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Enzymes in œnology :

Production, Regulation, applications

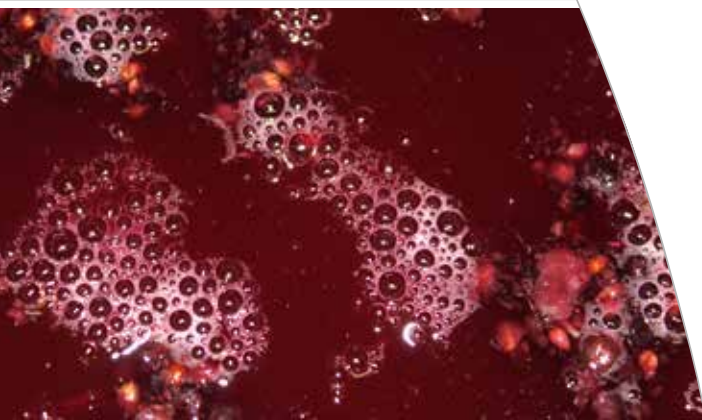


INTRODUCTION

The booklet “Itinéraires” about enzymes in œnology aims to provide elements of understanding and/or information on the nature of enzymatic preparations, their production, composition, œnological interest and applications in pre and post fermentative operations.

This booklet may seem dense, but its structure in 4 chapters allows the readers (producers, technicians, institutionals, ...) to easily find the desired information.

It is important to state in the preamble that all of this work began twelve years ago with the aim to clarify the composition of the enzyme preparations and actions on the grapes, musts and wines. The regulatory environment through the OIV and technical references have evolved, this is the current state of knowledge what is presented here.



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Chapter 1

Enzymes and their Production

What are Enzymes?

Enzymes are proteins and biological catalysts, which authorise biochemical reactions under conditions of temperature and of biological media. In the presence of adequate concentrations of their substrate, and under suitable physico-chemical conditions (pH, T°), enzymes convert this substrate to form one or more product(s).

The specificity of enzyme action is usually compared to a key that only opens one door. Each enzyme is specific to a type of reaction (hydrolysis, decarboxylation, or oxidation) and substrate (chemical structure, spatial configuration, etc.). Enzymes are therefore linked to their substrate in a combination called an 'enzyme-substrate complex' (see diagram 1), which can then catalyze a reaction. An example of enzyme action is cutting a substrate into two products before separating off from them.

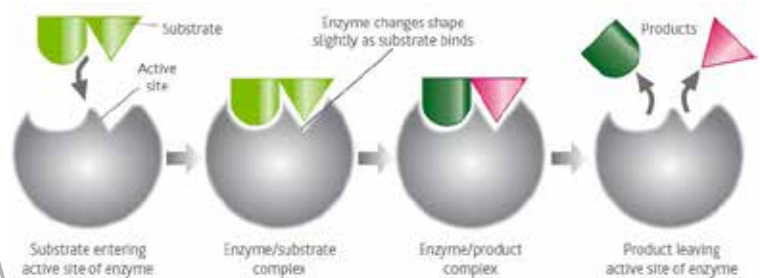


Figure 1 : Enzyme Action Mechanism

The Production of Industrial Enzymes

Although enzymes are present in all living organisms, the exploitable source of enzymes used by industrialists is microscopic bacteria and fungi. These microorganisms are taken from various natural environments (forests, mountains, fjords, etc.). All enzyme manufacturers have their own strains. Enzymes of oenological interest come mainly from microorganisms belonging to the *Aspergillus* and *Trichoderma* species. Each of these species includes many strains, whose ability to produce enzymes varies greatly; for example, there are almost 10,000 known strains of *Aspergillus niger*. The first stage in enzyme manufacture is to identify the most suitable species and strain to achieve the desired technological objective. This search for the

most suitable microorganism involves three possible forms of technology :

- Optimization of selected strains by controlled fermentation conditions.
- Mutagenic improvement of strains (gene mutation caused by certain conditions) which, for example, improves productivity.
- Genetic engineering, which involves inserting a gene coding for an enzyme of industrial interest into a microorganism. This route is not currently favored for oenological applications.



How can you tell if enzymes are from a genetically modified organism?

The International Organization of Vine and Wine (OIV) requires enzyme manufacturers to inform their customers if the enzymes supplied have been produced by GMOs. This information must be given either on the product label or on the technical documentation (resolution OIV-OENO 485-2012).



The industrial production process involves four main stages (diagram 2)



Figure 2 : Main Production Stages for Commercial Enzyme Preparations

Stage 1 : Fermentation

Enzymes are produced by microorganisms under fermentation conditions. Microorganisms are placed in culture, either in a liquid substrate (submerged fermentation) or on a solid substrate (solid-state fermentation). Temperature, hygrometry, pH, and nutrition conditions are essential for fermentation to produce the desired enzymes. All enzyme manufacturers have developed specific expertise to control these external factors, which condition the final quality of enzymes produced. The nutrition source, called the 'substrate', also forms part of manufacturers' expertise. The substrate is the source of carbon, nitrogen, vitamins, and minerals needed to produce enzymes. Each strain has a specific substrate formula.

Stage 2 : Separation

Flocculation and filtration are generally used to separate enzymes from the microorganism that produced them. The resulting enzyme-containing liquid then undergoes various filtration stages.

Stage 3 : Concentration and Purification

The clarified enzyme-containing liquid must be concentrated to give a sufficiently active product. Concentration is carried out using membrane technology and/or evaporation. At this stage, the producer can proceed to a purification step if necessary.



Stage 4 : Formulation

At the end of the concentration stage, the producer can formulate, ie put the enzyme concentrate in a storable form, marketable and suitable for industrial use. During this step, the producer can also proceed to the blend of enzymes of different fermentations. Enzymes are sold in either granulated or liquid form: manufacturers either subject the concentrated solution to a drying and granulation process, or package the enzyme solution directly after stabilization (liquid enzymes).

The granulation process requires the use of an inert carrier material to stabilize the enzymes. Maltodextrin and sodium citrate are commonly used as inert carriers for oenological enzyme preparations. Liquid formulations are stabilized to prevent the development of microbial contaminations and stabilize enzyme

activity. Potassium chloride (KCl), glycerol and ammonium sulfate are the preferred substances for oenological enzymes since they are compatible with compounds found in wine.

To know

The quantity of glycerol supplied by packaged liquid preparations is about 200 times lower than the glycerol content produced by yeast during alcoholic fermentation. At this dose, it has no impact on the taste of wine.



What is the difference between liquid and granulated preparations ?

Liquid enzyme preparations must be stored at low temperature (ideally at 4°C) to maintain their activity and must be used within 24 months of manufacture. They can be added directly to must or wine.

Granulated preparations can be stored in their original packaging for up to 4 years after their manufacture date and must be rehydrated before use.



The Formulation of Oenological enzyme Preparations

In response to the complex structure of grape plant cell wall, oenological enzyme preparations often contain several enzymes acting in synergy. For this reason, **pectolytic** enzymes used to break down pectic substances in grapes always contain a mixture of pectin lyase (PL), pectin methylesterase (PME), and polygalacturonase (PG) activity. These activities are produced simultaneously by microorganisms and replicate the action of endogenous grape enzymes. Oenological research and natural mechanisms at work in grapes to convert them into wine form the essential core knowledge needed to formulate specific oenological enzymes.

Removing **cinnamoyl esterase** activity from certain enzyme preparations via a purification process is the result of improved understanding of the free phenol acid and volatile phenol formation process achieved in the 1990s. Following the discovery of these mechanisms for converting phenol acids into ethylphenols and vinylphenols, oenological enzyme manufacturers developed enzyme formulations with low cinnamoyl esterase activity (FCE: Free from Cinnamoyl Esterase). Cinnamoyl esterase (CE) activity is produced naturally in smaller or larger

quantities by pectinase-producing microbial strains. It encourages the formation of volatile phenol substrates responsible for aromatic deviations: medicinal taste in white wines; horse sweat and stable aromas in red wines contaminated with *Brettanomyces*.

Removing this activity via purification requires complex physical treatment that can last several days: simultaneous removal (never absolute) of **cinnamoyl esterase** and **anthocyanases (betaglucohydrolases)**, which damage wine color (especially in red wines). This purification stage usually results in around a 30% loss of activity, which is taken into account in the product's final concentration.

What is an enzymatic preparation formulator?

An oenological enzyme formulator purchases concentrated liquid or granulated enzyme preparations, which contain various activities of oenological interest, from an enzyme manufacturer (who has the required fermentation technology). The final blend of these enzyme preparations depends on the desired oenological effect and the formulator's expertise.

Chapter 2

The Oenological Role of Enzymes

In this chapter are essentially treated the enzymes involved in the degradation of plant cell wall. In the process of transforming grapes into wine (alcoholic fermentation, malolactic fermentation), many activities are also involved but are not described here.

Enzyme Activity in Grapes and Wine

Grape berries contain two of the three main oenological pectolytic enzymes: a pectinmethylesterase (PME) [R1; R2] and an endopolygalacturonase (endo-PG) [R1], although the latter has low activity.

Pectinmethylesterase occurs mainly in grape-berry skin. It is 2-3 times less abundant in the pulp and almost non-existent in pips. PME activity varies according to grape variety, and increases as the fruit matures [R3].

Polygalacturonase (PG) promotes the physiological maturation of cell walls of the grapes. It works in concert with the PME by acting on the molecules derived from the hydrolysis caused by it. The activities of PME and PG decrease late maturation and have low tolerance of winemaking conditions (alcohol, pH, ...).

In addition to these pectolytic enzymes, most activities come from **glycosidases**. The most significant activity is β -D-glucosidase, but β -L-arabinofuranosidases and β -L-rhamnosidases are also present. Synthesis of these enzymes also increases as the fruit matures [R4]. Nevertheless, grape β -D-glucosidase has low activity in must pH and is inhibited by glucose [R5].

In 1999 and in 2001, a team of French researchers and a team of Australian researchers studied respectively the activity of cell-wall modifying enzymes during grape-berry (*Vitis vinifera* L.) development. Pectinmethylesterase activity was observed. By contrast, although polygalacturonase mRNA was present, no activity was detected. The researchers also demonstrated the activity of other oenological enzymes, such as β -galactosidase, but found no trace of galactanase, cellulase, and xylanase [R6; R7].

Vines also produce **β -D-glucanases** during fungal attacks [R5].

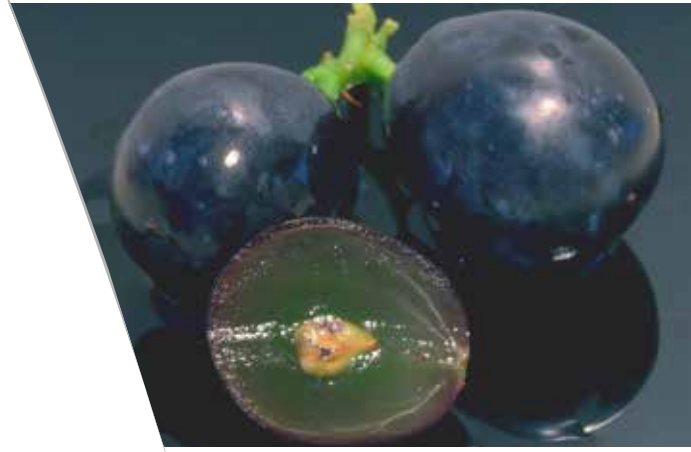
Enzymes are not produced only by grape berries; they are also synthesized by **yeasts and other endogenous microorganisms**. These are predominantly non-alcohol tolerant yeasts (*Hanseniaspora*, *Kloeckera* and *Candida*) rather than *Saccharomyces cerevisiae*. In particular, they secrete glycosidases, cellulases, esterases, etc. into both their periplasmic space and the medium. Unlike grape-berry glycosidases, those produced by yeast are not inhibited by glucose [R8].

Botrytis cinerea, which causes noble rot on grapes, produces a highly active pectinmethylesterase, as well as polygalacturonases [R3].

Most enzymes found in industrial preparations also occur in grapes, yeast and spoilage flora, with the exception of pec-

tin lyases and certain glycosidases such as apiosidase. Nevertheless, use of these enzymes is still justified since they are present in insufficient quantities and are not active enough under winemaking conditions. They have poor tolerance of low temperature and must pH (2.8-3.8), and are inhibited by high contents in glucose, SO₂, red-wine tannins, and alcohol [R9].





The Main Oenological Enzyme Activities

Although this list of enzymes is not exhaustive, it corresponds to the most well-known enzyme activities.

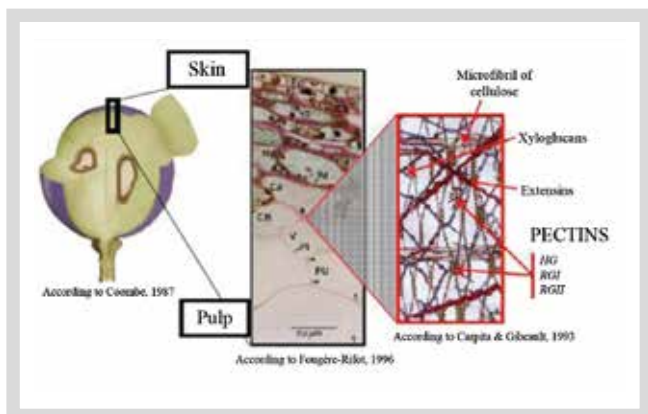


Figure 3 : Diagram of an undifferentiated plant cell and model of the cell-wall structure [R10; R11; R12]

Pectolytic Enzymes

Pectolytic enzymes break down pectic polysaccharides comprising plant cell walls. They include enzymes that break down homogalacturonans, and enzymes that break down other pectin components: type I and II rhamnogalacturonans, and their side chains (galactans, arabinans, and arabinogalactans). (Figure 3)

> Pectinmethylesterase (PME) (figure 4)

Pectinmethylesterases release methanol and polygalacturonic acid from pectin molecules. They have high affinity for polygalacturonic acid methyl esters. Their optimum activity is at pH 3.5-4.5 (fungal enzymes) and pH 7-9 (plant enzymes), and at a temperature approaching 37°C [R13].

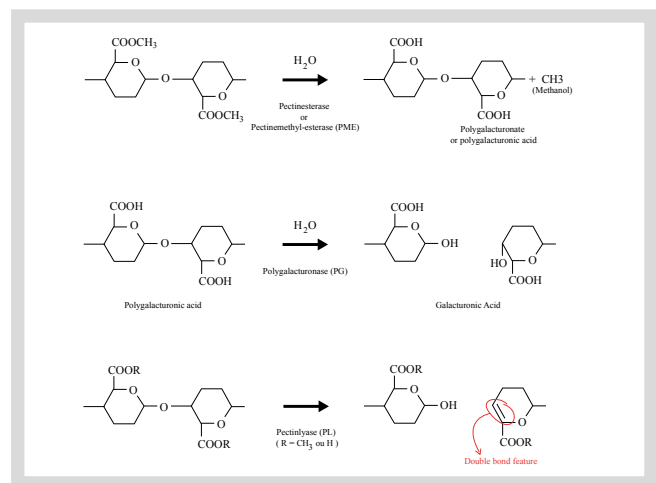


Figure 4 : Mode of action of Pectinases

> Polygalacturonases (PG) (figure 4)

These enzymes catalyze reactions that break chemical bonds in galacturonic acid molecules. There are various types of polygalacturonases, which act in different ways (endogenous and exogenous):

- Exopolygalacturonases act recurrently on the non-reducing extremity of chains. This reaction produces either galacturonic acid or digalacturonic acid. They have high affinity for non-methylated pectins and are inhibited by rhamnose or side chains. They are isolated mainly from fruit and vegetables and their optimum pH is 4.5-5.5.
- Endopolygalacturonase reaction products are galacturonic acid monomers, dimers, and trimers. The reaction yield depends largely on the level of methylation; if methanol has caused more than 75% esterification, pectins are no longer hydrolyzed by the only PG.

PG requires prior use of PME, which reduces the level of methylation [R14].

> Endopectin Lyases (PL) (figure 4)

These enzymes work in a special way, catalyzing the cleavage of glycosidic bonds. This reaction results in the formation of a double bond. Unsaturated compounds obtained in this way have maximum absorbance at around 235 nm, making them easy to detect. Fungal endopectin lyases have optimum activity at pH 5-5.5.

Unlike PGs, they have higher affinity as the level of pectin methylation increases.

> Galactanases

There are as many types of galactanases as their various substrates (galactans, type I and II arabinogalactans). Fungal galactanases have optimum activity at around pH 4 and at a temperature of 40°C.

Hemicellulolytic Enzymes or Hemicellulases

Hemicelluloses are a class of polysaccharides very varied, both in terms of constituent monosaccharides at the level of branches. Xyloglucans, xylans and arabinoglucuronoxylans have been described in the polysaccharide constitution of the cell walls of grapes [R15].

> Xylanases

These enzymes hydrolyze bonding in variously substituted xylans (arabinoxylans, arabinoglucuronoxylans, etc.). Given the nature of xylan substituents, the use of other enzymes acting in synergy with xylanases is required to break them down completely.

There are two main xylanase families:

- **Exoxylanases**, which produce mainly D-xylose from the non-reducing extremities of xylans.
- **Endoxylanases**, which produce xylose and xylobiose via random action within chains.

Xylan complexes are secreted by many fungi, bacteria and yeasts. Fungal xylanases have optimum activity at pH 4-5 and at temperatures of 40-60°C [R15 ; R16]



Cellulolytic Enzymes or Cellulases [R13] (figure 5)

These enzymes break down cellulose. This action is carried out by an enzyme complex [R17] consisting of :

- **Endoglucanases**, which randomly catalyze osidic bonds in cellulose. They are also involved in breaking down xyloglucans in the hemicellulose group.
- **Exoglucanases**, which release cellobiose and glucose respectively. They also work in relay on fragments obtained by the action of endoglucanase.
- **β -glucosidase** which breaks down cellobiose in glucose. This enzyme is also involved in releasing aromatic precursors.

Since cellulose has a crystalline structure, it is only broken down by enzymes to a limited extent.

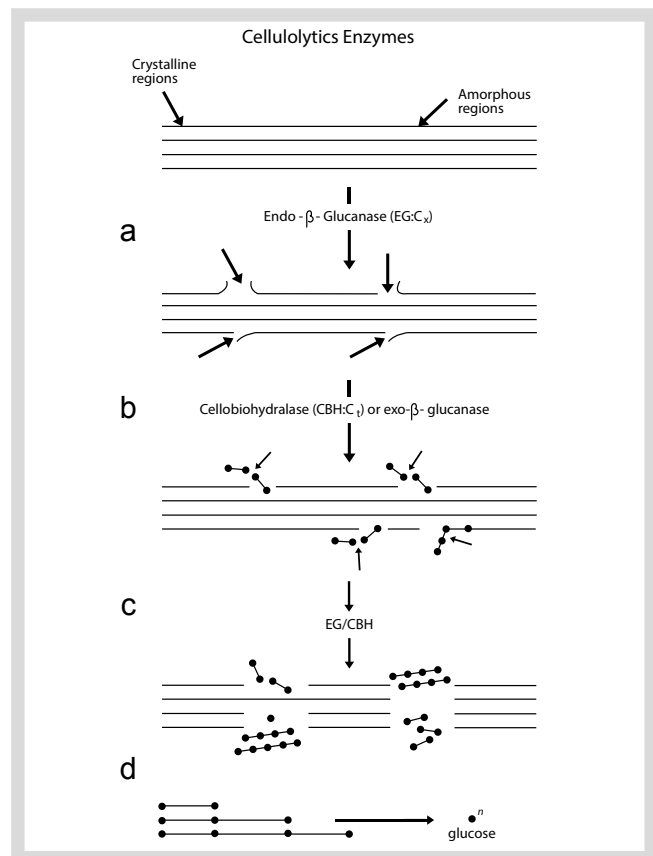


Figure 5 : schematic representation of the synergistic action (a,b,c,d) of enzymes during the cellulolysis

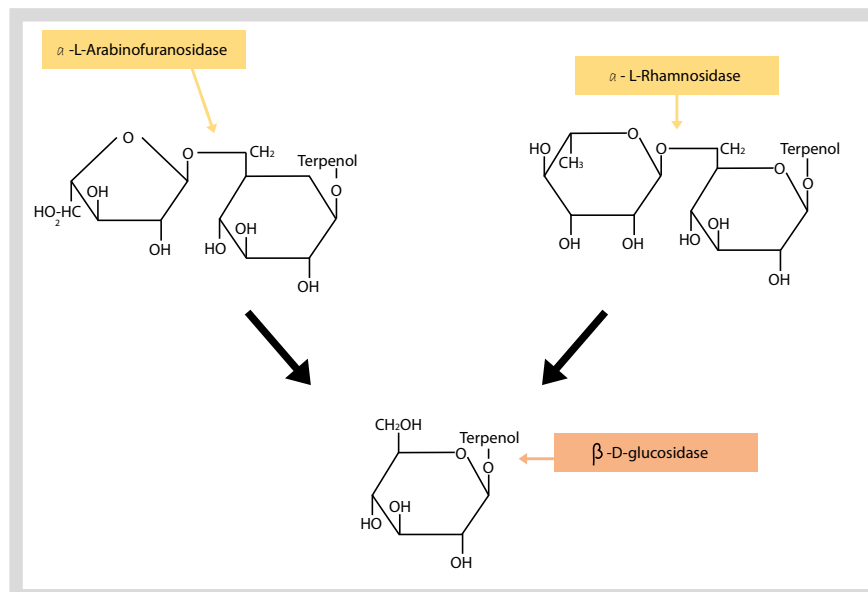


Figure 6 : Monoterpenols release mechanism from their glycosidic precursors.

Glucosidases

Grapes contain terpenols bound to diglycosides [R18]. An enzyme complex is involved in the release of terpenol [R19]. The grape aroma characterizes the grape variety and is therefore a key element of the aromatic profile, the typically and, therefore, the quality of the wines. Certain aromatic compounds are found both in free form and associated with carbohydrate residues. Free aromas can be detected by smell while bound form is odorless. In the grape berry, the combined fraction is predominant compared to the free form. Thereby releasing the bound form it is possible to increase the potential of aromatic wines.



> α -L-arabinofuranosidase (figure 6)

This enzyme enables arabinoxylans, arabinans, and arabinogalactans to be broken down completely. It catalyzes inter-oxidic bonding in arabinofuranosyl-glucoside precursors of complex aromas.

> α -L-rhamnosidase (figure 6)

This enzyme enables sequential hydrolysis of rhamnosyl-glucoside precursors of complex aromas.

> β -D-glucosidase (figure 6)

Diglycosides release aglycone after their terminal sugar has been cut by an α -L-arabinofuranosidase and an α -L-rhamnosidase working in sequence. Volatile substances (monoterpenols, norisoprenoids, and volatile phenols) are released from monoglycosides.



Lysozyme

Lysozyme is an enzyme occurring in egg whites. It is a natural preservative and has been used medicinally and by the food industry for many years. In particular, its specific action against Gram+ bacteria, including lactic acid bacteria, has led to its use in cheese. Sulfur dioxide is generally used to ensure the microbial stability of wine. Many research studies have demonstrated [R22, R23] that lysozyme could be used to ensure the microbial stability of wine since it inhibits lactic acid bacteria. This would enable lower doses of SO₂ to be used. Lysozyme inhibits malolactic fermentation (MLF) in white wines, stabilizes red wines after MLF, and protects wines in which alcoholic fermentation is sluggish.

Ureases

Urea is the main precursor of ethylcarbamate, which in the light of high doses administered to laboratory animals is regarded as potentially carcinogenic. Ethylcarbamate is produced naturally in all fermented drinks and food. The maximum authorized content in wine is regulated in some importing countries. In order to limit the presence of this molecule, enzyme-catalyzed breakdown of urea is emerging as a solution to remove ethylcarbamate formation at source. This enzyme, produced by the strain *Lactobacillus fermentum*, is not used anymore in wineries.

Non-cellulolytic β -glucanases

Exo-(1→3)- β -glucanases and (1→6)- β -D-glucanases break down glucans produced by *Botrytis cinerea*, which occur in musts infected with gray or noble rot, and also the polysaccharides of the yeasts cell walls [R20]. Their optimum temperature is 30-50°C and optimum activity is between pH 4.5 and pH 6.

Proteases (under evaluation)

The use of bentonite to limit protein breakdown depletes the organoleptic qualities of wine. The use of proteolytic enzymes as an alternative has been assessed. Although exogenous proteases remain extremely active under the conditions (of pH and alcohol content) in must and wine, they have no effect on their protein content. Nevertheless, new research [R21] is renewing interest in the use of these enzymes, demonstrating the conditions under which they act (prior heat denaturation of proteins). Although this is not the only solution to stabilize proteins, protease treatment of musts and white wines may enable a much lower dose of bentonite to be used later. The OIV is also examining the broader interest of proteases for other oenological applications in musts and wines.



Table 1 below shows the theoretical relationships between enzyme activities and technological effects. Then, the supply in œnological enzymes today is structured around three main families:

- Pectolytic preparations are the most widely used in enology. They have three main activities: polygalacturonase, pectin lyase, and pectin methylesterase. These activities are involved in breaking down the primary chain of grape pectin,

hence their appreciable effect in maceration and depectinization/clarification. Pectolytic enzyme preparations also contain other hemicellulase, cellulase, and β -glucosidase activities, which are present in lower concentrations than those mentioned above.

- Aroma-releasing, glycosidase-based preparations, whose function is to release grape varietal aromas, especially aromas in the terpenol family.

- Glucanase-based preparations, whose specific function is to break down yeast parietal polysaccharides and glucans excreted by *Botrytis cinerea*.

Enzymatic activities		Pre-fermentative steps				Post-fermentatives steps		
		Settling	Clarification	Pressing	Extraction / Maceration	Release of aromas	Improving filterability	Ageing on lies
Pectolytic	Polygalacturonase (PG)	■	■	■	■		■	
	Pectine Lyase (PL)	■	■	■	■		■	
	Pectine Méthyl Estérase (PME)	■	■	■	■		■	
	Galactanase		■				■	
Hemicellulotyc	Xylanase			■	■			
Cellulases	Endo - (1→4) - β - D-Glucanase			■	■		■	■
	Exo - (1→4) - β - D-Glucanase			■	■		■	■
Glycosidases	β - D - glucosidase			■	■	■		
	α - L - arabino-furanosidase			■	■	■		
	α - L - rhamnosidase			■	■	■		■
Glucanases	exo (1→3) et (1→6) β - D - glucanases		■				■	■

Table1 : enzymes and technological rules

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Chapter 3

Enzyme Regulations

General Framework

In the vast majority of cases, the use of food enzymes is regarded as a processing aid. In other words, enzymes are involved in the manufacturing process of foodstuffs but have no function in the finished product. They are subject to regulations that vary greatly from one country to another. The JECFA, the scientific body that assesses food safety for the *Codex Alimentarius* (an organization linked to WHO and responsible for drawing up worldwide food standards), assesses and draws up specifications for enzyme preparations, as well as nomenclature for each enzyme activity. This nomenclature is recognized by the 188 member countries of the *Codex Alimentarius*.

In the EU, enzymes, like all substances used in the preparation of foodstuffs, are part of the FIAP (Food Improvement Agents Package) assessment and marketing authorization program. This European program launched in 2008 has drawn up specific measures relating to enzyme production and marketing procedures: Regulations (EC) 1332/2008 and (EC) 1331/2008. An ambitious toxicological assessment plan for each enzyme activity on the European market is also envisaged, which will lead to a positive list of authorized enzymes by 2020.

Alongside this European legislation, which remains the regulatory reference applied by the 28 member states, the OIV has drawn up and described enzyme activities used in winemaking. Monograph OENO 365-2009* on enzyme preparations was revised in 2012. The purpose of this revision was to incorporate the latest knowledge about enzymes, which is described in detail in the new datasheet of the Code of Oeno-

logical Practices adopted in June 2013 (OENO 498/2013*). The monograph on enzyme preparations also makes recommendations for labelling and production methods. Each recognized enzyme activity in an oenological enzyme preparation is also described in a specific monograph. Among other things, these monographs describe analytical methods that can be used to measure the various enzyme activities.

Enzyme preparations are currently recognized in wine for the following applications :

- improving filterability in musts (OENO 14/04)* and wines (OENO 15/04)
- release of aromas in musts (OENO 16/04) and wines (OENO 17/04)
- release of yeast compounds into wines (OENO 18/04)
- clarification in musts (OENO 11/04) and wines (OENO 12/04)
- maceration in musts (OENO 13/04)
- hydrolysis of glucans produced by *Botrytis cinerea* (OENO 03/85)
- urea hydrolysis (OENO 2/95)
- protein hydrolysis (adoption process underway in 2014).

Modified
by OENO
498/2013



Focus on organic wine production [R24]

Specifications for the manufacture of organic enzymes are constantly changing. Only the use of pectolytic enzyme preparations for clarification is currently authorized. Betaglucanase and lysozyme are currently prohibited.

*: Resolution number, available on the OIV website (<http://www.oiv.int>)

Measuring Enzyme Activity

The enzyme activities presented above can be assayed using protocols adopted by the OIV. The OIV recommends that these measurements be expressed in nanokatal (nanomoles of product formed/seconds under specified protocol conditions) and/or in U/g preparation, except for the overall rheological measurement. These methods have been developed in order to have a better understanding of the components of commercial enzyme preparations [R25].

They also help link the profile of enzyme activities in these preparations to their stated technological effects.

These methods are based on determining products released by enzymes and/or on substrate breakdown by enzymes. Although the suggested methods are meant to be 'straightforward' to use (essentially spectrophotometric measurements), they require expertise in analysis and sound

knowledge of enzymes, especially to interpret the results.

The following table (Table) gives the methods adopted by the OIV and/or currently being adopted. The detailed protocol for each method is available on the OIV website.

Table 2 :
Summary of Methods for Measuring Enzyme Activities Described Above

Enzyme Activity	Measurement Principle	Assay principle	Date when resolution adopted by the IOV
Pectin Methylesterase (PME)	Measurement of methanol released	Klavons and Bennet (1986) : assay of formaldehyde formed	Adopted in June 2008 (OENO 9/2008) revised in 2012 (OIV-OENO 363-2012)
	Measurement of carboxyl groups formed from pectin	Acid-base titration by measuring volume of 0.01M sodium hydroxide added	Adopted in 2012 (OIV-OENO 363-2012)
Pectin Lyase (PL)	Measurement of conjugated double bond relocation	Absorbance variation at 235 nm	Adopted in June 2009 (OENO 314/2009), revised in 2012 (OIV-OENO 491-2012)
Endo- and exopolygalacturonase (PG)	Measurement of galacturonic acid released	Assay of reducing sugars released (Method used by Nelson, 1944)	Adopted in June 2008 (OENO 10/2008), revised in 2012 (OIV-OENO 364-2012)
		2-cyanoacetamide assay	Adopted in 2012 (OIV-OENO 364-2012)
	Halving of the viscosity of a standard solution	Measurement of viscosity reduction over a given time	Adopted in 2009 (OIV-OENO 351-2009)
Endo (1-4) β -D- Xylanase	Measurement of xylose released	Assay of reducing sugars released (Nelson's method, 1944)	Proposal in progress
Endo- α (1,5) arabinanase	Measurement of azurine released	Assay at 590 nm	Adopted in 2012 (OIV-OENO 412-2012)
Endo (1-4) β -D- galactanase	Measurement of galactose released	Reducing sugars assay (Nelson's method, 1944)	Adopted in June 2009 (OENO 313/2009), revised in 2012 (OIV-OENO 490-2012)
Endo (1-4) β -D- glucanase	Mesure du glucose libéré	Reducing sugars assay (Nelson's method, 1944)	Adopted in June 2008 (OENO 8/2008), revised in 2012 (OIV-OENO 4868-2012)
Endo (1-3, 1-6) β -D- glucanase	Measurement of glucose released	Assay using DNS/Phenol method	Adopted in June 2010 (OENO 340/2010), revised in 2013 (OIV-OENO 488-2013)
Cinnamoyl esterase (CE)	Measurement of substrate breakdown (chlorogenic acid)	Assay of absorbance variation at 350 nm	Adopted in June 2007 (OENO 6/2007), revised in 2013 (OIV-OENO 487-2013)
	Measurement of ethyl cinnamate breakdown	HPLC measurement	
β -D- glucosidase	Measurement of paranitrophenol released	Absorbance assay at 400 nm	Adopted in June 2007 (OENO 5/2007), revised in 2012 (OIV-OENO 489-2012)
β -D- galactosidase			
α -L- rhamnosidase			
α -L- arabinofuranosidase			
β -D- xylosidase			

It turns out that the correlation between the profile of enzymatic activities [R26; R27 ; R28 ; R29] and technological effect has proved hard to establish. Although there appears to be a well-established relationship in specific preparations (for releasing aromas, filtration, maturation on lees, etc.), this relationship is harder to demonstrate in commercial preparations mainly containing

pectolytic enzymes (for pressing, settling, clarification, extraction, etc.). It is worth recalling that since the preparations used are biological by nature, they are a complex combination of activities whose synergy cannot easily be quantified. Furthermore, raw material quality (grape variety, maturity, sanitary state, etc.) and winemaking conditions also significantly influence the final result of

enzyme action. For this reason, although these measurements improve understanding of products used, technological tests will always be required to assess the performance of preparations.

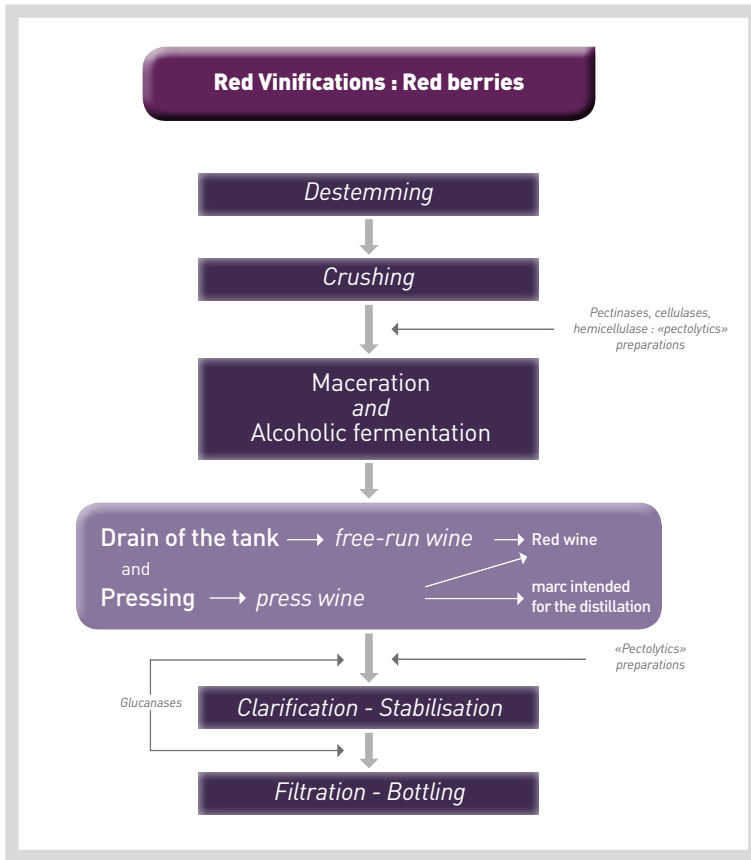


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Chapter 4

Use of Enzyme Preparations in Winemaking

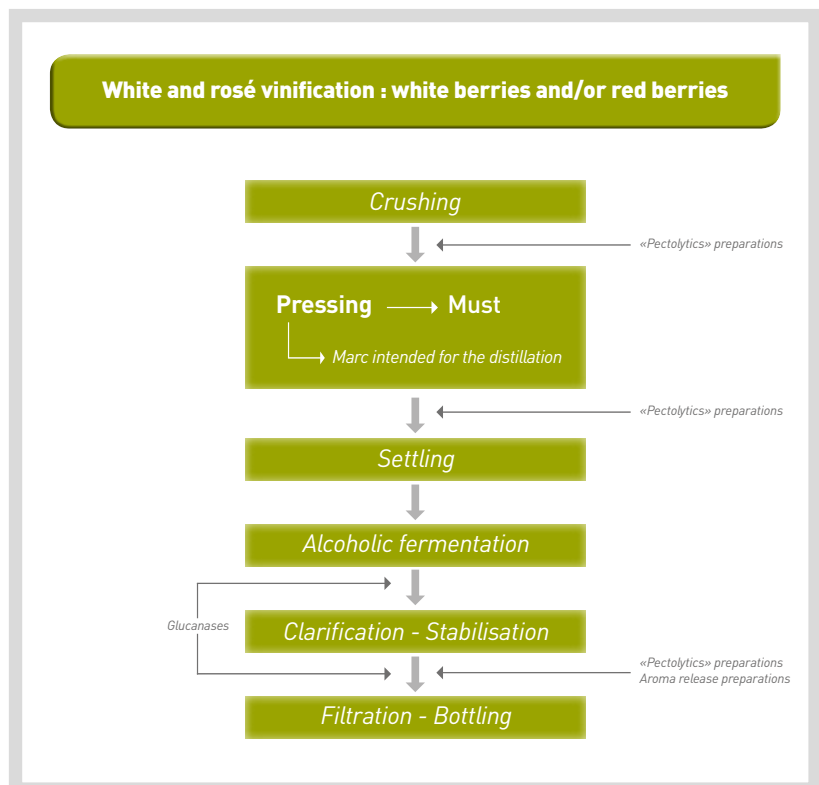


The main œnological applications of enzymes can be summarized by looking at the suggested routes for making white/rosé and red wines (figures 7 and 8). The following paragraphs detail all pre- and post-fermentation procedures.

Figure 7 : Itinerary of red wine making



Figure 8 : Itinerary of white and rosé wine making



Pre-fermentation Procedures

Pressing

Goals [R30] : The use of enzymes enables grape-berry polysaccharides to be broken down, which facilitates the release of juice and aromatic precursors. Pectolytic enzymes are used to facilitate pressing, especially to treat reputedly pectin-rich vine varieties that have viscous pulp. This early use of enzymes is usually sufficient to clarify juice before settling.

Use : Upon harvest when pressing is carried out, or even better, during the maceration stage between harvesting and pressing. Enzymes used in the grape harvest must be very homogeneous and used in accordance with correct procedures. It is important to protect the grapes and must (inert gas blanketing, addition of SO₂ or other processing aids).

Measuring Technological Efficiency : Pressing yield (hL/ton of grapes) is the most relevant indicator. It is worth assessing the free-run volume, pressed-juice volume, and quantity of marc. A 'straightforward' test can be used to assess if the enzymes are acting properly: the pectin/glucan test (see insert p.21).

Experimental and Other Results : In technical terms, enzyme action is expressed by the release of larger amounts of juice (5-10% increase in the extraction rate), as well as by a higher proportion of 'quality' free-run juice or low-pressure collection. This effect varies according to the vine variety, maturity, and enzyme action time [R27; R28 ; R26 ; R31].

Furthermore, since this enzyme action weakens cell walls, it is also recommended for facilitating the extraction of aromatic precursors from grape-berry skin into juice [R32].

In practical terms, early use of enzymes helps shorten the pre-fermentation stages [R33], thereby optimizing the equipment used for this task. Even more convincing results are achieved if pressing is combined with maceration of grape skins over a variable time period.

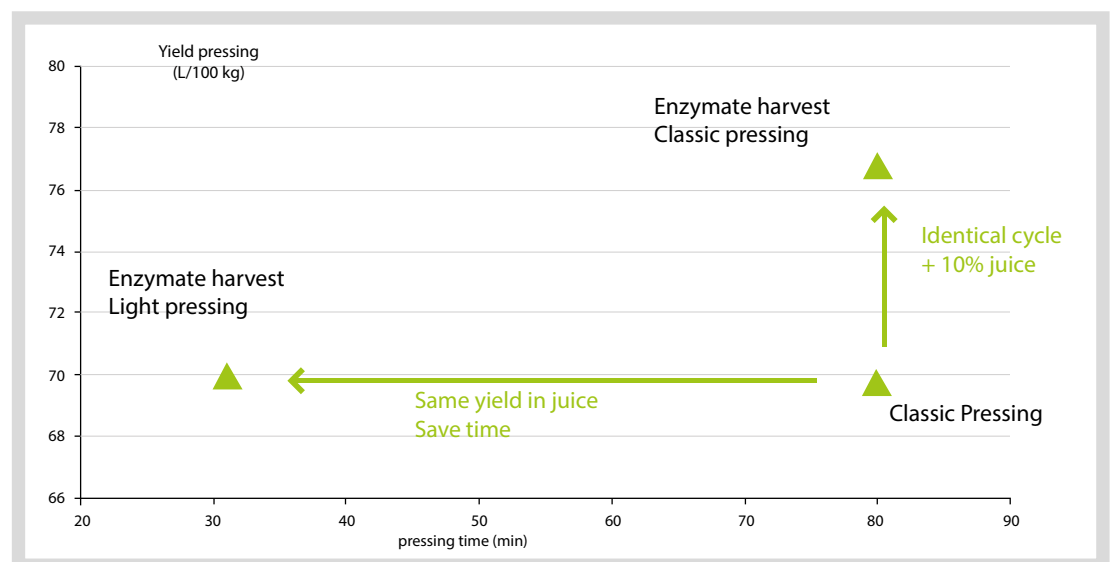


Figure 9 : Changes in total juice yield measured after pressing, depending on pressing duration and whether or not pectolytic enzymes were added – Grenache, IFV Nîmes, 2001.

Settling

One of first œnological uses of enzymes was for must settlement during white-wine production.

Static Settling

Goals : Must is naturally turbid after pressing; its lees content varies according to the intensity of mechanical treatment to which the grape harvest is subjected. Gross lees (size >200 microns) have a negative effect. Excessive quantities of fine lees (size <20 microns) harm the quality and aromatic finesse of wines. The efficiency of enzymes added in this stage depends on the vine variety, grape maturity, and harvesting and pressing conditions. Although settling is usually spontaneous, pectinases must be added to prompt it in some vine varieties. The addition of enzymes always speeds up this process, which is of interest to wineries working with many vat rotations.



Use : As soon as it comes out of the press if used in the juice. Enzymes can also be added very early on, while the press is filling up, and even on the grapes (see 'Pressing'). They must be used in accordance with correct procedures, and it must be possible to protect the initial juice (SO₂ and appropriate temperature). The enzyme dose used should be adjusted to the initial must load, time available for settling, and must temperature. The initial must load can be estimated by measuring the clarification capacity (insert p.23).

Measuring Technological Efficiency : Settling intensity can be assessed by measuring turbidity, which is expressed in NTUs (*Nephelometric Turbidity Units*); this is measured using a turbidimeter (sometimes called a nephelometer). A 'straight-forward' test can be used to assess if the enzymes are acting properly: the pectin/glucan test (see insert).

Pectin/glucan test

Demonstrating the presence of pectins in must and wine :

Solution used : ethanol 96°C acidified with 0.5% concentrated hydrochloric acid.

Gently mix 2 volumes of ethanol per 1 volume of must or wine in a test tube.

- If a gel ring forms at the surface of the test tube after about ten minutes, *a lot of pectins* are present.
- If bubbles formed by shaking rise to the surface with difficulty (they are held back by insoluble pectin), *small amounts of pectins* are present.
- If the mixture is completely clear and sparkling after 10 minutes, *no pectin* is present.

Demonstrating the presence of glucans :

To detect large amounts of glucans (>15 mg/l) :

Gently mix 4 volumes of ethanol per 6 volumes of must or wine in a test tube.

- If white or gray strands appear, *glucans are present*.

To detect smaller amounts of glucans (3-15 mg/L) :

Mix 30 ml of must or wine with 20 ml of ethanol. After leaving to react for 30 minutes, centrifuge for 20 minutes at 3000 r/min. Remove the supernatant, dilute the precipitate in 6 ml of water and add 4 ml of ethanol.

- If white or gray strands appear, *small amounts of glucans* are present.

Experimental and Other Results : Clarification kinetics are improved noticeably by the use of enzymes. Indeed, their use is required for some vine varieties, whatever the vintage [R26; R27] (figure n°10). Nevertheless, it is important to control turbidity in order to avoid reaching levels that may cause fermentation problems and hence impair the quality of finished wines. The greatest success after adding these enzymes is observed in healthy grapes and/or musts obtained from healthy grapes. The presence of *Botrytis cinerea*, especially the glucans it produces, significantly limits good sedimentation of lees. In the tests carried out, pectin-enrichment was greater than hydrolysis, especially when commercial preparations were added to grapes rather than pressed juice; this may explain the reduction in lees settlement. Furthermore, wines produced from enzyme-treated grapes had more intense colors (overall, absorbance at 420 nm was twice as high as the control method). We have repeat results proving the opposite. Pressing management must be adapted to enzyme use. Otherwise, winemakers will be using two extraction tools – enzymes and presses – on top of one another.

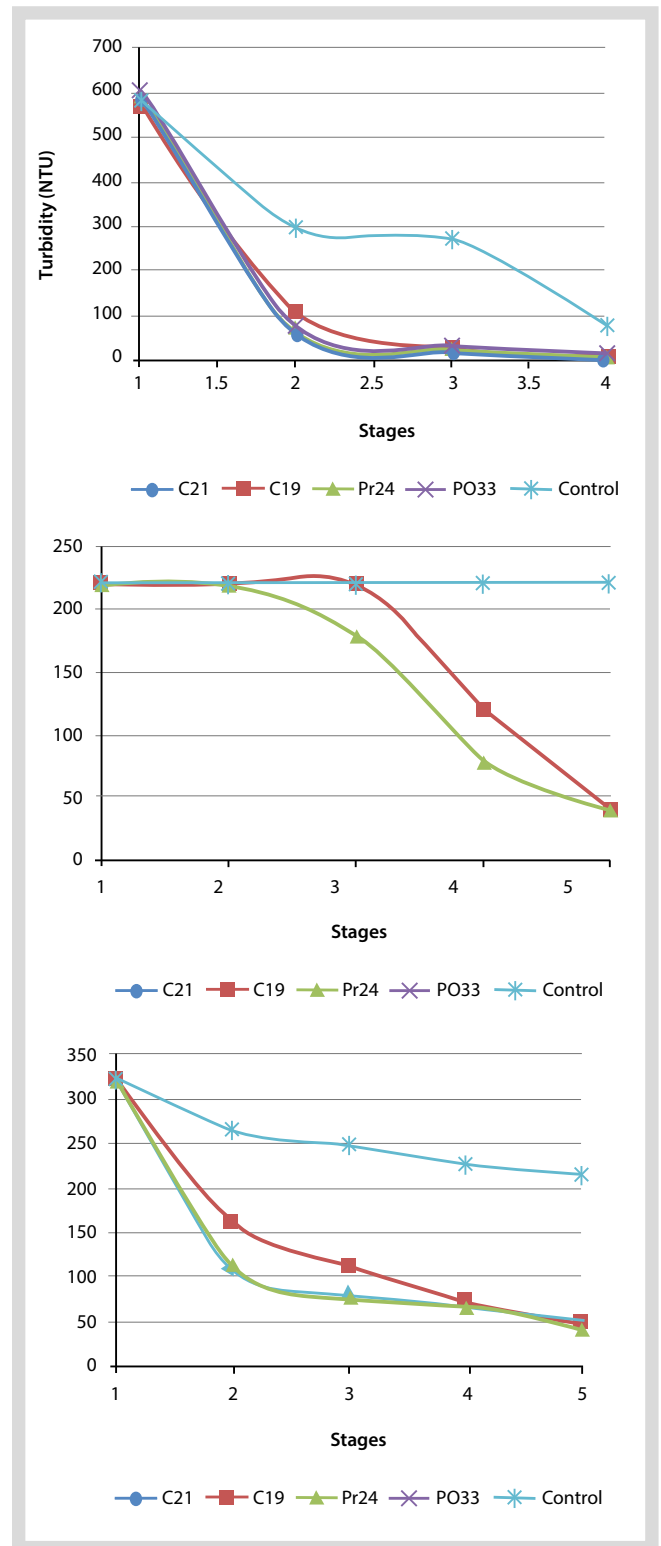
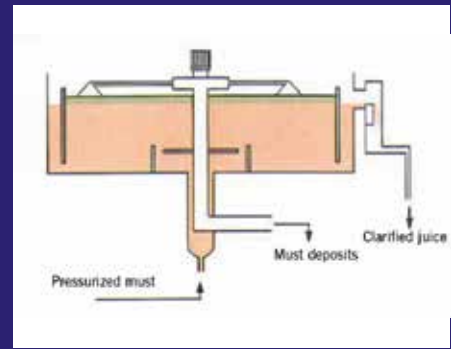


Figure n°10 : Clarification kinetics (Turbidity NTU) for 3 different vine varieties (from left to right: Chenin, Melon B, and Sylvaner), harvested in 2004 and using 4 enzyme preparations (Stage 1: pressed juice (T0); stage 2: 1st sampling point; stage 3: 2nd sampling point; stage 4: end of settling; stage 5: after racking).

Dynamic Settling: A Specific Example of Flotation

Clarification via flotation gives continuous settling at high flow rate and results in adequate clarification. In particular, it removes some of the polyphenols, thus reducing oxidizable material by the same degree. Nevertheless, complete depectinization of musts is required for effective setting and flotation. The use of pectolytic enzymes helps achieve this result within a reasonable time. Enzymes can be added when

must leaves the crusher and/or press. The settling intensity can be assessed by measuring turbidity, which is expressed in NTUs and measured using a turbidimeter. A 'straightforward' test can be used to assess if the enzymes are working properly: the pectin/glucan test (see insert p.21).



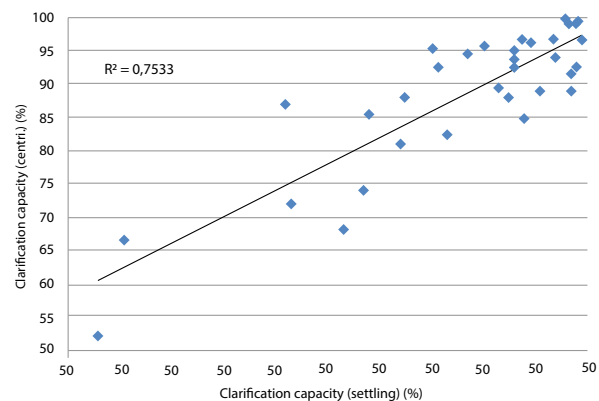
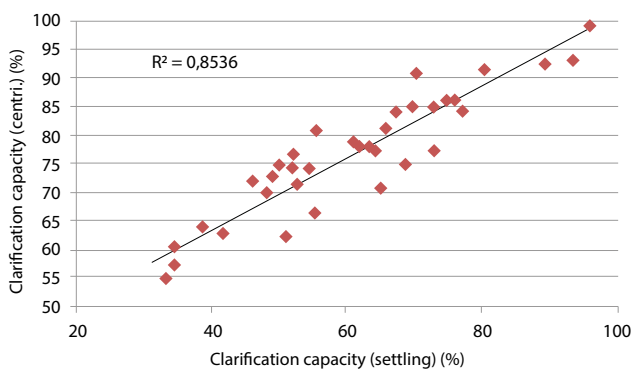
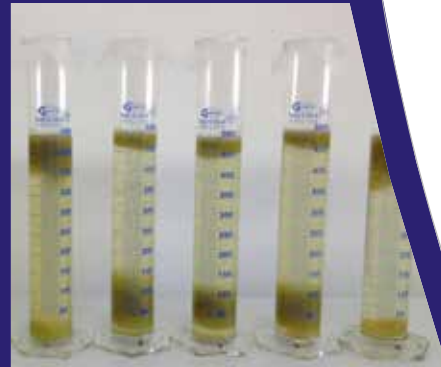
Clarification Capacity

The measurement of juice clarification capacity via centrifugation appears to be a useful aid for deciding whether or not enzymes are making a contribution.

Juice clarification capacity is measured in two different ways:

The first method measures the final turbidity of juice in a 250 ml-test tube after 24 hours at 20°C. The second method measures final turbidity of the same juice in a test tube after centrifugation at 2500 g for 10 minutes at 20°C. These measurements enable 'sedimentation' turbidity and 'centrifugation' turbidity

respectively to be calculated. Figures 11 & 12 shows that the smooth running of juice clarification can be predicted. This indicator can be used in combination with pectin and glucan detection, although these measurements are only possible in unaltered grape harvests, which require the use of alternatives such as glucanases (β 1.3; β 1.6)



Figures n°11 & 12 : Correlation between turbidity measurements after sedimentation and after centrifugation (left-hand figure: Grenache and Cinsault; right-hand figure: Chenin, Melon, and Riesling), 2003–2006

Extraction of phenolic compounds

Although this has become a common application, its results are much debated.

Goals : The use of pectolytic, cellulolytic, and hemicellulolytic enzymes during 'red' grape maceration gives faster and more effective access to the intracellular content of grape skin. This usually results in wines that are more structured, easier to clarify and filter, and with a higher pressing yield.

Use : On grapes and/or at the start of soaking, with homogenization carried out by pumping over.

Measuring Technological Efficiency : The best assessment criteria are anthocyanin and tannin measurements. Color-linked qualitative criteria may be taken into account: optical density (OD) at 420 nm, 520 nm, and 620 nm.

Experimental and Other Results :

In terms of taste, enzyme-treated wines are more structured, rounder, fruitier, and have a more stable color [R34] [R35]. Although anthocyanins are fragile compounds, they are stabilized when they form complexes with tannins [R32]. Nevertheless, cellulase activities include β -D-glucosidases, which break down these very anthocyanins. It is therefore essential to use β -D-glucosidases with low affinity for flavonoids [R33 ; R37]. Research carried out by the IFV between 2002 and 2007 on 5 'red' vine varieties, using 7 different enzyme preparations, showed that the most convincing effect on color and tannins was obtained using enzyme preparations on under-ripe grapes [R27]. Very variable results were obtained using enzymes on mature grapes, and depended on the vine variety, vintage, and winemaking route.

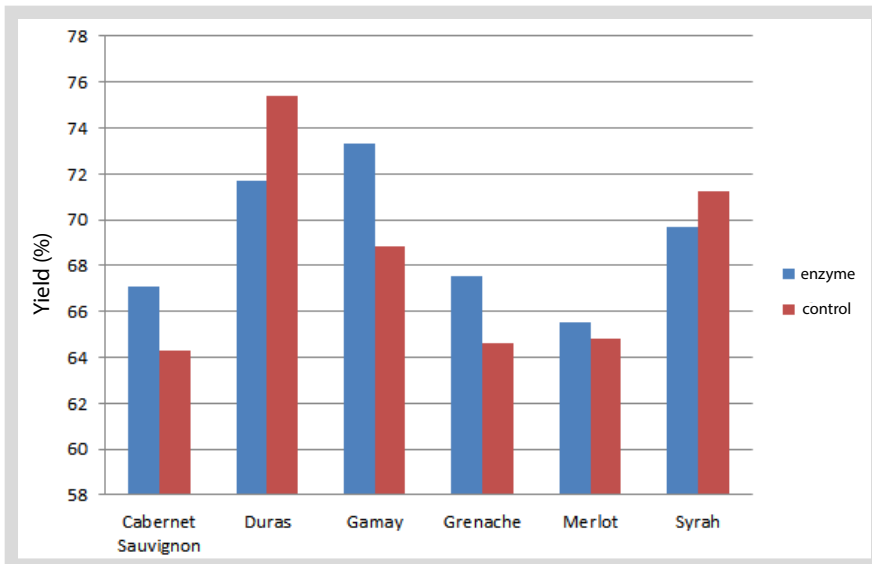


Figure n°13 : Pressing yields obtained for all tests on the same vine variety. A beneficial effect is observed in the following vine varieties : Gamay (6.2% increase/control); Cabernet Sauvignon (4.5% increase/control) ; and Grenache (4.3% increase/control)

Nevertheless, there was a higher yield of free-run juice using the enzyme-treatment method (Fig N°13), as well as improved clarification and filterability. Wines produced using enzyme treatment had a more tannic taste. More recent research has demonstrated the positive role of pectinases on the polyphenol and polysaccharide composition of 20-month-old Merlot wines [R38]. On three vintages, the enzyme wines were richer in polysaccharides such as RGII. More intense, modified color, as well as condensed tannins were observed two vintages over three. These results confirm that the enzyme preparations degrade the cell walls and releases polysaccharides. They confirm also that extraction of polyphenols that results depends on the vintage and the winemaking process.

> The Special Case of Cinnamoyl Esterase

Secondary enzyme activities in commercial preparations include cinnamoyl esterase, which can under certain conditions encourage organoleptic deviations to appear in wine. Cinnamoyl esterase hydrolyzes cinnamic acid esters (including tartaric esters, which are very common in *Vitis vinifera*) into phenol acids. Volatile phenols are then formed via the action of cinnamate decarboxylase from *Saccharomyces cerevisiae*. White wines contain vinylphenols responsible for gouache, medicinal, and clove odors [R39].

In red wines, ethylphenols (horse stable, horse sweat, leather, smoky, spicy odors) only occur in the event of accidental contamination by the yeast *Brettanomyces dekkeri*. It has nevertheless been proved that the use of commercial preparations contaminated with cinnamoyl esterase activity considerably increases the risk of these ethylphenols appearing if *Brettanomyces* is present [R40].

To overcome this problem, enzyme manufacturers have developed FCE (Free of Cinnamoyl Esterase) preparations using ultracentrifuge purification. Recent research [R41 ; R42] has provided a new approach to cinnamoyl esterase activity in wine. Cinnamoyl esterase

may have a positive action on wine color when added under specific conditions (treatment before alcoholic fermentation, and fermentation managed using a POF+ yeast). The formation of pyroanthocyanidin complexes by yeasts with a specific hydroxycinnamate decarboxylase function may inhibit cinnamic acids and improve anthocyanin stability. Combining enzymes and yeasts therefore requires a very specific approach for a precise oenological impact.

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Thermovinification

Goals : Thermovinification, i.e. heating harvested grapes, achieves two main objectives: color extraction within a short time and destruction of polyphenol oxidases present in the grape harvest (*Botrytis cinerea*). But heating also makes pectin more soluble and inactivates endogenous enzymes, resulting in must that is virtually impossible to clarify. Exogenous enzymes must be used to break down all or part of the soluble pectin, thereby facilitating pressing and clarification.

Use : Enzymes may be added upon taking delivery of grapes or after processing, when the temperature has started to drop. Optimal color extraction occurs at maceration temperatures of around 70-75°C, while the optimum temperature for pectinases is 45-55°C. At temperatures above 65°C, pectinases are still active but quickly inactivated. A compromise temperature must therefore be found.

Measuring Technological Efficiency : The most noticeable and desired effect in thermovinified wine is clarification. This can be assessed by measuring turbidity, which is expressed in NTUs (Nephelometric Turbidity Units). A 'straightforward' test can be used to assess if the enzymes are acting properly: the pectin/glucan test (see insert p.21). Other parameters, such as pressing yield and color extraction, can also be assessed.

Experimental and Other Results : Tests on Carignan wines have shown that enzyme-treated wines are three times less turbid than other wines, as well as rounder, fruitier, and less bitter and astringent [R43]. The same results were obtained from similar tests carried out in 13 cellars in the Languedoc-Roussillon region between 1997 and 1999 [R44]. Two different enzyme-incorporation times (on grapes and after heating) were tested. However, in many cases, the only enzymes can't degrade all compounds extracted from grapes heating. It will be necessary to use them in synergy with conventional clarifiers to achieve the desired level of clarification.



Post-fermentation Procedures

Clarification

Enzyme preparations intended for clarification are often described in the same group as enzymes used for settling, even though they do not work in the same way since native pectins are no longer present in the wine. A determining factor appears to be the presence of enzymes that break down side chains rather than areas made up of homogalacturonans; nevertheless, this varies according to each specific case [R5]. All research presented above has shown that the use of pectolytic enzymes during pre-fermentation procedures facilitates wine clarification. Nevertheless, clarification of some wines, especially 'red' press wines, is very long and difficult. Although endogenous and exogenous enzymes act on free-flow wine, pressing still extracts colloids from grape-harvest solids. It is therefore worth using enzymes to help clarify press wines, ensure subsequent good filtration (cf. "Improving filtration", p.28), and improve the organoleptic quality of wines.



Improving Aroma and Flavor

Release of Aromas

Goals : Commercial enzyme preparations for aromatic expression contain glycosidases that release bound aromas.

Use : The use of aroma-releasing enzyme preparations is recommended after fermentation. Bentonites should not be used. The duration of enzyme action depends on the desired product. It is therefore recommended to add bentonite in order to remove enzymes when their activity needs to be stopped [R45].

Measuring Technological Efficiency: Monitoring wine flavor is the most relevant criterion. It is also possible to analyse aromatic compounds, which depend on the vine variety used. It is also possible to carry out analyzes of aromatic compounds. These require knowledge regarding the choice of compounds to be assayed, which generally depends on the studied varieties.

Experimental and Other Results : Much research has already been carried out, especially on Muscat vine varieties (Muscat, Muscadelle, Gewürztraminer, etc.), whose distinctive primary aroma is made up of terpenes. Much lower concentrations of these terpenes are also present in other 'white' vine varieties (Sauvignon, Sémillon, Riesling, Chardonnay, etc.), as well as in several 'red' vine varieties. Research by *Guérin et al.*, 2009 [R25] has demonstrated the specificity of enzyme activities in these preparations. Nevertheless, use of these enzymes requires careful management to avoid an 'exuberant' sensory profile in wine. Furthermore, laboratory research on glycosides in Chardonnay and Gewürztraminer vines has shown that glycosides hydrolyze their substrate under optimum conditions of temperature, pH, alcohol and SO₂ content. Under winemaking conditions, however, the yield from hydrolysis is much lower, which may explain the absence of results (depending on the vine variety and vintage). Although analysis of aromatic compounds does not reveal higher concentrations of free aromas, enzymes always modify the sensory profiles of the resulting wines [R29].



Maturation on Lees

Much research has been carried out on the benefits for final-wine quality of maturation on lees [R46; R47; R48].

Goals : The main process involved is yeast autolysis, which uses endogenous enzyme activity in yeast cells to release their intracellular components (amino acids, peptides, nucleotides, polysaccharides, etc.). The benefit obtained is wines that are rounder in the mouth. Pectinase- and glucanase-based preparations (β , 1-4; 1-3 and 1-6) are used to speed up this process.

Use : Add at the end of alcoholic fermentation on fine and/or gross lees.

Measuring Technological Efficiency : The best assessment criterion remains the wine's taste. Polysaccharides measures (total, acid or neutral) are possible and allow assessing the action of enzymes.

Experimental and Other Results : Although these enzyme preparations are used mainly in white wines, their use in red wines is increasing. These preparations speed up yeast autolysis and improve clarification and filterability in wines after treatment [R49].

Improving Filtration

Given the presence of a complex colloid matrix, this remains the most important and also the most delicate stage. Despite various clarification processes during maturation, some protector colloids, which impair filtration, may still be present.

Goals : Improving wine filterability requires the use of β -glucanase-rich pectolytic enzyme preparations. These enzymes break down pectins and other colloids that clog filtration membranes (protein and polysaccharide macromolecules, glucans from possible *Botrytis cinerea* infection, microorganisms, etc.).

Use : At the end of alcoholic fermentation.

Measuring Technological Efficiency : Wine filterability can be assessed using the Vmax index and/or IC (Indice de Colmatage or fouling index) and/or CFLA (Filtration criteria Lamothe Abiet). This principle consists in measuring the filtration time of a given volume and/or volume filtered over an identical time [R50 ; R51].

Experimental and Other Results : Adding commercial enzyme preparations doubles the volume of wine filtered before clogging. The cost of the enzymes (approximately 0.8 euros/hl) is offset by lower filtration costs [R52]. Adding pectolytic enzyme preparations during pre-fermentation procedures improves clarification and filterability of the resulting wines. Nevertheless, enzyme preparations containing a high concentration of β -1,3-1,6 glucanases are required in the special case of wines produced from grapes infected with *Botrytis cinerea*; this is in order to hydrolyze glucanases in *Botrytis cinerea*. Likewise, the wines from the oenological processes partially degrading polysaccharides of grapes, such as thermovinification systematically present great difficulties of clarification and filtration and must be treated with enzymes.



Enological factors or inputs influencing enzyme activity

Temperature : enzymes are denatured at temperatures above 60°C. If the temperature is too low (under 5°C), enzymes are not active enough. The optimum temperature for enological enzymes is generally between 30°C and 60°C.

pH : virtually all enological enzymes are active between pH 2.8 and pH 5.0; their optimum range is pH 3-6.5.

SO₂ : enzyme activity is inhibited by free SO₂. Nevertheless, inhibition only occurs above 500 mg/L. It may be necessary to shift in time the addition of these two products.

Bentonite : this absorbs proteins and therefore inactivates enzymes. Light bentonite fining (5-10 g/hL) may stop enzyme action.

Enological tannins : research has shown that tannins combine with proteins via hydrogen bonds and hydrophobic interactions. It could be understood that such an association may exist with enzymes of commercial preparations.

Correct dose : the correct dose at which an enzyme is used must be worked out according to the quantity of substrate to be broken down, the time available to obtain the desired action, and the temperature (the level of enzyme activity at low temperatures can be compensated for by increasing the dose [acting for the same length of time]. Likewise, low enzyme doses can be compensated for by raising the temperature).



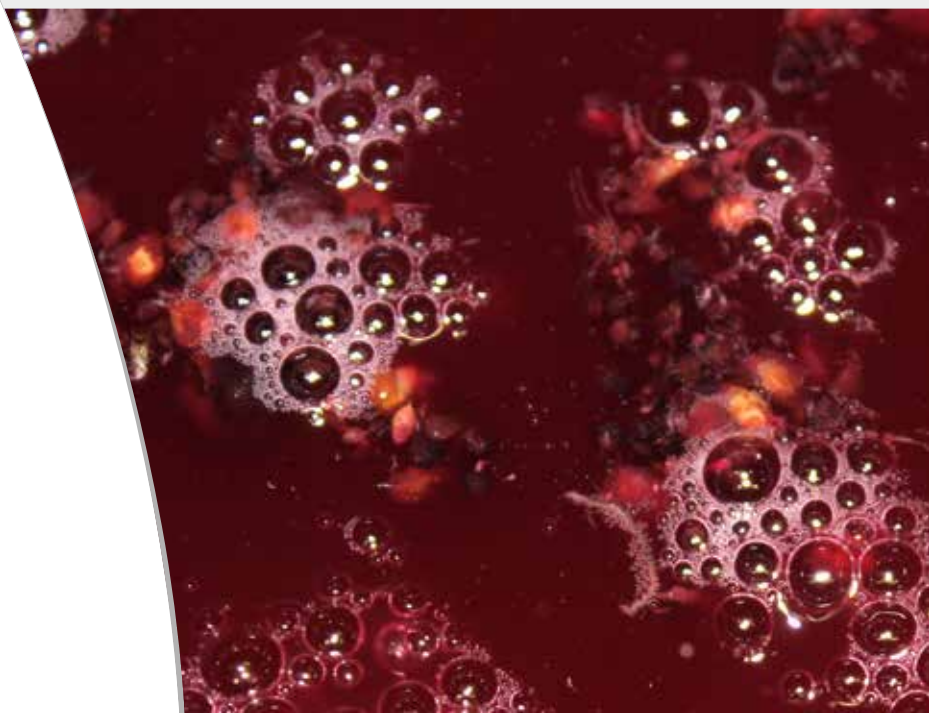
Conclusions

Enzymes are tools of Biotechnologies whose interest and efficiency are demonstrated. However, their use are not sufficiently powered, probably due to poor control of the tool that limits obtaining more conclusive results than conventional operations. In some cases, results are not measured by volume and / or quality of finished products, but by a gain of frigories, power consumption and productivity.

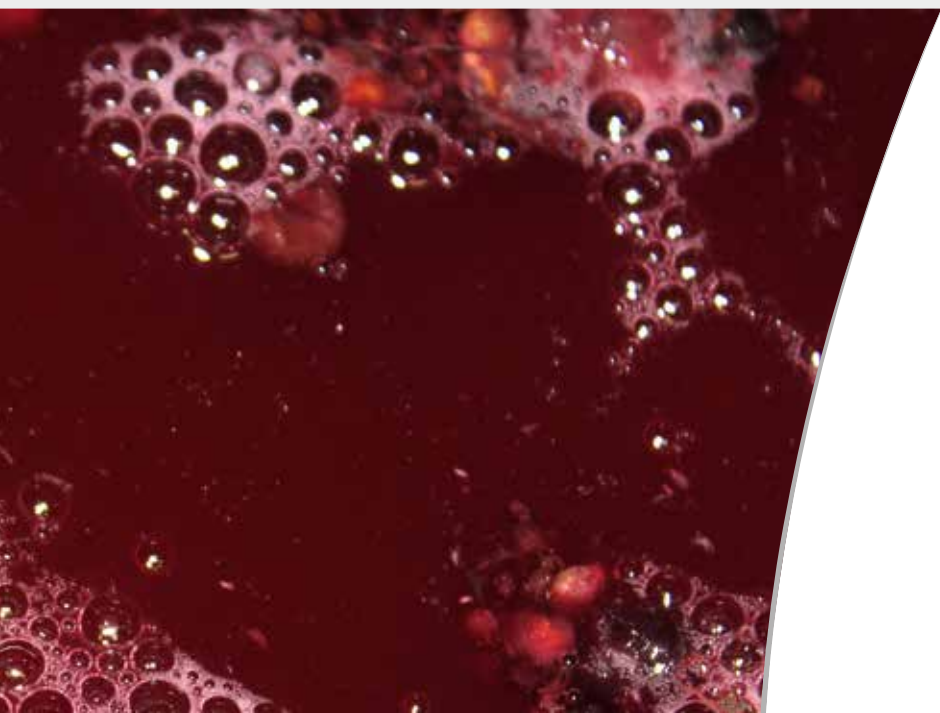
To accompany the development of this tool, the IFV makes a commitment in a next program on the use of the aroma release preparations and on a more thorough knowledge of the action of the pectolytics preparations on the polysaccharides of grapes.

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