the analysis. A 0-95% linear gradient of solvent A (0.1% formic acid in water) to B (0.1% formic acid in acetonitrile) was employed over 15 minutes, followed by a solvent B hold of 2 minutes, returning to 100% A in 2 minutes and a 6-minutes post-time solvent A hold. Metabolites were directly eluted into a High Capacity ion Trap HCTplus (Bruker-Daltonik, Bremen, Germany). Mass spectra for metabolite extracted samples were acquired in negative ion mode. ESI capillary voltage was set at 3000V (-) ion mode. The liquid nebulizer was set to 30 psig and the nitrogen drying gas was set to a flow rate of 9 l min^{-1}.

4.4.2 Total proteins content determination

Total proteins content was determined on base wine and on sparkling wine samples at 4, 8 and 12 months after *tirage* by the Bradford method (Bradford, 1976) using Bovine Serum Albumine (BSA) 2 mg ml^{-1} as standard to make the calibration curve. 100 µl of vacuum degassed sparkling wine was introduced into a 4 ml Poly(methyl methacrylate) (PMMA) couvette, then 3 ml of Bradford reagent were added. After 45 minutes of incubation, the absorbance was measured at 595 nm for both calibration curve's points and samples. Total proteins content was determined by comparing the absorbances of the calibration curve with those of the samples.

4.4.3 Foaming properties

Foaming properties were evaluated both in base wines and in sparkling wine samples at 4, 8 and 12 months after *tirage* in order to study the effect of β-glucanase addition and the differences between the selected yeast strains. Foam characteristics were determined

following the method of Pueyo et al. (1995) suitably modified, by measuring foam height (cm) and foam stability time (s).

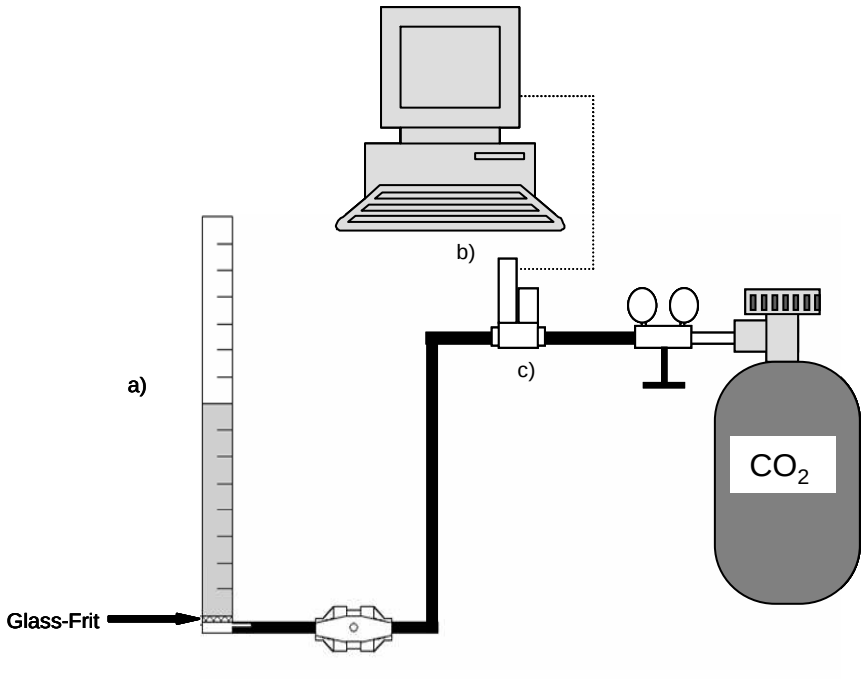
The equipment was composed by a graduated glass cylinder (530x15mm) (Steroglass S.r.l., San Martino in Campo, Perugia), with a glass frit in the bottom of the column (pore size 10μm). The column was attached to the mass flow meter and controller EL-Flow select, model F201CV-200-AGD-33-Z-MFC (Bronkhorst hi-tech; Ruurlo, Netherlands) connected to a CO_2 fitting. Instrument control and CO_2 flux setting was obtained by the Software Flow-Bus vs. 4.6® provided by Bronkhorst hi-tech (Figure 9).

Before each measurement, the glass cylinder was accurately washed three times with 10 ml of 95% ethanol, three times with 10 ml of Milli-Q water and finally with 50 ml of sample. Into the glass cylinder, 10 ml of degassed sparkling wine was introduced and then a 75 ml min^{-1} of CO_2 flux was introduced into the column through the glass frit. Two parameters were measured:

§ Foam height, i.e. the maximum height (cm) reached by the foam column into the glass cylinder after CO_2 injection and reflecting wine capacity to form foam.

§ Foam stability time, i.e. the time (seconds) elapsed before all the bubbles disappear after CO_2 flux interruption.

Figure 9 *Equipment for foaming properties analysis. a) Graduated glass cylinder; b) Personal Computer; c) Gas flow meter and controller*



4.4.4 Sensory analysis

A panel of 10 judges with experience in tasting sparkling wines conducted sensory analysis of samples 12 months after *tirage* using the tasting card proposed by Hardy (1986, 1987a, 1987b, 1988), suitably modified. Statistical analysis of the obtained data was performed by the non-parametric Quade test (Ubigli, 2009).

4.5 Statistical analyses

For the preliminary characterization of Lallzyme MMX® preparation, kinetic parameters standard errors were determined by the non linear regression analysis of the Michaelis-Menten kinetic equation using the software GraphPad Prism 4®. Standard errors were reported as bars in figures or as SD in tables.

For sparkling wines instead, a PCA (Principal Component Analysis) bi-plot was performed to determine samples distribution using the software The Unscrambler®, considering free amino acids and total proteins as variables.

For sensory analysis, statistical analysis of the obtained data was performed by the non-parametric Quade test (Ubigli, 2009).

5 RESULTS AND DISCUSSION

5.1 Preliminary characterization of Lallzyme MMX® oenological preparation

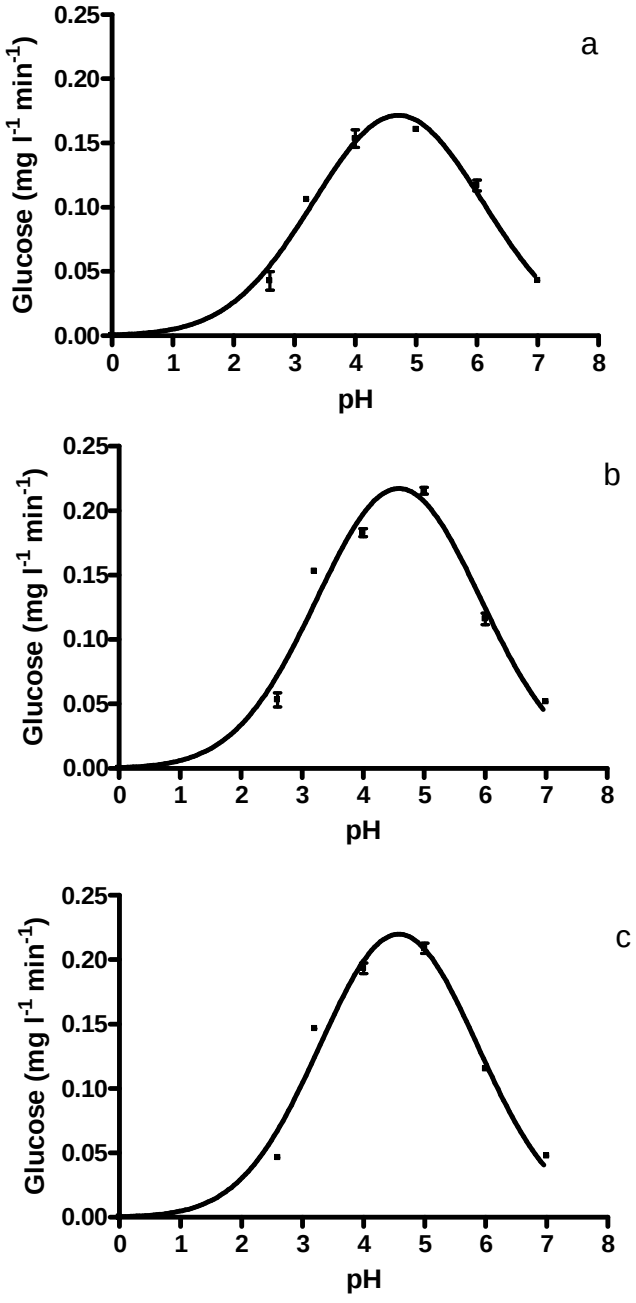
5.1.1 Effect of pH on enzyme activity

In order to promote wine enrichment in yeast polysaccharides, it is possible to use specific enzymatic preparations mainly containing pectolitic and β-glucanasic activities. Pectinases partially hydrolyse grape polysaccharides, while β-glucanases cause the hydrolyses of both *Botrytis cinerea* and yeast cell wall polysaccharides. The hydrolysis of yeast polysaccharides gives rise to the release of parietal macromolecules such as mannoproteins. β-glucanase is a natural enzyme also produced by different yeast strains, such as *Saccharomyces cerevisiae*, the activity of this enzyme varies during the life cycle of cells, but it has been detected during yeast autolysis. For this reason, the addition of β-glucanases to the wine just intensifies a pre-existing enzymatic activity, responsible of yeast autolysis, a slow process that may take several months to occur. The addition of exogenous β-1,3-glucanases reduces the time required for the autolysis process and enhances the release of polysaccharides, mannoproteins and intracellular compounds that have a positive effect on wine quality.

The effect of the addition of these enzymatic preparations on wine quality has been scarcely investigated; moreover, a preliminary study of the enzymatic preparation Lallzyme MMX® and its effect on different secondary fermentation yeast strains is not currently available. Considering its further applications in sparkling wine production, in this study, pH influence on β-glucanase activity was evaluated and the lytic activity at wine pH of two yeast strains was also determined. In Table 3, β-glucanase activity vs pH is reported for Lallzyme MMX®, both in absence (E) and in presence of EC1118 (T^{EC}) and BCS103 (T^{BCS}) yeast strains. The

optimum pH value is 5.00, both in absence (Figure 10a) and in presence (Figure 10b, 10c) of yeasts.

Figure 10 *β-glucanase activity (mg l^{-1} min^{-1} of glucose) vs. pH determined on E: Lallzyme MMX® alone (a); T^{EC}: in presence of EC1118 yeast strain (b) and T^{BCS}: in presence of BCS103 yeast strain (c)*



In addition, a higher activity was detected at all pH values in presence of yeast strains; in particular an increase of 34 and 30% for T^{EC} and T^{BCS} respectively was detected at the optimum pH (Table 3).

Table 3 *Activity (mg l^{-1} min^{-1} of glucose) at different pH of β-glucanase in presence and in absence of yeast strains*

pH	[a]E	[b]T^{EC}	[c]T^{BCS}	Relative activity (%)				
				[d]E	[d]T^{EC}	[d]T^{BCS}	[e](T^{EC} *vs* E)	[f](T^{BCS} *vs* E)
2.6	0,043 ± 0,007	0,053 ± 0,005	0,046 ± 0,002	27	25	21	123	108
3.2	0,106 ± 0,002	0,153 ± 0,002	0,147 ± 0,001	66	71	70	144	138
4.0	0,153 ± 0,007	0,183 ± 0,003	0,193 ± 0,004	96	85	93	120	126
5.0	0,1605 ± 0,0002	0,215 ± 0,003	0,209 ± 0,004	100	100	100	134	130
6.0	0,117 ± 0,004	0,116 ± 0,004	0,1151 ± 0,0003	73	54	55	99	98
7.0	0,043 ± 0,001	0,051 ± 0,002	0,048 ± 0,002	27	24	23	119	111

[a] enzymatic preparation alone
[b] enzymatic preparation plus EC1118 yeast strain
[c] enzymatic preparation plus BCS103 yeast strain
[d] Relative activity of E, T^{EC} and T^{BCS} samples compared to those at the optimum pH value
[e] Relative activity of T^{EC} sample compared to E sample
[f] Relative activity of T^{BCS} sample compared to E sample

At wine pH (3.20) the relative activity respect to the optimum was 66, 71 and 70% for E, T^{EC} and T^{BCS} respectively, indicating that even if wine pH is not the optimum value for β-glucanase, the residual activity was useful for the user's purposes. Moreover, the enhancement of activity due to yeasts presence was about 10% higher at wine pH than at the optimum (Table 3). These results show the effectiveness of the enzymatic preparation in yeast lysis enhancement, in particular for EC1118 strain, that leads to a consistent release of the yeast intracellular enzyme or a more efficient cell lysis, slightly evident at pH 3.20 than 5.00. No activity was detected in the control samples containing yeasts and substrate without

Lallzyme MMX® (Y^{EC} and Y^{BCS}), thus demonstrating that the increase of the activity noticed is due to the Lallzyme MMX® β-glucanase hydrolysis of yeast cell wall.

5.1.2 Enzyme kinetics

The data obtained up till now showed that β-glucanase is able to hydrolyse the cell wall of both yeast strains tested, and is particularly active on yeast EC1118. In order to evaluate at best the efficacy of the enzyme preparation is useful to determine kinetic parameters both in presence and in absence of yeast strains. The kinetic curves obtained always showed an hyperbolic trend, for this reason the elaboration was performed using the Michaelis-Menten equation. No enzymatic activity was detected in samples containing yeasts and substrate, without enzyme (Y^{EC} and Y^{BCS}).

The activity rate was higher for samples with yeasts at all substrate concentrations (Figures 11, 12). V_{max} values demonstrated an increase in product release rate in presence of yeasts, as indicated by the V_{max} (Table 4), even if no differences were detected between T^{EC} and T^{BCS}. The efficacy of EC1118 strain was indicated by k_M value (Table 4), which was significantly lower than BCS103 one and similar to those of the enzyme alone (E), indicating a better enzyme-substrate complex formation.

Figure 11 *β-glucanase activity vs. Substrate concentration of E, Lallzyme MMX® alone (▲) and T^{EC}, in presence of EC1118 yeast (■) in McIlvaine buffer, pH 5.00*

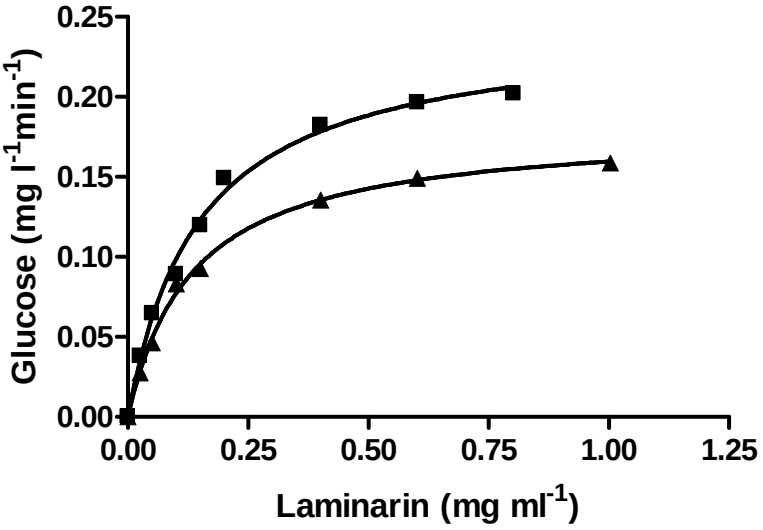


Figure 12 *β-glucanase activity vs. Substrate concentration of E, Lallzyme MMX® alone (▲) and T^{BCS}, in presence of BCS103 yeast (■) in McIlvaine buffer, pH 5.00*

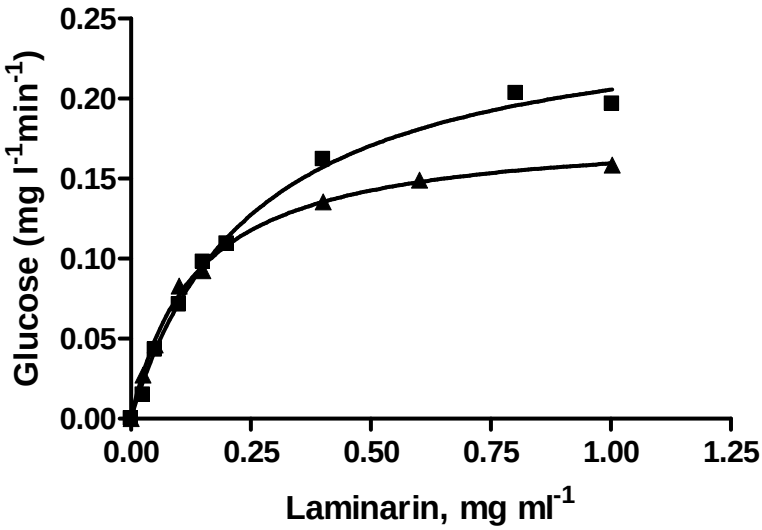


Table 4 *Kinetic parameters determined on E: Lallzyme MMX® alone; T^{EC}: in presence of EC1118 yeast strain and T^{BCS}: in presence of BCS103 yeast strain*

Kinetic parameters	E	T^{EC}	T^{BCS}
[a]Vmax	0,181 ± 0,003	0,244 ± 0,007	0,259 ± 0,010
[b]k_M	0,134 ± 0,008	0,148 ± 0,013	0,257 ± 0,024

[a] mg l^{-1} min^{-1} of glucose
[b] mg ml^{-1}

5.1.3 Effect of ethanol content on enzyme activity

It is common knowledge that ethanol may be a competitive inhibitor for many proteolytic enzymes, such as bromelain (Esti et al. 2011). Ethanol influence has just been assessed also on the activity of the enzymatic preparation Vinoflow G (Laffort Oenologie, Bordeaux Cedex, France), a pectinase and β-glucanase blend particularly active in promoting the degradation of pectic substances and the hydrolysis of β-glucans (Zinnai et al. 2010). The authors observed a decrease in activity of 50% in a model wine solution containing 13% v/v of ethanol. Since wine ethanol content generally varies between 11 and 18% v/v, an increasing knowledge about ethanol inhibitory effect on oenological enzymatic preparations activity is useful. Therefore, the effect of two different ethanol concentrations (11,3 and 12,7 % v/v), corresponding to those of a base wine and a sparkling wine respectively, was tested in tartaric buffer, which is the most similar to real wines. No significant differences were detected in tartaric buffer compared to the McIlvaine buffer at pH 3.20, as reported in Table 5.

Experimental findings showed in Table 6 demonstrate that ethanol addition inhibited enzyme activity and that the decrease slightly depends upon the tested ethanol concentrations both in absence and in presence of yeasts. In presence of yeast strains a positive effect on enzyme

activity is also observed (70 and 68% in presence and 62% in absence of yeasts). No significant differences were detected between the two strains tested.

Strain EC1118 showed a linear decrease of activity in presence of ethanol and thus, it was possible to extrapolate the E50, i.e. the 50% of maximum activity, corresponding to an ethanol concentration of 20% v/v, a higher value than those generally present in wines. For samples E and T^{BCS} instead, the decrease in enzyme activity is not linearly dependent from tested ethanol concentrations, but a similar E50 value can be extrapolated (Figure 13).

Table 5 *β-glucanase activity at pH 3.20 in McIlvaine and in Tartaric buffer*

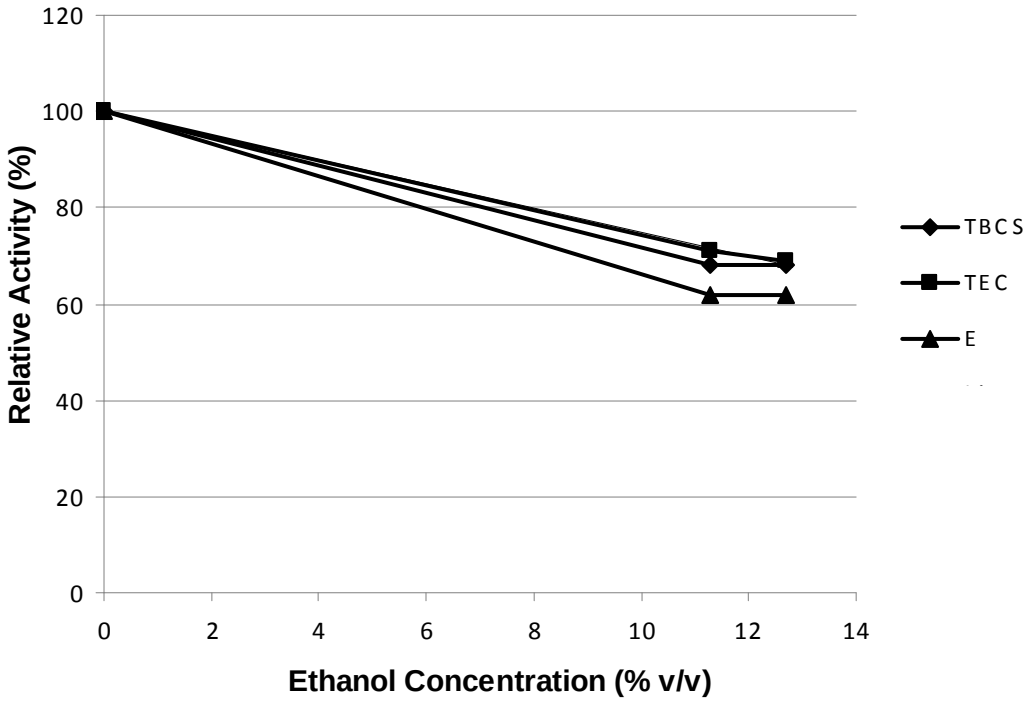
Samples	**McIlvaine buffer**	**Tartaric buffer**	**Tartaric *vs* McIlvaine (%)**
E	0,106 ± 0,002	0,117 ± 0,002	110
T^{EC}	0,153 ± 0,002	0,172 ± 0,002	112
T^{BCS}	0,147 ± 0,001	0,156 ± 0,001	107

Table 6 *Effect of different ethanol concentrations on Lallzyme MMX® activity at pH 3.20*

Samples	Ethanol (% v/v)	Enzyme activity (glucose, mg l^{-1} min-1)	Relative activity (%)
[a]**E**	0	0,117 ± 0,002	100
E	11.30	0,072 ± 0,004	62
E	12.70	0,072 ± 0,002	62
[b]$\mathbf{T^{EC}}$	0	0.172 ± 0.002	100
$\mathbf{T^{EC}}$	11.30	0.122 ± 0.017	71
$\mathbf{T^{EC}}$	12.70	0.118 ± 0.005	69
[c]$\mathbf{T^{BCS}}$	0	0,156 ± 0,001	100
$\mathbf{T^{BCS}}$	11.30	0,107 ± 0,003	68
$\mathbf{T^{BCS}}$	12.70	0,107 ± 0,002	68

[a] enzymatic preparation alone
[b] enzymatic preparation plus EC1118 yeast strain
[c] enzymatic preparation plus BCS103 yeast strain

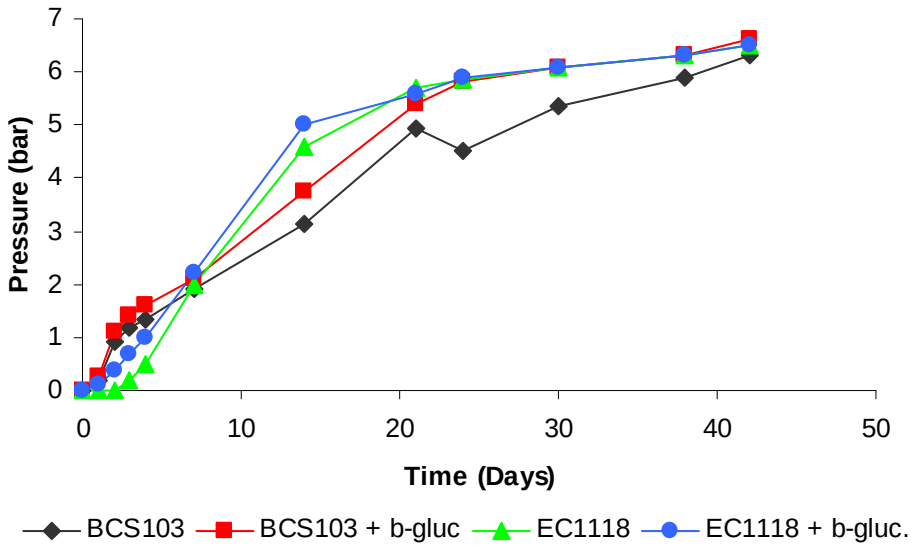
Figure 13 *Relative activity vs. ethanol concentration determined for E: Lallzyme MMX® alone; T^{EC}: in presence of EC1118 yeast strain and T^{BCS}: in presence of BCS103 yeast strain*



5.2 Sparkling wines production by the traditional method

All sparkling wines produced by the traditional method completed the secondary fermentation in 42 days, as indicated by internal bottle pressure monitoring. In all thesis, as reported in Figure 14, secondary fermentation started later for sparkling wines produced with EC1118, both in absence and in presence of Lallzyme MMX®. However bottle pressure constantly increased for all thesis, more rapidly for EC1118 strain both in absence and in presence of Lallzyme MMX®, even if at the end of the process, all thesis had the same pressure value. Roscetto base wine contained 11.3 % v/v of alcohol, while the obtained sparkling wines reached an alcohol content of 12.7 % v/v.

Figure 14 *Internal sparkling wine bottles pressure (bar), measured by aphrometers during secondary fermentation*



5.3 Direct evaluation of yeast lysis

5.3.1 Scanning Electron Microscopy and Transmission Electron Microscopy analyses

The observation of yeast cells in sparkling wines allows to detect structural and ultrastructural changes occurring during ageing over lees. Many studies aimed at analyzing the products released during autolysis are available, while less attention has been paid to this aspect.

In Figure 15 yeast cells images show that 4 months after *tirage* an internal cytoplasmic disorganization was present, as shown by TEM images (Figure 15b) for EC1118 and BCS103 both in absence and in presence of Lallzyme MMX® respect to time 0 (Figure 15a). SEM images instead showed no differences 4 months after *tirage* respect to time 0. As above mentioned in fact, cell lysis is a slow process, especially in the restrictive conditions in which secondary fermentations occur. In presence of Lallzyme MMX®, for both strains, no differences were still detectable.

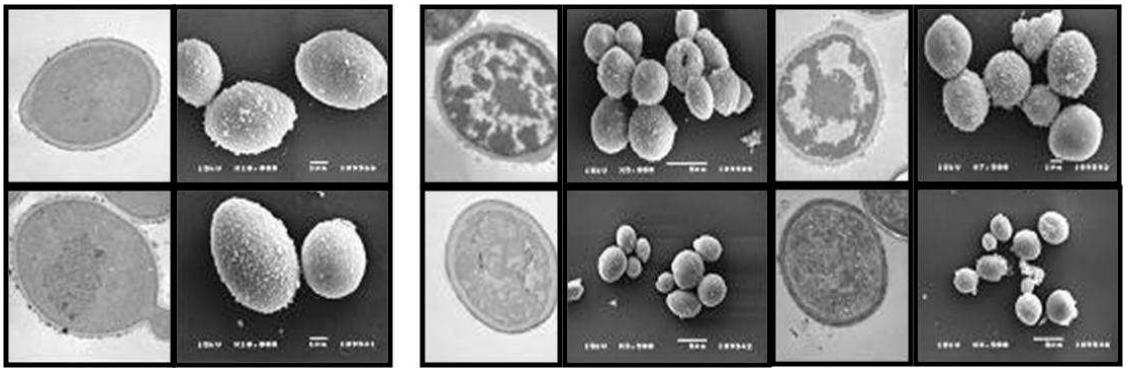
After 8 months of ageing, the cytoplasmic disorganization was more evident and cell wall started to be collapsed in presence of Lallzyme MMX® (Figures 16a, 16b). SEM images showed a slight row cells surface, indicating the beginning of the autolysis process for both yeasts, cells lysis was mildly more evident in sparkling wines produced with Lallzyme MMX® addition.

After 12 months of ageing in sparkling wines, yeast cells were collapsed, explaining the smaller size than time 0, both in presence and absence of β-glucanase, as shown by SEM images in Figures 17a and 17b. SEM observations revealed a rough cells surface, the cell wall was unbroken, but presented many ridges or folds, both in presence and in absence of Lallzyme MMX®, indicating the cell lysis.

TEM observations showed, at 12 months, a clear cytoplasmic wrinkling and a membrane detachment from cell wall for both yeasts.

Figure 15 *TEM and SEM images of BCS103 and EC1118 yeast cells at time 0 (a); TEM and SEM images of yeast cells in sparkling wines 4 months after tirage, in absence (EC1118 and BCS103) and in presence (EC1118+β-gluc.; BCS103+β-gluc.) of Lallzyme MMX® (b)*

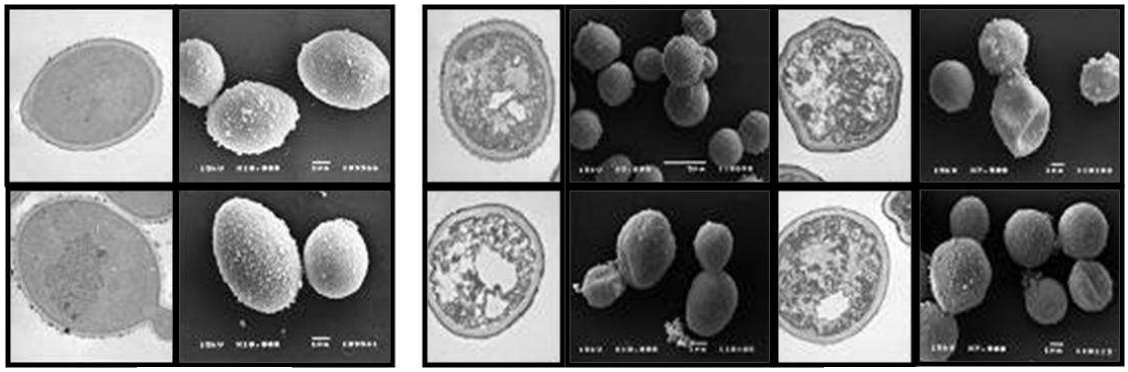
(a) EC1118 EC1118 EC1118+β-gluc. **(b)**



BCS103 BCS103 BCS103+β-gluc.

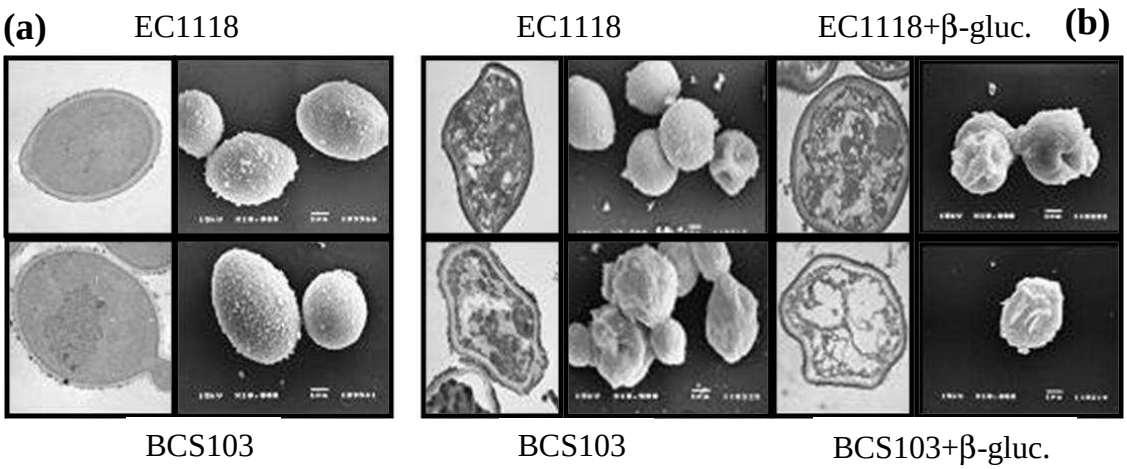
Figure 16 *TEM and SEM images of BCS103 and EC1118 yeast cells at time 0 (a); TEM and SEM images of yeast cells in sparkling wines 8 months after tirage, in absence (EC1118 and BCS103) and in presence (EC1118+β-gluc.; BCS103+β-gluc.) of Lallzyme MMX® (b)*

(a) EC1118 EC1118 EC1118+β-gluc. **(b)**



BCS103 BCS103 BCS103+β-gluc.

Figure 17 *TEM and SEM images of BCS103 and EC1118 yeast cells at time 0 (a); TEM and SEM images of yeast cells in sparkling wines 12 months after tirage, in absence (EC1118 and BCS103) and in presence (EC1118+β-gluc.; BCS103+β-gluc.) of Lallzyme MMX® (b)*



5.4 Indirect evaluation of yeast lysis

5.4.1 Free amino acids analysis

During autolysis in sparkling wines, free amino acids content varies a lot. During the ageing of sparkling wine over lees, Martínez-Rodríguez et al. (2002), found that the yeast strain used for refermentation influenced the content of free amino acids and peptides. They also detected four main stages in the ageing of wines with yeasts: in the first one, amino acids and proteins diminish and peptides are released; in the second stage, there is a release of nitrogen compounds used as nutrients for the viable cells which in this stage coexist with dead cells. An intracellular protease activity is also detected and proteins are degraded to peptides which then are hydrolyzed to amino acids. In the third stage, no viable cells are present and proteins and peptides are released, this is due to the enzymatic activity still present in wine. The last phase occurs after about 270 days after *tirage*, authors found a decrease of amino acids in some of

the wines studied. A similar trend was observed in the free amino acids content of the studied sparkling wines.

In Table 7, free amino acidic composition of base wine and sparkling wines after 4, 8 and 12 months of ageing is reported. The most abundant amino acid in base wine (time 0) was lysine (7,84 mmol l^{-1}), which made up 74% of total free amino acids, followed by Gaba (γ-aminobutyric acid), 7% and methionine (4%). 4 months after *tirage*, a decrease in amino acids sum was detected, probably due to their assimilation by the yeasts for the synthesis of proteins and for their accumulation as a reserve in vacuoles. The most evident decrease was for lysine (that is no more present), serine, alanine, asparagine, threonine, methionine and leucine. 8 months after *tirage* instead, an increase in amino acids sum was detected for all samples, indicating that autolysis started.

The increase was particularly manifest for EC1118 strain and slightly greater in presence of Lallzyme MMX® for both yeasts.

Table 7 *Free amino acids content in base wine and sparkling wines after 4, 8 and 12 months of ageing*

Base Wine		Sparkling wines											
A.A.		**EC1118**			**EC1118 + β-gluc.**			**BCS103**			**BCS103 + β-gluc.**		
Months	**0**	**4**	**8**	**12**	**4**	**8**	**12**	**4**	**8**	**12**	**4**	**8**	**12**
Lys	7,84	0,00	22,4	13,6	0,00	22,5	0,00	0,00	0,00	0,00	0,00	0,00	22,54
Ser	0,06	0,00	0,03	0,03	0,02	0,04	0,03	0,00	0,04	0,00	0,00	0,00	0,04
Pro	0,07	0,16	0,07	0,03	0,12	0,00	0,10	0,03	0,17	0,09	0,20	0,22	0,00
Cys	0,05	0,60	0,00	0,00	0,04	0,03	0,03	0,01	0,03	0,05	0,04	0,04	0,03
Gaba	0,76	0,75	0,15	0,00	0,72	0,85	0,85	0,59	0,00	0,02	0,65	0,70	0,85
Ala	0,37	0,06	0,55	0,42	0,06	0,05	0,06	0,03	0,06	0,00	0,05	0,06	0,05
Gly	0,07	0,61	0,04	0,03	0,02	0,04	0,04	0,32	0,03	0,60	0,55	0,64	0,04
Asn	0,22	0,08	0,00	0,09	0,00	0,00	0,00	0,01	0,00	0,01	0,01	0,00	0,00
Gln	0,00	0,00	0,03	0,03	0,03	0,04	0,04	0,03	0,04	0,00	0,00	0,03	0,04
Hys	0,03	0,05	0,41	0,22	0,40	0,45	0,47	0,03	0,47	0,05	0,05	0,06	0,42
Thr	0,38	0,02	0,08	0,04	0,08	0,10	0,09	0,03	0,09	0,03	0,02	0,02	0,08
Arg	0,05	0,30	0,34	0,21	0,28	0,35	0,35	0,20	0,33	0,30	0,25	0,30	0,33
Val	0,00	0,00	0,04	0,03	0,42	0,46	0,44	0,21	0,62	0,42	0,40	0,42	0,39
Met	0,40	0,00	0,03	0,02	0,02	0,03	0,04	0,03	0,03	0,03	0,02	0,02	0,03
Leu	0,21	0,21	0,09	0,06	0,06	0,08	0,22	0,07	0,22	0,20	0,17	0,19	0,09
Phe	0,02	0,02	0,11	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00
Ile	0,00	0,05	0,01	0,01	0,05	0,06	0,06	0,04	0,04	0,00	0,05	0,06	0,06
Tyr	0,03	0,04	0,04	0,03	0,00	0,00	0,00	0,00	0,00	0,03	0,03	0,00	0,00
Trp	0,01	0,10	0,01	0,00	0,09	0,12	0,12	0,08	0,12	0,11	0,08	0,94	0,11
Asp	0,04	0,02	0,08	0,05	0,54	0,56	0,54	0,31	0,72	0,33	0,50	0,52	0,58
Glu	0,01	0,01	0,04	0,04	0,04	0,05	0,05	0,04	0,05	0,01	0,01	0,04	0,05
[a]**Sum**	**10,6**	**3,11**	**24,54**	**14,93**	**3,00**	**25,83**	**3,53**	**2,07**	**3,10**	**2,30**	**3,11**	**4,29**	**25,74**

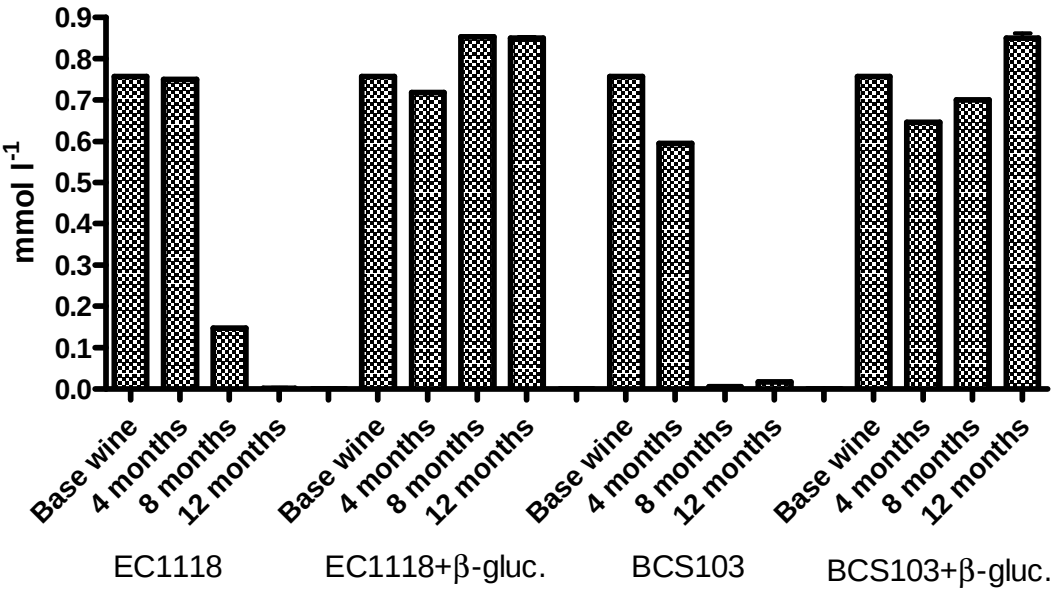
[a] = Free amino acids sum

As an autolysis consequence, a great release of lysine (an essential amino acid) was detected for EC1118 both in presence and in absence of enzyme and after 12 months for BCS103 + β-gluc. At 12 months of ageing, a decrease of amino acids was detected, probably due to deamination or decarboxylation reactions or to esters formation, except for BCS103 + β-gluc., were autolysis started later than all other samples, as indicated by high lysine content.

Gaba (γ-aminobutyric acid), that is not a constituent of proteins, was released by both yeasts in presence of Lallzyme MMX® during autolysis (Figure 18). In fact, is reported that *S. cerevisiae* can utilise Gaba as carbon source, so it builds up in the intracellular pool during the first months of fermentation (Martínez-Rodríguez et al., 2001a) and is released into the

extracellular environment during autolysis. These data demonstrate that autolysis occurred 8 months after *tirage*, even if each free amino acid showed a different behaviour.

Figure 19 *Gaba (γ–aminobutyric acid) content in base wine and in sparkling wines 4, 8 and 12 months after tirage, both in absence and in presence of Lallzyme MMX®*



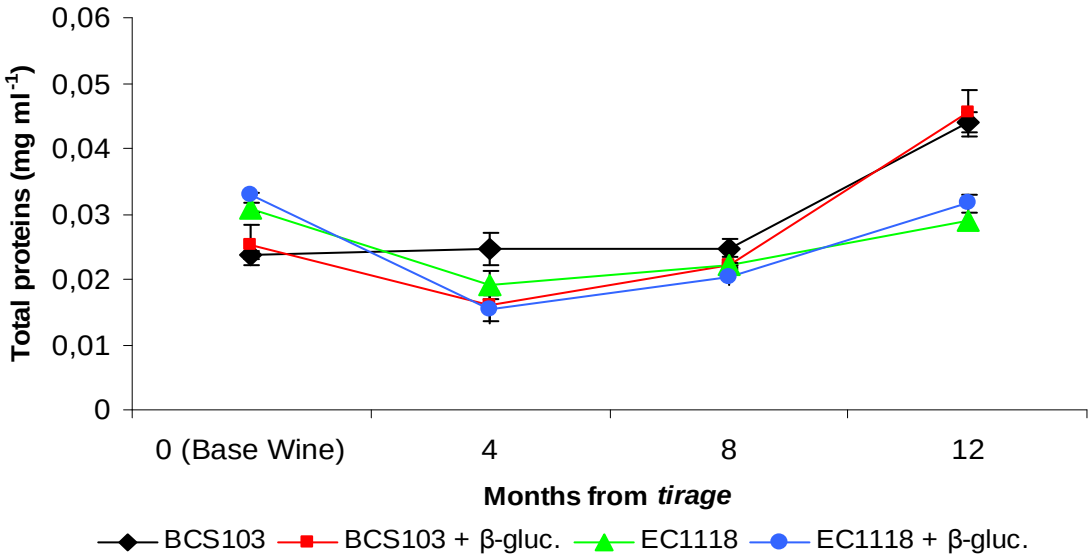
5.4.2 Total proteins content determination

Even if proteins are minor constituents of wine, they contribute to the quality of the product, in fact, they are responsible for the "body" sensation, they can blind volatile compounds retaining wine aroma and also have a positive effect on foam stability (Brissonnet and Maujean, 1991; Luguera et al., 1998). Proteins seem to be foam active substances because of their surface properties. They act as tensoactive compounds and enhance foam stability (García et al., 2009).

During the autolysis process, proteases hydrolyze yeast proteins to lower molecular weight compounds. Total proteins content was analysed with the Bradford method that quantify both polypeptides with a molecular weight higher than 3500 Da and proteins. Data (Figure 19),

indicated a decrease for all samples, except for BCS103, 4 months after *tirage*, probably due to proteins insolubilization because of alcohol content increase. An increment was instead observed between 8 and 12 months after *tirage* with higher values for BCS103 strain, both in absence and in presence of Lallzyme MMX®. This result indicated the occurrence of yeast lysis between 8 and 12 months of ageing.

Figure 19 *Total proteins of base wine (time 0) and sparkling wines at 4, 8 and 12 months after tirage in absence (EC1118 and BCS103) and in presence (EC1118+β-gluc; BCS103+β-gluc) of Lallzyme MMX®*



5.4.3 Foaming properties

Base wine and sparkling wines foam height and stability was analysed during the whole process. Foam height (Figure 20), decreased more slowly for EC1118 samples, but 12 months after *tirage*, all samples showed a similar result. The same behaviour was observed for foam stability (Figure 21), indicating that autolysis and protein content had no influences on foam quality.

Figure 20 *Foam height of base wine (time 0) and sparkling wines at 4, 8 and 12 months after tirage in absence (EC1118 and BCS103) and in presence (EC1118+β-gluc; BCS103+β-gluc) of Lallzyme MMX®*

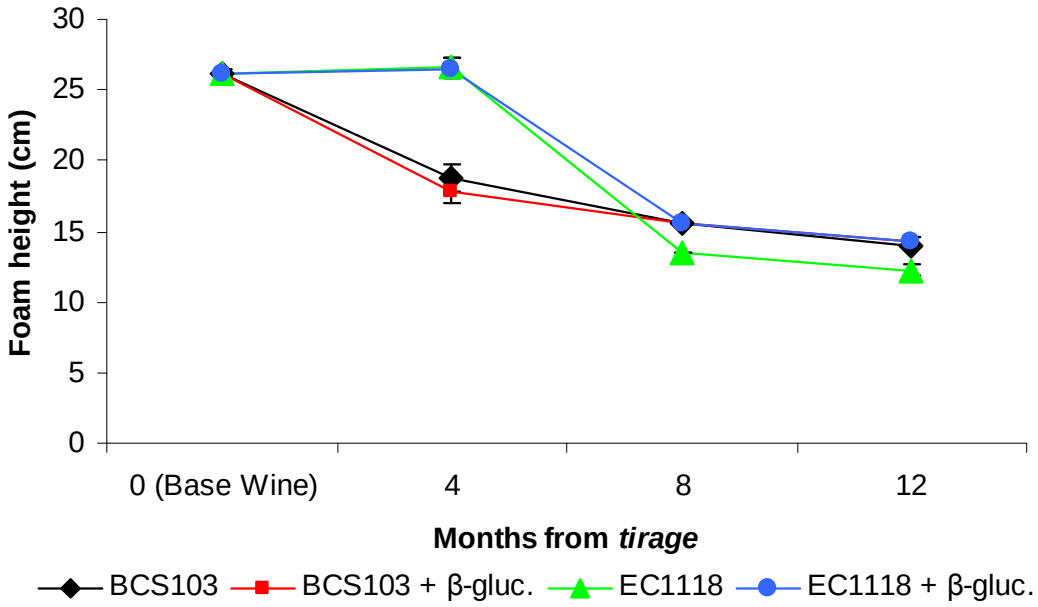
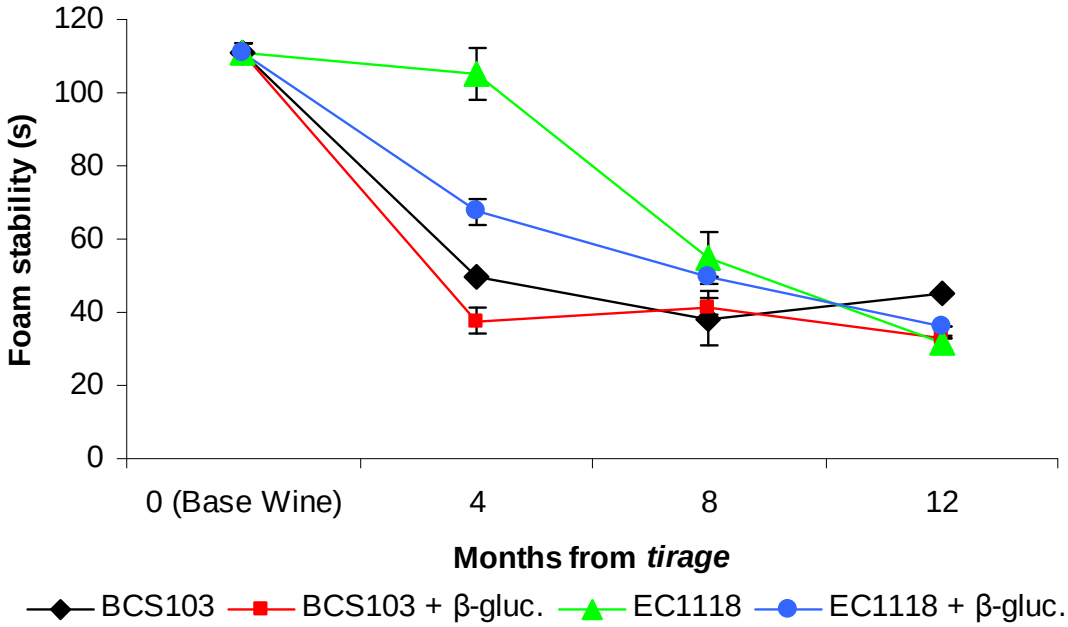
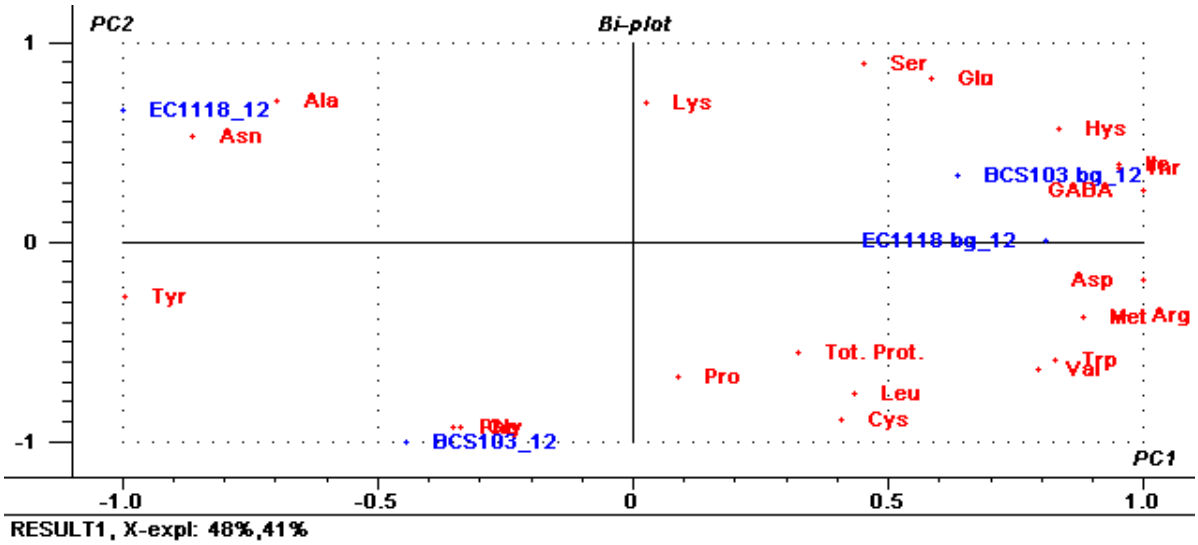


Figure 21 *Foam stability of base wine (time 0) and sparkling wines at 4, 8 and 12 months after tirage in absence (EC1118 and BCS103) and in presence (EC1118+β-gluc; BCS103+β-gluc) of Lallzyme MMX®*



To gain a greater knowledge, a PCA (Principal Component Analysis) was performed on 12 months samples (Figure 22). The first principal component (48% of variance) was best described by enzyme effect, indicating a similar behaviour for both yeasts in presence of Lallzyme MMX®. The second principal component (41% of variance) related better to the amino acidic pool that characterizes each lysed yeast strain in absence of enzyme. Lysine, alanine and asparagine seemed to be characteristics for EC1118 strain, whereas phenylalanine, glycine and proline for BCS103. Gaba, as above explained, was particularly affected by the enzymatic treatment, also hystidine seemed to be particularly influenced by the enzyme, such as isoleucine and threonine. Since threonine and serine are cell mannoproteins constituents (Alexandre et al., 2006), threonine appearance could reflect yeast lysis, even if serine increase was not particularly manifest.

Figure 22 *Samples distribution 12 months after tirage according to the PCA (bi-plot) considering free amino acids and total proteins as variables*



5.4.4 Sensory analysis

Sensory analysis performed on sparkling wines 12 months after *tirage* did not show differences between samples, even if all sparkling wines were considered as good.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

This study focuses on the characterization of the enzymatic preparation Lallzyme MMX® containing β-glucanase for further applications in sparkling wines production. The effect of the β-glucanase activity on yeast lysis of two secondary fermentation yeast strains was investigated. Even if pH 3.20, the average value commonly found in wines, is not the optimum pH for the enzyme β-glucanase, it showed a satisfactory activity, useful for oenological purposes.

Moreover, a lytic activity on yeast cell wall glucans was pointed out. This was more clearly evident for the strain EC1118, which confirmed its aptitude for sparkling wines production by in bottle alcoholic fermentation and prolonged ageing on lees; yeast strain BCS103 instead, was less affected by the enzyme lytic action.

Ethanol inhibitory effect on enzyme activity was detected, as already reported from Zinnai et al. (2010), however, the lytic effect of β-glucanase was not compromised and sufficient for user's purposes.

V_{max} values showed that the maximum rate was higher for samples with yeast (T^{EC} and T^{BCS}), respect to that with enzyme alone (E), this effect may be due to a consistent release of the yeast intracellular enzyme or a more efficient cell lysis. No significant differences were detected between V_{max} values of both strains in presence of Lallzyme MMX® (T^{EC} and T^{BCS}). k_M value indicated the efficacy of EC1118 strain, this parameter for T^{EC} sample resulted to be significantly lower than that of T^{BCS} and similar to the experiment of β-glucanase alone (E), thus indicating a better enzyme substrate complex-formation.

All sparkling wines reached the desired pressure and completed secondary fermentation without drawbacks. However, secondary fermentation started later for sparkling wines produced with EC1118 strain, both in absence and in presence of Lallzyme MMX®.

Nevertheless, bottle pressure constantly increased for all thesis, more rapidly for EC1118 strain both in absence and in presence of Lallzyme MMX®, even if at the end of the process, all thesis had the same pressure value.

Direct evaluation of yeast lysis by TEM and SEM observations was useful to describe and monitor yeast lysis during ageing. Free amino acids content showed that autolysis occurred 8 months after *tirage* for all sparkling wines, except for sparkling wine produced with BCS103 strain in presence of Lallzyme MMX®, for this sample in fact, cell lysis occurred later. Free amino acids content was also useful to show the effect of the enzymatic treatment on yeast lysis, particularly manifested for some amino acids, such as Gaba, hystidine, threonine and isoleucine 12 months after *tirage*.

Also total proteins content showed that autolysis occurred already 8 months after *tirage*, even if this parameter seemed to have no positive influences on foaming properties, which presented a decreasing trend during sparkling wines ageing. Sensory analysis showed no significant differences between samples even if all sparkling wines were considered as good. Future perspectives may look at increase knowledge about enzyme inhibitors both in synthetic buffers and in wine, aimed to better understand enzyme behaviour in real wine.

7 REFERENCES

Alcaide-Hidalgo, J.M., Pueyo, E., Polo, M.C., Martínez-Rodríguez, A.J. (2007). Bioactive peptides released from Saccharomyces cerevisiae under accelerated autolysis in a wine model system. *J. Food Sci*., 72 (7), 276-279.

Alexandre, H., Guilloux-Benatier, M. (2006). Yeast autolysis in sparkling wine – a review. *Aust. J. Grape Wine Res*., 12, 119-127.

Alexandre, H., Heintz, D., Chassagne, D., Guilloux-Benatier, M., Charpentier, C., Feuillat, M. (2001). Protease A activity and nitrogen fractions released during alcoholic fermentation and autolysis in enological conditions. *J. Ind. Microbiol. Biotechnol.*, 26, 235-240.

Andrés-Lacueva, C., Lamuela-Raventós, R.M., Buxaderas, S., De la Torre-Boronat, M.C. (1997). Influence of variety and aging on foaming properties of cava (sparkling wine). J. *Agric. Food Chem*., 45, 2520–2525.

Babayan, T.L., Bezrukov, M.G. (1985). Autolysis in yeasts. *Acta Biotechnologica*, 2, 129-136.

Bartrà, E. (1995). Aspectos microbiológicos de la elaboración del cava. Microbiología SEM, 11, 43–50.

Bayly, J. C., Douglas, L. M., Pretorius, I. S., Bauer, F. F., & Dranginis, A. M. (2005). Characteristic of Flo 11-dependent flocculation in Saccharomyces cerevisiae. *FEMS Yeast Res.*, 5, 1151–1156.

Bester, M.C., Pretorius, I.S., Bauer, F.F. (2006). The regulation of Saccharomyces cerevisiae FLO gene expression and Ca2+-dependent flocculation by Flo8p and Mss11p. *Curr. Genet*., 49(6), 375–383.

Bidan, P., Divies, Ch., and Dupuy, P., (1980). France Patent no. 2432045.

Bidan, P., Feuillat, M., Moulin, J.P. (1986). Les vins mousseux. Rapport de la France. 65ème Assemblée Générale de l'OIV. *Bulletin OIV*, 59, 563–626.

Bikerman, J.J. (1938). The unit of foaminess. *Trans. Faraday SOC.*, 34,634.

Bradford, M.M. (1976). A rapid and sensitive method for the quantization of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.* 72, 248–255.

Brissonnet, F., Maujean, A. (1991). Identification of some foam active compounds in champagne base wine. *Am. J. Enol. Vitic*., 44, 97-102.

Caridi, A. (2006). Enological functions of parietal yeast mannoproteins. *Atonie van Leeuwenhoek*, 89, 417-422.

Castellari, L., Ferruzzi, M., Magrini, A., Giudici, P., Passarelli, P., Zambonelli, C. (1994). Unbalanced wine fermentation by cryotolerant vs. non-cryotolerant Saccharomyces strains. *Vitis*, 33, 49–52.

Cebollero, E., Carrascosa, A.V., Gonzalez, R. (2005). Evidence for yeast autophagy during simulation of sparkling wine aging: A reappraisal of the mechanism of yeast autolysis in wine. *Biotechnol. Prog*., 21, 614-616.

Cebollero, E., Gonzalez, R. (2006). Induction of autophagy by second-fermentation yeasts during elaboration of sparkling wines. *Appl. Environ. Microb*., 72 (6), 4121-4127.

Cebollero, E., Gonzalez, R. (2007). Autophagy: From basic research to its application in food biotechnology. *Biotechnol. Adv*., 25, 396-409.

Charpentier, C. (2000). Yeast autolysis and yeast macromolecules? Their contribution to wine flavour and stability. *Proceedings of the ASEV 50th Anniversary Annual Meeting*, Seattle, Washington, June 19-23, 2000, 2001, ISBN 0963-0711-4-9, pp. 271-277.

Charpentier, C., Aussenac, J., Charpentier, M., Prome, J.C., Duteurtre, B., Feuillat, M. (2005). Release of nucleotides and nucleosides during yeast autolysis : kinetics and potential impact on flavor. *J. Agric. Food Chem.*, 53, 3000-3007.

Charpentier, C., Feuillat, M. (1993). Yeast autolysis. In *Wine Microbiology and Biotechnology*, Graham G. Fleet ed., pp. 225-242.

Charpentier, C., Freyssinet, M. (1989). The mechanism of autolysis in wine. *Yeast*, 5, 181–186.

Chung, S. (1986). Contribution à l'étude de la formation des composés volatils au cours de l'autolyse des levures de vinification. Thesis, Université de Bourgogne.

Colagrande, O., Silva, A., Fumi, M.D. (1994). Recent applications of biotechnology in wine production. *Biotechnol. Prog.*, 10, 2-18.

Coloretti, F., Zambonelli, C., Tini, V. (2006). Characterization of flocculent Saccharomyces interspecific hybrids for the production of sparkling wines. *Food Microbiol.*, 23, 672–676.

Connew, S. (1998). Yeast autolysis. A review of current research. *Aust. NZ Wine Ind. J.*, 13, 61-64.

D'Auria, M., Emanuele, L., Mauriello, G., Racioppi, R. (2003). On the origin of "Goût de Lumière" in champagne. *J. Photoch. Photobio. A*, 158, 21-26.

De Person, M., Sevestre, A., Chaimbault, P., Perrot, L., Duchiron, F., Elfakir, C. (2004). Characterization of low-molecular weight peptides in champagne wine by liquid chromatography/tandem mass spectrometry. *Anal. Chim. Acta*, 520, 149-158.

Desportes, C., Charpentier, M., Duteurtre, B., Maujean, A., Duchiron, F. (2000). Liquid chromatographic fractionation of small peptides from wine. *J. Chromatogr. A*, 893, 281-291.

Diviès, C., Cachon, R., Cavin, J.F., Prévost, H. (1994). Theme 4 : immobilized cell technology in wine production. *Crit. Rev. Biotechnol.*, 14 (2), 135-153.

Dubourdieu, D., Villetaz, J.C., Desplanques Morales, C., Ribéreau-Gayon, P. (1981). Dégradation enzymatique du glucane de Botrytis cinerea. Application à l'amélioration de la clarification des vins issus de raisins pourris. *Connaiss Vigne Vin*, 15, 161-177.

Dussaud, A., Robillard, B., Carles, B., Duteurtre, B. (1994). Exogenous lipids and ethanol influences on the foam behaviour of sparkling base wines. *J. Food Sci.*, 59, 148-151.

Efremenko, E., Stepanov, N., Martinenko, N., Gracheva, I. (2005). Cultivation conditions preferable for yeast cells to be immobilized into Poly(vinyl alcohol) and used in bottled sparkling wine production. *Chem. Ind. Chem. Eng. Q.*, 12 (1), 18-23.

Esti, M., Benucci, I., Liburdi, K., Garzillo, A.M.V. (2011). Effect of wine inhibitors on free pineapple stem bromelain activity. *J. Agric. Food. Chem.*, 59 (7), 3391-3397.

Feuillat, M. (1998). Autolyse de levures. In *Œnologie: Fondements Scientifiques et Technologiques*, C. Flanzy ed., pp. 444-454.

Feuillat, M. (2003). Yeast macromolecules: Origin, composition, and enological interest. *Am. J. Enol. Vitic.*, 54 (3), 211-213.

Feuillat, M., Charpentier, C. (1982). Autolysis of yeasts in Champagne. *Am. J. Enol. Vitic.*, 33, 6-13.

Fornairon-Bonnefond, C., Camarasa, C., Moutounet, M., Salmon, J.M. (2002). New trends on yeast autolysis and wine aging on lees : a bibliographic review. *J. Int. Sci. Vigne Vin.*, 36, 49-69.

Francioli, S., Torrens, J., Riu-Aumatell, M., Lopez-Tamanez, E., Buxaderas, S. (2003). Volatile compounds by SPME-GC as age markers of sparkling wines. *Am. J. Enol. Vitic.*, 54, 158-162.

Fumi, R.D., Ragg, E., Battistotti, G., Colagrande, O. (1994). Alginate immobilized Saccharomyces cerevisiae cell alteration during alcoholic fermentation. *It. J. Food Sci.*, 6, 325.

Fumi, M.D., Trioli, G., Colagrande, O. (1987). Preliminary assessment on the use of immobilized yeast cells in sodium alginate for sparkling wine processes. *Biotechnol. Lett.*, 9, 939.

Fumi, M.D., Trioli, G., Colombi, M.G., Colagrande, O. (1988). Immobilization of Saccharomyces cerevisiae in calcium alginate gel and its application to bottle fermented sparkling wine production. *Am. J. Enol. Vitic.*, 39, 267.

Gallart, M., López-Tamames, E. Buxaderas, S. (1997). Foam measurements in wines: comparison of parameters obtained by gas sparging method. *J. Agric. Food Chem.*,45,4687-4690.

Gallart, M., Tomás, X., Suberbiola, G., López-Tamames, E. (2004). Relationship between foam parameters obtained by the gas-sparging method and sensory evaluation of sparkling wines. *J. Sci. Food Agric.*, 84, 127-133.

García, M.J., Alexandre, J.L., Álvarez, I., Lizama, V. (2009). Foam aptitude of Bobal variety in white sparkling wine elaboration and study of volatile compounds. *Eur. Food Res. Technol.*, 229, 133-139.

Giovani, G., Rosi, I. (2007). Release of cell wall polysaccharides from Saccharomyces cerevisiae thermosensitive autolytic mutants during alcoholic fermentation. *Int. J. Food Microbiol*., 116(1), 19–24.

Girbau-Sola, T., Lopez-Barajas, M., Lopez-Tamames, E., Buxaderas, S. (2002a). Foam aptitude of Trepat and Monastrell red varieties in Cava elaboration 2: second fermentation and aging. *J. Agric. Food Chem.*, 50 (20), 5600-5604.

Girbau-Sola, T., Lopez-Tamames, E., Bujan, J., Buxaderas, S. (2002b). Foam aptitude of Trepat and Monastrell red varieties in Cava elaboration 1: base wine characteristics. *J. Agric. Food Chem.*, 50 (20), 5596-5599.

Gòdia, F., Casas, C., Solàg, C. (1991). Application of immobilized yeast cells to sparkling wine fermentation. *Biotechnol. Progr.*, 7, 468-470.

Gonzalez, R., Martínez-Rodríguez, A.J., Carrascosa, A.V. (2003). Yeast autolytic mutants potentially useful for sparkling wine production. *Int. J. Food Microbiol*., 84, 21-26.

Gonzalez-Ramos, D., Cebollero, E., Gonzalez, R. (2008). A re-combinant Saccharomyces cerevisiae strain overproducing mannoproteins stabilizes wine against protein haze. *Appl. Environ. Microb.*, 74 (17), 5533-5540.

Gryta, M. (2002). The assessment of microorganism growth in the membrane distillation system. *Desalination*, 142, 79–88.

Hardy, G. (1986). La dégustation des vins effervescents élaborés en method champenoise. *Revue des Œnologues*, 42, 15 (1ère partie).

Hardy, G. (1987a). La dégustation des vins effervescents élaborés en method champenoise. *Revue des Œnologues*, 43, 13–19 (2ème partie).

Hardy, G. (1987b). La dégustation des vins effervescents élaborés en method champenoise. *Revue des Œnologues*, 44, 43–50 (3ème partie).

Hardy, G. (1988). La dégustation des vins effervescents élaborés en method champenoise. *Revue des Œnologues* XII, 48, 24–26 (4ème partie).

Hardy, G. (1990). Importancia del prensado en la calidad de los vinos base en el método Champenoise. *ACE Rev. Enol.*, 20, 17-22.

Hernawan, T., Fleet, G.H. (1995). Chemical and cytological changes during the autolysis of yeast. *J. Ind. Microbiol.*, 14, 440-450.

Houg, M.D., Maddox, I.S. (1970). Yeast autolysis. *Process Biochem.*, 5, 50-52.

Humbert-Goffard, A., Saucier, C., Moine-Ledoux, V., Canal-Llaubères, R.M., Dubourdieu, D., Glories, Y. (2004). An assay for glucanase activity in wine. *Enzyme Microb. Tech.*, 34, 537–543.

Jackson, R.S. (2008). Specific and Distinctive Wine Styles. In *Wine Science Principles and applications, third edition*, Academic Press, Elsevier ed., pp. 538-576.

Jin, Y.L., Speers, R.A. (1999). Flocculation of Saccharomyces cerevisiae. *Food Res. Int.*, 31(6–7), 421–440.

Kirtadze, E., Nutsubidze, N. (2009). Metabolic potential of alcoholic fermentation yeasts. *Bull. Georg. Nat. Acad. Sci.*, 3 (1), 112-116.

Klis, F.M., Mol, P., Hellingwerf, K., Brul, S. (2002). Dynamics of cell wall structure in *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.*, 26, 239-256.

Kourkoutas, Y., Bekatorou, A., Banat, I.M., Marchant, R., Koutinas, A.A. (2005). Immobilization technologies and support materials suitable in alcohol beverages production: a review. *Food Microbiol.*, 21, 377–397.

Kourkoutas, Y., Kanellaki, M., Koutinas, A.A., Tzia, C. (2004). Effect of fermentation conditions and immobilization supports on the wine making. *J. Food Eng.*, 69, 115-123.

Kourkoutas, Y., Manojlović, V., Nedović, V.A. (2010). Immobilization of microbial cells for alcoholic and malolactic fermentation of wine and cider. In *Encapsulation Technologies for Active Food Ingredients and Food Processing*, N.J. Zuidam and V.A. Nedovic´ eds., p. 327.

Lebeau, T., Jouenne, T., Junter, G.A. (1998). Diffusion of sugars and alcohols through composite membrane structures immobilising viable yeast cells. *Enzyme Microb. Technol.*, 22, 434–438.

Leroy, M.J., Charpentier, M., Duteurtre, B., Feuillat, M., Charpentier, C. (1990). Yeast autolysis during Champagne ageing. *Am. J. Enol. Vitic.*, 41 (1), 21-28.

Liger-Belair, G. (2005). The physics and chemistry behind the bubbling properties of Champagne and sparkling wines: A State-of-the-Art Review. *J. Agric. Food Chem.*, 53 (8), 2788–2802.

Lipke, P.N., Ovalle, R. (1998). Minireview. Cell wall architecture in yeast: new structure and new challenges. *J. Bacteriol.*, 180, 3735-3740.

López-Barajas, M., López-Tamames, E., Buxaderas, S, Thomás, X., De Latorre, MC. (1999). Prediction of wine foaming. *J.Agric. Food Chem.*, 47, 3743-3748.

Luguera, C., Moreno-Arribas, M.V., Pueyo, E., Bartolomé, B., Polo, M.C. (1998). Fractionation and partial characterization of protein fractions present at different stages of the production of sparkling wines. *Food Chem.*, 63 (4), 465-471.

Machet, F., Robilard, B., Duteurtre, B. (1993). Application of image-analysis to foam stability of sparkling wine. *Sci. Alim.*, 13 (1), 73-87.

Manners, D.J., Masson A.J., Patterson, J.C. (1973a). The structure of a β-(1→3)-D-glucan from yeast cell walls. *Biochem. J.*, 135, 19-30.

Manners, D.J., Masson A.J., Patterson, J.C. (1973b). The structure of a β-(1→6)-D-glucan from yeast cell walls. *Biochem. J.*, 135, 31-36.

Martínez-Rodríguez, A.J., Carrascosa, A.V., Barcenilla, J.M., Pozo-Bayón, M.A., Polo, M.C. (2001a). Autolytic capacity and foam analysis as additional criteria for the selection of yeast strains for sparkling wines production. *Food Microbiol*., 18, 183-191.

Martínez-Rodríguez, A.J., Carrascosa, A.V., Martín-Àlvarez, P.J., Moreno-Arribas, M.V., Polo, M.C. (2002). Influence of the yeast strain on the changes of the amino acids, peptides and proteins during sparkling wine production by the traditional method. *J. Ind. Microbiol. Biot.*, 29, 314-322.

Martínez-Rodríguez, A.J., Carrascosa, A.V., Polo, M.C. (2001c). Release of nitrogen compounds to the extracellular medium by three strains of Saccharomyces cerevisiae during induced autolysis in a model wine system. *Int. J.Food Microbiol.*, 68, 155–160.

Martínez-Rodríguez, A.J., Gonzalez, R., Carrascosa, A. V. (2004). Morphological changes in autolytic wine yeast during ageing in two model systems. *J. Food Sci.,* 69(8), 233–239.

Martínez-Rodríguez, A.J., Polo, M.C. (2000). Characterization of the nitrogen compounds released during yeast autolysis in a model wine system. *J. Agric. Food Chem*., 48, 1081-1085.

Martínez-Rodríguez, A.J., Polo, M.C. (2003). Effect of the addition of bentonite to the tirage solution on the nitrogen composition and sensory quality of sparkling wines. *Food Chem.*, 81 (3), 383-388.

Martínez-Rodríguez, A.J., Polo, M.C., Carrascosa, A.V. (2001b). Structural and ultrastructural changes in yeast cells during autolysis in a model wine system and in sparkling wines. *Int. J. Food Microbiol*., 71, 45-51.

Martínez-Rodríguez, A.J., Pueyo, E. (2009). Sparkling Wines and Yeast Autolysis. In *Wine Chemistry and Biochemistry*, Springer ed., pp. 61-80.

Martynenko, N.N., Gracheva, I.M. (2003). Physiological and biochemical characteristics of immobilized champagne yeasts and their participation in champagnizing processes: a review. *Appl. Biochem. Microb.*, 39 (5), 439–445.

Martynenko, N.N., Gracheva, I.M., Sarishvili, N.G., Zubov, A.L., El'-Registan, G.I., Lozinsky, V.I. (2004). Immobilization of champagne yeasts by inclusion into cryogels of polyvinyl alcohol: means of preventing cell release from the carrier matrix. *Appl. Biochem. Micro.*, 40 (2), 158-164.

Masino, F., Montevecchi, G., Arfelli, G., Antonelli, A. (2008). Evaluation of the combined effects of enzymatic treatment and aging on lees on the aroma of wine from Bombino Bianco grapes. *J. Agric. Food Chem*., 56, 9495–9501.

Maujean, A., Poinsaut, P., Dantan, H., Brissonnet, F., Cossiez, E. (1990). Etude de la tenue et de la qualité de la mousse des vins effervescents. *Bull. OIV*, 711, 405-426.

Maujean, A., Seguin, S. (1983). Contribution à l étude des goûts de lumière dans les vins de Champagne. *Sci. Aliments*, 3, 589-601.

McIlvaine, T.C. (1921). A buffer solution for colorimetric comparison. *J. Biol. Chem.,* 49, 183-186.

Moreno-Arribas, M.V., Pueyo, E., Nieto, F.J., Martín-Álvarez, P.J., Polo, M.C. (2000). Influence of the polysaccharides and the nitrogen compounds on foaming properties of sparkling wines. *Food Chem*., 70, 309-317.

Moreno-Arribas, M.V., Pueyo, E., Polo, M.C. (2002). Analytical methods for the characterization of proteins and peptides in wines. *Anal. Chim. Acta*, 458, 63-75.

Nunez, Y.P., Carrascosa, A.V., González, R., Polo, M.C., Martínez-Rodríguez, A.J. (2005). Effect of accelerated autolysis of yeast on the composition and foaming properties of sparkling wines elaborated by a champenoise method. *J. Agric. Food Chem.*, 53, 7232-7237.

Nunez, Y.P., Carrascosa, A.V., González, R., Polo, M.C., Martínez-Rodríguez, A.J. (2006). Isolation and characterization of a thermally extracted yeast cell wall fraction potentially useful for improving the foaming properties of sparkling wine *J. Agric. Food Chem.*, 54, 7898-7903.

Obiols, J.M., de la Presa-Owens, C., Buxaderas, S., Bori, J.L., de la Torre-Boronat, M.C. (1998). Protocolo de evaluación de la formación de la efervescencia y espuma en un vino espumoso. *ACE, Revista d'Enología*, 15, 3–8.

Orlean, P. (1997). Biogenesis of yeast wall and surface components. In: *Molecular and cellular biology of the yeast Saccharomyces, vol. 3. Cell cycle and cell biology*, Pringle, J., Broach, J., Jones, E. ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 229–362.

Palomero F., Benito S., Morata A., Tesfaye W., González M.C., Suárez-Lepe J.A. (2009) Effect on the autolysis process and the colouring matter of several commercial preparations with β-glucanase action in red winemaking. *Eur. Food Res. Technol*., 229, pp. 85–592

Palomero, F., Morata, A., Benito, S., González, M.C., Suárez-Lepe, J.A. (2007). Conventional and enzyme-assisted autolysis during ageing over lees in red wines: Influence on the release of polysaccharides from yeast cell walls and on wine monomeric anthocyanin content. *Food Chem*., 105, 838-846.

Parodi, G. (2002). L'affinamento dei vini con l'ausilio di preparati enzimatici. *VigneVini*, 5, 54–58.

Pellerin, P., Tessarolo, L. (2001). Optimizing the ageing of wine on lees. *Austr. Grapegr. Winemaker*, 444, 14-15.

Pérez-Ortín, J.E., García-Marínez, J., Alberola, T.M. (2002). DNA chips for yeast biotechnology. The case of wine yeasts. *J. Biotechnol.*, 98, 227-241.

Pérez-Serradilla, J.A., Luque de Castro, M.D. (2008). Role of lees in wine production: A review. *Food Chem.*, 111, 447-456.

Poinsaut, P. (1991). Le Mosalux, appareil de mesure du pouvoir moussant d'un vin. *Revue des Œnologues*, 59, 35-43.

Pozo-Bayón, M.Á., Martínez-Rodríguez, A., Pueyo, E., Moreno-Arribas, M.V. (2009). Chemical and biochemical features involved in sparkling wine production: from a traditional to an improved winemaking technology. *Trends Food Sci. Tech.*, 20, 289-299.

Pozo-Bayón, M.Á., Pueyo, E., Martín-Álvarez, P.J., Martínez-Rodríguez, A.J., Polo, M.C. (2003). Influence of yeast strain, bentonite addition, and ageing time on volatile compounds of sparkling wines. *Am. J. Enol. Vitic.*, 54, 273-278.

Pretorius, I.S., 2000. Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast*, 16, 675–729.

Pretorius, I.S., Høj, P.B. (2005). Grape and wine biotechnology: Challenges, opportunities and potential benefits. *Aust. J. Grape Wine R.*, 11, 83-108.

Pueyo, E., Martín-Álvarez, P.J., Polo, M.C. (1995). Relationship between foam characteristics and chemical composition in wines and cavas (sparkling wines). *Am. J. Enol. Vitic*., 46, 518-524.

Ramon-Portugal, F., Silva, S., Taillandier, P., Strehaiano, P. (2003). Immobilized yeasts: actual oenologic utilizations. *Wine Internet Technical Journal*. (1), (www.vinidea.net).

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B., Lonvaud, A. (2004). Alcune vinificazioni particolari. In *Trattato di enologia I, microbiologia del vino e vinificazioni*, Edagricole ed., pp. 489–528.

Robillard, B., Delpuech, E., Viaux, L., Malvy, J., Vignes-Adler, M., Duteurtre, B. (1993). Improvements of methods for sparkling base wine foam measurements and effect of wine filtration on foam behaviour. *Am. J. Enol. Vitic.*, 44 (4), 387-392.

Senée, J., Viaux , L., Robillard, B., Duteurtre, B., Vignes-Adler, M. (1998). The endogenous particles of a sparkling wine and their influence on the foaming behaviour. *Food Hydrocolloid.*, 12, 217-226.

Suzzi, G., Romano, P., Zambonelli, C. (1984). Flocculation of wine yeasts: frequency, differences and stability of the character. *Can. J. Microbiol.*, 30, 36–39.

Tabera, L., Munoz, R., Gonzalez, R. (2006). Deletion of BCY1 from the Saccharomyces cerevisiae genome is semidominant and induces autolytic phenotypes suitable for improvement of sparkling wines. *Appl.Environ. Microb.*, 72(4), 2351–2358.

Takeshige, K., Baba, M., Tsuboi, S., Noda, T., Ohsumi, Y. (1992). Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction. *J. Cell Biol.*,119, 301–11.

Tini, V., Zambonelli, C., Beneveli, M., Castellari, L. (1995). I ceppi autolisogeni di Saccharomyces cerevisiae in spumantizzazione. *Industrie delle Bevande*, XXIV, 113–118.

Tiţa, O., Jâşcanu, V., Tiţa, M., Sand, C. (2003). Economical comparative analysis of different bottle fermentation methods. (AVA) *Agrárgazdaság, vidékfejlesztés és agrárinformatika az évezred küszöbén, április,* 1-2.

Todd, B.E.N., Fleet, G.H., Henschke, P.A. (2000). Promotion of autolysis through the interaction of killer and sensitive yeasts: potential application in sparkling wine production. *Am. J. Enol. Vitic.*, 51 (1), 65-72.

Torresi, S., Frangipane, M.T., Anelli, G. (2011) Biotechnologies in sparkling wine production. Interesting approaches for quality improvement: A review. *Food Chem.*, 129 (3), 1232-1241.

Trevelyan, W.E. (1978). Effect of procedures for the reduction of nucleic acid content of SCP on the DNA content of Saccharomyces cerevisiae. *J. Sci. Food Agric.*, 29, 903-908.

Trione, D., Martínez, A. (2001). Elevage sur lies des vins rouges: la voie enzymatique. *Rev. Oenol.*, 101, 19–21.

Ubigli, M (2004). Guida all'assaggio degli spumanti. In *I profili del vino,* Edagricole ed., pp. 103-116.

Ubigli, M (2004). I test. In *I profili del vino,* Edagricole ed., pp. 117-171.

Ucke, C., Schlichting, H.J. (1997). Why does champagne bubble? *Phys. Technol. Quest.*, 2, 105-108.

Ugliano, M. (2009). Enzymes in winemaking. In *Wine Chemistry and Biochemistry,* Springer ed., pp. 103-126.

Van Rensburg, P., Pretorius, I.S. (2000). Enzymes in winemaking: Harnessing natural catalysts for efficient biotransformations – a Review. *S. Afr. J. Enol. Vitic.*, 21, Special Issue: 52-73.

Vázquez-Garcidueñas, S., Leal-Morales, C.A., Herrera-Estrella, A. (1998). Analysis of the b-1,3-glucanolytic system of the biocontrol agent Trichoderma harzianum. *Appl. Environ. Microb.*, 64, 1442-1446.

Veliky, I.A., Williams, R.E. (1981). The production of ethanol by Saccharomyces cerevisiae immobilized in plycation-stabilized calcium alginate gels. *Biotechnol. Lett.*, 3, 275.

Wada, M., Kato, J., Chibata, I. (1979). A new immobilization of microbial cells: immobilized growing cells using carrageenan gel and their properties. Eur. J. Appl. Microbiol. Biotechnol., 8, 241-247.

Whitehurst, R.J., Van Oort, M. (2009). Enzymes in potable alcohol and wine production. In *Enzymes in Food Technology*, Wiley ed., pp 195-207.

Wright, R. (2000). Transmission Electron Microscopy of Yeast. Microsc. Res. Techniq., 51, 496–510.

Yokotsuka, K., Yajima, M., Matsudo, T. (1997). Production of bottle fermented sparkling wine using yeast immobilized in double-layer gel beads or strands. *Am. J. Enol. Vitic.*, 48 (4), 471-481.

Zambonelli, C. (2006a). Influenza del lievito sulla qualità degli spumanti. Azione del ceppo. In *Microbiologia e biotecnologia dei vini. I processi biologici e le tecnologie della vinificazione*, Edagricole ed., pp. 207–208.

Zambonelli, C. (2006b). Le caratteristiche enologiche dei lieviti. Le modalità di sviluppo nei mezzi liquidi. In: *Microbiologia e biotecnologia dei vini. I processi biologici e le tecnologie della vinificazione* Edagricole ed., pp. 144-151.

Zambonelli, C. (2006c). La rifermentazione in bottiglia. In *Microbiologia e biotecnologia dei vini. I processi biologici e le tecnologie della vinificazione*, Edagricole ed., p. 207.

Zambonelli, C., Rainieri, S., Chiavari, C., Montanari, G., Benevelli, M., Grazia, L. (2000). Autolysis of yeasts and bacteria in fermented foods. *Ital. J. Food Sci*., 1 (12), 9-21.

Zhao, J., Fleet, G.H. (2003). Degradation of DNA during the autolysis of *Saccharomyces cerevisiae*. *J. Ind. Microbiol. Biotechnol.*, 30, 175-182.

Zhao, J., Fleet, G.H. (2005). Degradation of RNA during the autolysis of Saccharomyces cerevisiae produces predominantly ribonucleotides. *J. Ind. Microbiol. Biotechnol.*, 8, 415-423.

Zinnai, A., Venturi, F., Quartacci, M.F., Andreotti, A., Andrich, G. (2010). The kinetic effect of some wine components on the enzymatic hydrolysis of β-glucan. *S. Afr. J. Enol. Vitic.*, 31, 9-13.

Zoecklein, B. (2002). A review of Méthode champenoise production. *Virginia Tech.* Reprinted 2002. Publication 463-017W.

8 GLOSSARY

5'-AMP - Ribonucleotide adenosine 5′-monophosphate

5'-GMP - Ribonucleotide guanosine 5′-monophosphate

5'-IMP - Ribonucleotide inosine 5′-monophosphate

5'-UMP- Ribonucleotide uridine-5'-phosphate

Aphrometer - A device used to measure the pressure within a bottle of sparkling wine.

A.S. - (Specific Activity): Is the enzymatic activity per milligram of total proteins (expressed as $mg_{glucose}\ l^{-1}\ min^{-1}\ mg_{proteins}^{-1}$). Specific activity gives a measurement of the purity of the enzyme.

Autolysis - Enzymatic self-degradation of cells constituents thus causing the release of many parietal and cytoplasmic compounds with proved foaming and sensory properties.

Autophagy - Autophagy is a catabolic process omnipresent in eukaryotic cells that involves cytoplasm degradation in the vacuole or lysosome lumen. This degrading process seems to be essential for cells adaptation to external and internal stress factors.

β-glucanases - EC number 3.2.1.58, The activity of these enzymes determine the hydrolysis of glucans and the release of cell wall mannoproteins, without involving the cell wall directly. As a consequence of this degradation, the cell wall becomes less rigid and polysaccharides are released.

Base wine.- This is the still wine used for secondary fermentation. Base wine should always presents typical characteristics like moderate alcohol content, pale colour; fruity aroma; low residual sugar content; low volatile acidity and should usually have been subjected to tartaric stabilization. Moreover, is convenient for base wines to show quite higher total acidity.

Bidule - This is a hollow polyethylene cup usually 17mm Ø and 14 mm high. It helps the disgorging process, prevents leakage and metal contact from the crown cup.

BSA – BSA is a serum albumin protein derived from cows. It is often used as a protein concentration standard.

Disgorging or dégorgement - This procedure is performed by inserting the neck of the bottle in a solution which freezes the sediment trapped in the *bidule*. Bottles are then placed neck-up, uncorked and the pressure within the bottle ejects both *bidule* and ice plug.

Dosage solution or liqueur d'expédition - this is a solution composed of wine, sugar, brandy, SO_2, citric acid, copper sulphate, with a different formula for each firm, used to fill sparkling wines bottle after disgorging.

EC number - the Enzyme Commission number (EC number) is a numerical classification scheme for enzymes, based on the chemical reactions they catalyze. As a system of enzyme nomenclature, every EC number is associated with a recommended name for the respective enzyme.

Enzymatic preparation - they are generally composed of a mix of different enzymes like pectin-lyases, pectinesterases, polygalacturonases and cellulases. Otherwise, it is possible to obtain a pure culture. They are obtained by fermentation of selected culture of fungi like *Aspergillus niger* or *Trichoderma harzianum*, by extraction from bacterial cultures such as *Lactobacillus fermentum* or from eggs' white (lysozyme). Since enzymes naturally present in wines are often unable to sufficiently catalyse the different bio-transformations occurring during winemaking, exogenous enzymatic preparations have been introduced.

Flocculation - this is a distinctive yeast feature consisting in cells aptitude to form compact lumps which form a deposit in the bottle. This characteristic is a frequently occurring trait in *S. cerevisiae*.

HM (foamability) - this parameter (mm) represents the maximum height reached by sparkling wine foam 1 or 2 minutes after CO_2 injection.

HS (Height stability) - this foam parameter (mm) is obtained maintaining the same conditions of pressure and flow of CO_2, in this circumstance, sparkling wine foam collapses to stabilize at a level HS.

International Unit (U) - one U is defined as the amount of enzyme that catalyzes the conversion of 1 micro mole of substrate per minute (25°C, pH value and substrate concentration that yield the maximal substrate conversion rate).

K_M.- representing the amount of substrate corresponding to ½ V_{max}. This parameter reflects the efficiency of the enzyme-substrate complex formation.

Laminarin - it is a storage β(1→3)-glucan (a polysaccharide of glucose) found in the brown alga *Laminaria digitata*.

Mannoproteins - glycoproteins located in the outermost layer of the yeast cell wall, where they are connected to a matrix of amorphous β-1,3-glucan by covalent bonds. When found in wine, they exist as polysaccharide and protein moieties.

Principal Component Analysis (PCA) - this is a mathematical procedure that uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables called principal components. The number of principal components is less than or equal to the number of original variables.

Riddling or rémuage - during this phase of sparkling wines production, gravity slowly conveys yeasts sediment into the neck of the bottle, facilitating the subsequent lees removal.

Scanning Electron Microscopy (SEM) - sample surface is scanned with a high-energy beam of electrons in a raster scan pattern. The electrons interact with the atoms that compose the sample and produce signals containing informations about the sample's surface topography, composition, and other properties such as electrical conductivity.

Sparkling wine - a sparkling wine is a special wine obtained by a secondary fermentation of a still wine, called base wine. Depending on the production technology, they can be classified into sparkling wines produced by traditional in-bottle secondary fermentation and sparkling wines produced by secondary fermentation in hermetically-sealed tanks.

TDN - 1,1,6-trimethyl-1,2-dihydronaphthalene

Traditional method - this method for sparkling wines production requires a second in-bottle alcoholic fermentation of a base wine and a prolonged ageing in contact with lees. During this process, the characteristic endogenous CO_2 is produced with the consequent release of carbon dioxide when the bottle is uncorked.

Transmission Electron Microscopy (TEM) - this microscopy technique consists in the transmission of an electron beam through an ultra thin sample, the electron beam interacts with the sample while passes through it. The formed image is then magnified and focused onto an imaging device, on a layer of photographic film.

TS (Foam stability time) - this foam parameter (s) represents the time elapsed before all sparkling wine bubbles disappear after gas flux interruption.

V_{max} - corresponding to the maximum velocity at which an enzyme catalyzes a reaction, expressed as mg l^{-1} min^{-1} of glucose.

Yeasts Immobilisation - this technique consists yeast cells occlusion in polysaccharide gels such as agar, alginate and carrageenan. In sparkling wines production, a certain

amount of immobilised yeasts is easily inserted into the bottles and simply removed with the ice plug during disgorging.

APPENDIX

Lists of published papers and poster communications.

1 S. Torresi, M.T. Frangipane, R. Massantini, A.M.V. Garzillo (2012). Yeast lysis during bottle-fermented sparkling wines ageing in presence of a β-glucanase oenological preparation. Part 1–preliminary study of β-glucanase oenological preparation. *Submitted to Food Chemistry.*

Abstract: This study focuses on a preliminary characterization of a commercial enzymatic preparation Lallzyme MMX® containing β-glucanase, poly-galacturonase and pectinase activities for further applications in sparkling wines production by the traditional method. The effect of the β-glucanase activity of the Lallzyme MMX® preparation on yeast lysis enhancement of two different yeast strain, Saccharomyces cerevisiae r.f. bayanus BCS103® and Saccharomyces cerevisiae r.f. bayanus Lalvin EC1118, was tested.

The pH effect on enzyme activity showed that the optimum pH value for β-glucanase was 5.00; nevertheless, the residual activity at wine pH is about 66%, which is sufficient for the user's purposes. A higher activity was detected in presence of the EC1118 yeast strain, thus showing the enzyme's lytic activity on glucans of yeast cell wall. Ethanol influence on enzyme activity was also tested at two ethanol concentrations, 11,3 and 12,7% v/v, corresponding to those of a base wine and a sparkling wine respectively. We detected a slight inhibition effect, that did not compromise the lytic activity of beta-glucanase. Kinetic parameters (kM and Vmax), obtained by a Michaelis-Menten kinetic using Laminarin as substrate, demonstrate that EC1118 strain presents a more efficient enzyme substrate complex formation than BCS103, because of its lower kM value. Further studies will assess enzyme lytic activity during induced autolysis in synthetic wine and during sparkling wines production by the traditional method.

2 S. Torresi, M.T. Frangipane, M. Contini, A.M.V. Garzillo. Yeast lysis during bottle-fermented sparkling wines ageing in presence of a β-glucanase oenological preparation. Part 2 –β-glucanase applications in sparkling wine. *Submitted to Food Chemistry.*

Abstract: Sparkling wines are special wines produced by an in-bottle secondary fermentation of a base wine followed by a long-lasting ageing in contact with lees. The autolysis of yeasts takes place during ageing, it's an enzymatic self degradation of cell

wall constituents and parietal and cytoplasmic compounds with a great influence on sparkling wine's quality are released.

This work dealt with the analysis of exogenous β-glucanase addition effects on yeasts morphology, foaming properties, chemical composition changing and sensory quality implications on sparkling wines

Scanning and transmission electron microscopy observations were useful to describe yeast lysis during ageing. Free amino acids content showed that autolysis occurs 8 months after tirage for all sparkling wines and also the effect of enzymatic treatment on yeast lysis, particularly manifested for some amino acids 12 months after tirage. Total Proteins had no positive influences on foaming properties. Sensory analysis showed no significant differences between samples, but all sparkling wines were judged as good.

3 M. T. Frangipane, S. Torresi, D. De Santis, R. Massantini (2012). Effect of drying process in chamber at controlled temperature on the grape phenolic compounds. *Italian Journal of Food Science*, 24, pp.1-7.

Abstract: The aim of this paper was to study the phenolic compounds evolution during the off-vine drying process in chamber at controlled temperature and to assess whether this dehydration technique might be used to avoid strong oxidations. Grapes were placed in perforated plastic boxes inside a chamber; fans were installed to ensure humidity and temperature control. Phenolic compounds were characterized by HPLC. Dehydration was fast and regular. This technique ensures phenols protection, artificially dried grapes maintained a higher phenolic concentration than those just harvested. This grape drying technique may ensure the production of sweet wines in which phenols content is preserved.

4 S. Torresi, M. T. Frangipane, G. Anelli (2011). Biotechnologies in sparkling wine production. Interesting approaches for quality improvement: a Review. *Food Chemistry*, 129 (3), pp. 1232-1241.

Abstract: Sparkling wines are produced by a secondary fermentation of a base wine followed by a prolonged ageing in contact with lees. In the traditional method, the refermentation takes place in the same bottle used for market distribution. The autolysis of yeasts occurs during the ageing of sparkling wines, thus causing the release of many parietal and cytoplasmic compounds which have a great influence on sparkling wine's quality. The modern technologies for the production of sparkling wine are very different from the one developed by Abbot Dom Pierre Pérignon in the 17th century. Over the

years, many advances have been made, this paper aims at reviewing the recent knowledge in the application of biotechnologies for the improvement of sparkling wine's quality, in particular the factors affecting foaming properties and sensory qualities. Future perspectives and trends are also considered.

5 Torresi S. (2011) Yeast lysis during bottle-fermented sparkling wines ageing in presence of a β-glucanase oenological preparation. *Proceedings of the 16th Workshop on the developments in the Italian PhD Research on Food Science Technology and Biotechnology*, University of Milano e Piacenza, Lodi, 21-23 September, pp. 186-190.

Abstract: This PhD thesis dealt with the study of yeast lysis in bottle-fermented sparkling wines ageing in presence of a β-glucanase oenological preparation. A preliminary characterization of the enzymatic preparation in synthetic buffers was performed. An analysis of enzyme effects on yeasts morphology, foaming properties, chemical composition changing and sensory quality implications on the obtained sparkling wines was also conducted.

6 Torresi S. (2010) Study of a beta-Glucanase preparation in synthetic wine to enhance yeast lysis. *Proceedings of the 15th Workshop on the developments in the Italian PhD Research on Food Science Technology and Biotechnology*, University of Napoli I – Federico II, Portici, 15-17 September, pp. 301-302.

Abstract: In this paper, the first activity of the PhD Thesis project is reported. A kinetic study of a beta-Glucanase preparation has been conducted in synthetic wine in order to assess its activity also in presence of Saccharomyces cerevisiae EC1118 yeast strain. Moreover, ethanol and pH effects on enzyme activity have been determined.

7 Torresi S. (2009) Biotechnological applications to optimize sparkling wine quality. *Proceedings of the 14th Workshop on the developments in the Italian PhD Research on Food Science Technology and Biotechnology*, University of Sassari, 16-18 september, pp. 448-449.

Abstract: This PhD thesis research project is directed to improve the quality of a sparkling wine produced by the classic method (Champenoise) using selected yeasts and enzymatic preparations, in order to obtain a product in which the autolysis process is favoured and foaming and sensory properties are optimized.