THE PURIFICATION OF ENZYMATIC PREPARATIONS FOR OENOLOGY. EFFECTS ON THE QUALITY OF RED AND WHITE WINES.

Rose-Marie Canal-Llaubères
Novozymes France, 23, parvis des Chartrons, 33074 Bordeaux Cédex -France

Introduction

The enzymatic preparations produced by some strains of non-genetically modified micro-organisms contain numerous enzymatic activities. These filamentous non-pathogenic fungus are isolated from the soil where they do contribute to the microflora. They are ubiquitous. The enzymatic activities spectrum can be explained by the adaptation of the micro-organism to its natural medium. The strains used for oenology, Aspergillus niger and Trichoderma harzianum, besides the activities for which they are grown (pectinases for Aspergillus niger, beta-1,3-1,6 glucanases for T. harzianum), contain other types of enzymatic activities, such as cellulases, beta-glucosidases, xylanases, galactanases and proteases, only to list the more known ones. Some activities are undesirable for the oenological applications. It is the case of cinnamyl-esterase (CE), that can be found in A. niger. As a consequence, it seemed fully justified to have preparations with low levels of CE activity, and even no CE activity at all. The fermentative conditions in the industry are chosen in order to favour the production of activities that could be defined as “main activities”. These activities do characterize the enzymatic preparation. The enzymatic spectrum of each preparation depends on the strain and on the culture conditions though. They are specific to each producer. In order to obtain preparations containing only one type of activity, it is necessary to use genetically modified micro-organisms (GMO).

After presenting the strains and the fermentative conditions, this article will review the enzymatic activities responsible for the formation of volatile phenols in wines and the role that the purification plays on the undesirable enzymatic activities.

NB : we prefer to keep the term enzyme for a protein with a well-defined catalytic ability (for instance : pectin esterase). The enzymatic preparations contain several enzymes or enzymatic activities.

Which ones are the fungus producing enzymes?

The fungus strains usually used in the biotechnology industry for the production of enzymes in oenology belong to the family of Ascomycetes (like the strains of Saccharomyces sp. yeasts). They are non-pathogenic and colonize the natural mediums. Aspergillus niger (figure 1) is a microscopic filamentous fungus.

Figure 1: Diagram of Aspergillus niger

Trichoderma harzianum (figure 2) is isolated from the soil. It is a part of the natural environment of this soil. Some strains are known for acting as a fungicide which protects the culture from Botrytis or from the grey rot (mildew).
Aspergillus and Trichoderma are the two microorganisms used in the production of enzymatic preparations for oenology (pectinases, glucanases). The other preparations are extracted from the white of egg (lysozyme) or from Lactobacillus fermentum (urease) culture. The use of enzymatic preparations in oenology obeys to three regulation levels (Goutel, 1996): communal, national and international. This latter is based on the O.I.V. resolutions.

The production of the preparations

The enzymatic preparations are usually produced through fermentation. In the industry, the selected strains are cultivated on agricultural substrates such as soy flour or potato starch. The most commonly used method is culture in immersed medium. Canal Llaubères (2002) describes the various steps of the production from the fermentation to the final formulation step. The result of the fermentation is an enzymatic preparation containing several activities. This preparation has no producing micro-organism. The electrophoresis gel of a pectinase preparation, represented on figure 3, shows the various lanes which compose the preparation. Each lane corresponds to an enzymatic activity (from left to right: lane 1 pectinase produced by an *Aspergillus niger* strain, lane 2 and 3 pectinase produced by another *Aspergillus niger* strain, lane 4 pectin lyase A produced by a GMO, and lane 5 markers of molecular weights.)

This figure allows to view the complexity of the enzymatic preparations obtained from a non-genetically modified micro-organism (no GMO) compared to a one component (or one enzyme) enzymatic preparation produced from a GMO. It is important to note that the enzyme is not modified. The productive micro-organism is modified by genetic mutation in order to lead to synthesis of the desired type of enzymatic activity. Let’s recall that the enzyme is a catalyst with a proteolytic nature, and that is this produced by any lively cell. As every other protein, these ones are biodegradable.
Undesirable enzymatic activities

Among the known enzymatic activities, some have been identified as being able to develop organoleptic faults (loss of freshness in white wines, appearance of phenolic character, more or less marked in white and red wines). It is the case for the cinnamyl-esterase activity contaminant in the preparations of pectinases produced from *Aspergillus niger* strains. The enzymatic preparations produced by *Trichoderma harzianum* are naturally free of the CE activity. The level of activity depends on the micro-organism strain and on the fermentative conditions.

Purification and characterization of the cinnamyl-esterase activity

As soon as pectinases have been used in oenology in the middle of the 70’s for the musts decanting, Burkhardt (1976) notes that their use leads to a rapid loss of the bouquet of the German white wines. He attributes these organoleptic faults to the presence of an enzymatic activity “depsidase” produced by *Aspergillus niger* and contained in the enzymatic preparation used. This activity would be able to hydrolyse the tartaric hydroxy-cinnamoyls compounds of the white musts. Maurer (1987) qualifies this activity as “chlorogenase” but defines it in the same way. Numerous authors have looked into this problem without ever bringing an answer on the identification of the volatile substances responsible for the loss of typicity of those wines. The works of Chatonnet and al. (1992) allowed pointing out the role played by the pectinases preparations on the content in volatile phenols in the wines. Before that, Chatonnet and al. (1989) had demonstrated that the *Saccharomyces cerevisiae* played a role in the formation of these compounds. Barbe (1995) purifies and characterizes the cinnamyl-esterase activity. It is a glycoprotein with a molecular weight of about 240 000 Da, formed by 2 sub-units of 120 000 Da each.

The formation of volatile phenols

The volatile phenols, vinyl- and ethyl, contribute to the aroma of white and red wines. From a certain threshold onwards, these compounds can bring heaviness and mask the fruity character of the wines. They can even fault the wines, by developing medicinal notes or ink, gouache, or clove notes (vinyl-4-phenol, vinyl-4-guaïacol) and stable or sweat notes (ethyl-4-phenol, ethyl-4-guaicol). These compounds come from the metabolism of the phenol acids submitted to the action of micro-organisms during fermentation.

1. Formation of vinyl-phenols

Barbe (1995) demonstrated the evolution of the contents in vinyl-4-phenol and in vinyl-4-guaicol during the alcoholic fermentation, with or without the use of pectolytic preparations. The presence of vinyl-phenols can only be observed in the white wines. Their formation depends on enzymatic reactions from yeast which make the cinnamate decarboxylase (CD) (Albagnac, 1975). The gene POF1 (phenolic off-flavour) encoding for the synthesis of this protein has been cloned by Meaden et Taylor (1991). The CD activity is commonly present in *Saccharomyces* and in most of the active dry yeasts for oenology (Grando et al., 1993). Thus strains deprived of the CE activity (or POF) have been selected in order to avoid the vinyl-phenols formation. In red wines, this activity is inhibited by the phenolic compounds of the red grape (Chatonnet et al., 1989). The vinyl-phenols content is bigger in white wines enzyme-treated with pectinase presenting the CE activity. The increasing rate compared to a control wine can reach 50% (fig. 6). The enzymatic preparations do not contain a decarboxylase cinnamate activity. Figure 4, shows the hydrolysis of the
tartaric esters of the must cinnamic acids, under the action of a secondary esterase-type activity which is to be found in the enzymatic preparations. In the ripe grape, the concentration in precursors is from 2 to 5 times higher than the one of the free phenol acids.

Figure 4: Formation of vinyl-phenols in white wine. Stage 1: hydrolysis of the tartaric hydroxycinnamoyl derivatives by the CE activity (pectinase preparation, *Botrytis cinerea*).

The appearance of vinyl-phenols in wines is described in figure 5. These compounds are formed by the decarboxylation of the must free cinnamic acids and of the cinnamic acids formed by the hydrolysis of tartaric acids by an esterase present in the pectolytic preparations, under the action of the cinnamate decarboxylase of *Saccharomyces cerevisiae*.

Figure 5: Formation of vinyl-phenols in white wine. Stage 2: hydrolysis of the cinnamic acids by the CD activity (*Saccharomyces cerevisiae*).

As pectolytic preparations with a low CE activity were created, it allowed the limitation of vinyl-phenols formation in white wines. The results obtained in
Sauvignon blanc are shown in fig. 3. The use of classic pectolytic preparations (non-purified by the CE activity) for must settling leads to 2-to-4-time-higher vinyl-4-phenol contents in wines when compared to the vinyl-4-phenol content in the control wines. The use of preparations with a low CE activity limits, below the perception threshold (770µg/l), the vinyl-4-phenol content in the wine. These results have been confirmed on other grape varieties (Muscat).

Figure 6.: Effect of enzymatic preparations on the vinyl-phenol content in Sauvignon wines (12-hour-skin maceration at 15°C under CO₂, must settling with 1 g/hl pectinase, NTU 100, inoculation with 10 g/hl active dried yeasts pof+).

2. Formation of ethyl-phenols

In red winemaking, it is quite usual to find wines with a phenolic character. The *Saccharomyces* yeast is not responsible for the formation of volatile phenols, as the CD activity is inhibited by the phenolic compounds (Chatonnet et al., 1997). In red wines, the contaminating yeast *Brettanomyces* is the yeast that is responsible for their formation. It develops leather notes and, beyond a certain threshold, stable or sweat notes. Thus, according to Chatonnet, around 1/3 of the analysed Bordeaux red wines present this phenolic character. In the same way, Gerbaux underlines that the Pinot noir wines are sensitive to the *Brettanomyces* contamination. Thus 50% of the matured cuvees and 25% of the bottled wines are contaminated. This yeast is able to decarboxylate the must free phenol acids and of the ones which are released during pectinase use (figure 7). Unlike *Saccharomyces*, the cinnamate decarboxylase of *Brettanomyces* is not inhibited by the polyphenols. It allows the formation of vinyl-phenols that are then reduced into ethyl-phenols by means of a **vinyl-phenol reductase** (VPR) (figure 7).

Recent works from Gerbaux et al. (2002) show that some practises, such as a final hot maceration or as the use of pectinase on the Pinot Noir grape variety may increase the risk of formation of these compounds with contaminating yeasts and of going beyond the perception threshold (400µg/l with an ethyl-4-phenol / ethyl-4-guaiacol ratio of 10/1). Thus the use of low CE activity pectolytic preparations is justified in red winemaking as well.
Control of the CE activity

The literature mentions numerous esterase activities in *Aspergillus* sp. Besides, it shows a great confusion in the list of the fungus esterase activities (tannase, depsidase, chlorogenase, hydroxyl-cinnamic acid ester hydrolase) and eludes to various levels of qualitative effect. After the identification and the characterization of the activity responsible for the hydrolysis of the tartaric esters of the cinnamic acids, studies showed that it is possible to get partly rid of the cinnamyl esterase activity of the pectolytic preparations by ultra-filtration on membrane limiting size of 100 000 Da. Most part of the CE activity is found in the natant, as the supernatant has quite a weak CE activity. The method of production most commonly used is based on a pH treatment of the preparation before it is formulated in order to distort the CE activity. This process has a quantitative effect on the other enzymatic activities as well and this reduces the output. Barbe studies (1995) allowed the validation of this purification method which determines a minimum content in CE for which the production of vinyl-phenols remains below the sensory threshold.

Conclusion

The enzymatic preparations for oenology and especially the pectinase can contain undesirable activities such as the cinnamyl esterase of *Aspergillus niger*. Its presence can, in some cases, be detrimental to the quality of white wines and red wines, having them lose their aromatic freshness or enhancing the phenolic character up to unpleasant notes. The precaution principle leads the manufacturers to purify the preparations from this activity and propose pectolytic preparations with a low cinnamyl esterase activity (FCE). Their use on grapes (maceration) or on must (clarification) allows the limitation of the hydrolysis of the tartaric esters of the phenol acids. Thus the original composition of the red and white musts is not modified when the producer uses these enzymatic preparations.
Bibliography


