Universal Journal of Microbiology Research 3(2): 17-25, 2015 http://www.hrpub.org
DOI: 10.13189/ujmr.2015.030202

Enzyme Contribution of Non-*Saccharomyces* Yeasts to Wine Production

Sergi Maicas*, Jose Juan Mateo

Department of Microbiology and Ecology, Faculty of Biology, University of Valencia, Spain

Abstract The fermentation of grape must to produce wine is a biologically complex process, carried on by yeasts and malolactic bacteria. The yeasts present in spontaneous fermentation may be divided into two groups, the *Saccharomyces* yeasts, particularly *S. cerevisiae*, and the non-*Saccharomyces* yeasts which include members of the genera *Rhodotorula*, *Pichia*, *Candida*, *Debaryomyces*, *Metschtnikowia, Hansenula* and *Hanseniaspora. S. cerevisiae* yeasts are able to convert sugar into ethanol and CO_2 via fermentation. They have been used for thousands of years by mankind for the production of fermented beverages and foods, including wine. Their enzymes provide interesting wine organoleptic characteristics. β-Glucosidase activity is involved in the release of terpenes to wine, thus contributing to varietal aroma. β-Xylosidase enzyme is also interesting in industry due to its involvement in the degradation of hemicellulose by hydrolyzing its main heteroglycan (xylan). The ability of yeasts to release proteases has been observed by many researchers because of their potential to degrade haze proteins in wine and to generate nutrient sources for microorganisms. Moreover, these enzymes are interesting in biotechnology, for use in food processing such as cheese, pickles or sausage.

Keywords Non-*Saccharomyces* Yeasts, Wine, Flavor, Proteases, β-Glucosidase, β-Xylosidase

1. Introduction

Grape musts naturally contain a mixture of yeast species and wine fermentation is not a "single-species" fermentation. The dominance of *S. cerevisiae* (inoculated or indigenous) in the fermentation is expected and desired. However, the indigenous non-*Saccharomyces* yeasts, already present in the must, and often in greater numbers than *S. cerevisiae*, are adapted to the specific environment and in an active growth state, which gives them a competitive edge [1].

It is well established that wine fermentations, as conducted by traditional methods (without inoculation), are not the result of the action of a single species or a single strain of yeast. Rather, the final products result from the combined actions of several yeast species which grow in succession throughout the fermentation process. Previous studies performed in various countries have described the isolation and identification of yeasts from grape surfaces, and quantitative data on the ecology of grape yeasts have concluded that the isolation process of the total yeast population from the grapes is complex and dependent on many factors [2, 3]. Fermentations are initiated by the growth of various species of *Candida, Debaryomyces, Hanseniaspora, Hansenula, Kloeckera, Metschnikowia, Pichia* and *Torulaspora*. Their growth is generally limited to the first two or three days of fermentation, after which they die off. Subsequently, the most strongly fermenting and more ethanol tolerant species of *Saccharomyces* take over the fermentation [4].

Non-*Saccharomyces* yeasts, as the name suggests, refers to all yeast species found in wine production barring *S. cerevisiae*, with the proviso that this only includes yeast with a positive role in wine production. Recognized spoilage yeasts, such as *Dekkera/Brettanomyces*, are normally left out of this description [5].

Although most fields of research are often focussed primarily on *S. cerevisiae*, non-*Saccharomyces* research can benefit from the techniques and knowledge developed by the *S. cerevisiae* and other yeast researchers [1]. *S. cerevisiae* yeasts are able to convert sugar into ethanol and CO_2 via fermentation. They have been used for thousands of years by mankind for the production of fermented beverages and foods, including wine. This yeast is adapted to the harsh conditions in grape musts and grapes (high sugar concentration, increasing alcohol concentration, acidity, presence of sulfites, anaerobiosis, and progressive depletion of essential nutrients, such as nitrogen, vitamins, and lipids). But *S. cerevisiae* is not only responsible for the metabolism of grape sugar to alcohol and CO_2 but has an equally important role to play in the formation of secondary metabolites, as well as in conversion of grape aroma precursors to varietal wine aromas [4, 6-9].

In the past, the influence of non-*Saccharomyces* yeasts in

wine was restricted and even eliminated by inoculation with pure *S. cerevisiae* cultures because they have long been regarded as spoilage yeasts [10]. However, in the past three decades, great interest has grown in the potential beneficial role of non-*Saccharomyces* yeasts in wine biotechnology [2, 3]. It has been shown that some of the metabolites that these yeasts produce may be beneficial and contribute to the complexity of the wine when they are used in mixed fermentations with *S. cerevisiae* cultures [11, 12]. It is believed that when pure non-*Saccharomyces* yeasts are cultivated with *S. cerevisiae* strains, their negative metabolic activities may not be expressed or could be modified by the metabolic activities of the *S. cerevisiae* strains [13]. Several strains belonging to different non-*Saccharomyces* species have been extensively studied in relation to the formation of some metabolic compounds affecting the bouquet of the final product. Moreover some of these yeast showed positive oenological properties and their use in the alcoholic fermentations has been suggested to enhance the aroma and flavor profiles. The non-*Saccharomyces* yeasts have the capability to produce and secrete enzymes in the wine, such as β-glucosidases, which release monoterpenes derived from their glycosylated form. These compounds contribute to the higher fruit-like characteristic of final product.

2. Yeasts as Enzyme Producers

Ethanol is the central product of alcoholic fermentation, and contributes to the final characteristics of wine. However, wine is a more complex liquid, which is finally produced by a high number of biochemical transformations Yeasts are responsible for a high number of these processes, which enhance the final wine [14]. The variety of flavour compounds produced by diverse non-*Saccharomyces* yeasts is known [7, 8]. The metabolic products generated from non-*Saccharomyces* growth include terpenoids, esters, higher alcohols, glycerol, acetaldehyde, acetic acid and succinic acid [15, 16]. The primary flavour of wine is derived from the grapes, while secondary flavours are derived from ester formation by yeasts during wine fermentation [17]. Several flavour and aroma compounds in grapes are present as glycosylated flavourless precursors [4]. These compounds may be hydrolysed by the enzyme β-glucosidase to form free volatiles that can increase the flavour and aroma of wine, but this enzyme is not encoded by the *S. cerevisiae* genome [18]. In contrast, non-*Saccharomyces* yeasts belonging to the genera *Debaryomyces*, *Hansenula*, *Candida*, *Pichia* and *Kloeckera* possess various degrees of β–glucosidase activity and can play a role in releasing volatile compounds from non-volatile precursors [19, 20]. Co-fermentation of Chardonnay grape juice with *Debaryomyces pseudopolymorphus* and *S. cerevisiae* resulted in an increased concentration of the terpenols: citronellol, nerol and geraniol in wine [21]. Similarly, cofermentation of Muscat grape juice with *Debaryomyces vanriji* and *S. cerevisiae* produced wines with increased concentration of several terpenols [22]. Equally, mixed cultures of Sauvignon Blanc grape juice with *C. zemplinina/ S. cerevisiae* and *T. delbrueckii/S. cerevisiae* produced wines with high concentrations of terpenols compared to wines only fermented with *S. cerevisiae* [23].

Another strategy to increase the release of bound volatile compounds is to exogenously add enzyme preparations that can act on nonvolatile precursors. Numerous studies have characterized and described the effect of β-glucosidase addition on grape juice or wine, focusing particularly in the inhibition of β-glucosidase activity by sugar, alcohol, pH and/or temperature. An intracellular β-glucosidase from *Debaryomyces hansenii*, which is not inhibited by glucose and ethanol, was used during fermentation of Muscat grape juice resulting in an increase in concentration of monoterpenols in the wine [24]. The concentration of volatile terpenes in Arien, Riesling and Muscat wines was also increased following addition of an enzyme extract from *Debaryomyces pseudopolymorphus*. Therefore, sensory differences were found between actions [25]. Over 160 esters have been distinguished in wine. These esters can have a helpful effect on wine quality, especially in wine from varieties with neutral flavours that are consumed shortly after manufacture [17]. Non-*Saccharomyces* can be divided into two groups, neutral yeasts (producing little or no flavour compounds) and flavour-producing species. Flavour-producing yeasts included *P. anomala* (*Hansenula anomala*) and *K. apiculata*. *Candida pulcherrima* is also known to be a high producer of esters [16]. The net accumulation of esters in wine is determined by the balance between the yeast's ester-synthesizing enzymes and esterases (responsible for cleavage and in some cases, formation of ester bonds) [7]. Although extracellular esterases are known to occur in *S. cerevisiae* [18] the situation for non- *Saccharomyces* needs further investigation. Different non- *Saccharomyces* yeasts produce different levels of higher alcohols (n-propanol, isobutanol, isoamyl alcohol, active amyl alcohol) [17]. This is important during wine production, as high concentrations of higher alcohols are generally not desired, whereas lower values can add to wine complexity.

Glycerol, the next major yeast metabolite produced during wine fermentation after ethanol, is important in yeast metabolism for regulating redox potential in the cell [26]. Glycerol contributes to smoothness (mouth-feel), sweetness and complexity in wines, but the grape variety and wine style will govern the extent to which glycerol impacts on these properties [13]. Although the quality of Chardonnay, Sauvignon Blanc and Chenin Blanc is not enhanced by increased glycerol concentrations [27], some wines might benefit from increased glycerol levels. Several non-*Saccharomyces* yeasts, particularly *L. thermotolerans* and *C. zemplinina*, can consistently produce high glycerol concentrations during wine fermentation [28]. Unfortunately, increased glycerol production is usually linked to increased acetic acid production [29], which can be detrimental to wine quality. Spontaneously fermented wines have higher glycerol

levels, indicating a possible contribution by non-*Saccharomyces* yeasts [30].

However, the use of some non-*Saccharomyces* yeast in mixed fermentations with *S. cerevisiae* can generate wines with decreased volatile acidity and acetic acid concentration [28]. Some non-*Saccharomyces* yeasts are able to form succinic acid [13]. This correlates with high ethanol production and ethanol tolerance. Succinic acid production could positively influence the analytical profile of wines by contributing to the total acidity in wines with insufficient acidity. Nevertheless, succinic acid has a 'salt-bitter-acid' taste and excessive levels will negatively influence wine quality. Other non-*Saccharomyces* metabolites can act as intermediaries in aroma metabolic pathways. Acetoin is considered a relatively odorless compound in wine [31]. However, diacetyl and 2, 3-butanediol (potentially off-flavours in wine) can be derived from acetoin by chemical oxidation and yeast-mediated reduction, respectively. This indicates that acetoin can play a role in off-flavour formation in wines. Definitely, high concentrations of acetoin produced by non-*Saccharomyces* yeasts can be utilized by *S. cerevisiae* in mixed and sequential culture fermentations [32].

Other compounds that are known to play a role in the sensory quality of wine include volatile fatty acids, carbonyl and sulphur compounds [17]. There are over 680 documented compounds in wine and a large number of these can, depending on concentration, contribute either positively or negatively to wine aroma and flavour. Volatile thiols greatly contribute to the varietal character of some grape varieties, particularly Sauvignon Blanc [9]. Some non-*Saccharomyces* strains, specifically isolates from *C. zemplinina* and *Pichia kluyveri* can produce significant amounts of the volatile thiols 3-mercaptohexan- 1-ol (3MH) and 3-mercaptohexan-1-ol acetate (3MHA), respectively, in Sauvignon Blanc wines [33]. Similarly, *T. delbrueckii*, *M. pulcherrima* and *L. thermotolerans* have also been described as able to release important quantities of 3MH from its precursor during Sauvignon Blanc fermentation [33]. Other non-*Saccharomyces* extracellular enzymatic activities, such as proteolytic and pectinolytic (polygalacturonase) enzymes, might also be beneficial to winemaking [34]. For example, proteolytic activity of some non-*Saccharomyces* yeast could lead to a reduction in protein levels with accompanying increase in protein stability of the end-product. Species found to produce the greatest number of extracellular enzymes are *C. stellata*, *H. uvarum* and *M. pulcherrima*. Non-*Saccharomyces* yeasts have also been reported to affect the concentration of polysaccharides in wine [35]. Polysaccharides can positively influence wine taste and mouth-feel by increasing the perception of wine 'viscosity' and 'fullness' on the palate [36]. The early death of some non-*Saccharomyces* yeasts during fermentation can also be a source of specific nutrients for *S. cerevisiae* enabling it to ferment optimally. These nutrients include cellular constituents such as cell wall polysaccharides (mannoproteins). For this method of nutrient supply to be effective, any killer or other inhibitory effects by the non-*Saccharomyces* yeasts against *S. cerevisiae* should be known [37] so that the subsequent *S. cerevisiae* fermentation is not adversely affected.

2.1. Glycosidases

Research over the last decades has revealed that a great number of plant tissues flavour compounds are glycosilated and accumulate as non-volatile and flavourless glycoconjugates [38]. Although results in literature had long suggested the occurrence of glycosidically bound flavor compounds in plants, the first clear evidence was found in 1969 by Francis & Allock in rose flowers [39]. The work of Cordonnier & Bayonove [40] suggesting the occurrence in grapes of monoterpenes, important flavour compounds, as glycoconjugates on the basis of enzymatic works was later confirmed by identification of glycosides [41]. These findings opened a new field of intensive research on the chemistry of glycoconjugated flavour compounds to exploit this important flavour source present in both plants and fruit tissues. Some aglycones are already odorous when released from glycosides. They can therefore contribute to the floral aroma of some wines [38], grapes [42], apricots [43], peaches [44] and tea [45]. This is the case of monoterpenes such as geraniol, nerol and linalool which possess mainly floral attributes and low odour thresholds (100-400 ppb) [46].

Terpene compounds belong to the secondary plant constituents, of which the biosynthesis begins with acetyl-CoA [47]. Microorganisms are also able to synthesize terpene compounds [48] but the formation of terpenes by *Saccharomyces cerevisiae* has not yet been observed [46]. Several authors have shown that terpenes play a significant role in the varietal flavour of wines by means of their transformation to other compounds [49].

Terpene glycosides can also be hydrolysed by an enzymatic way, a more interesting way because it produces a more "natural" flavour in the wine [38, 50]. The glycosidase flavour potential from grape remains unfortunately quite stable during winemaking and in young wines as well. So, to enrich wine flavour by release of free aromatic compounds from natural glycoside precursors, particularly pathways are required. Mainly, enzymatic hydrolysis of glycosides is carried out with various enzymes which act sequentially according to two steps: firstly, α-L-rhamnosidase, α-L-arabinosidase or β-D-apiosidase make the cleavage of the terminal sugar and rhamnose, arabinose or apiose and the corresponding β-D-glucosides are released; subsequently liberation of monoterpenol takes place after action of a β-D-glucosidase [51]. Nevertheless, one-step hydrolysis of disaccharide glycosides has also been described; enzymes catalysing this reaction have been isolated from tea leaves [45] and grapes [52]. This one-step reaction occurs through the cleavage of the aglycone linkage which yields a disaccharide and aglycone, the identity of which have been confirmed by HPLC and GC/MS [52].

Enzymatic hydrolysis of glycoside extracts from Muscat, Riesling, Semillon, Chardonnay, Sauvignon and Sirah varieties have provoked the liberation not only of terpenes, but also C-13 norisoprenoids, such as 3-oxo-α-ionol and 3-hydroxy-β-damascenona [53]. These compounds are totally glycosilated in the grape and, opposite with terpenes, they are found in the same quantities in all the grape varieties, aromatics or neutral, and they are capable of awarding certain typicity to the wine flavour because they have lower threshold values than terpenes and they contribute characteristic aromatic features [54].

Yeasts of the *Hansenula* species isolated from fermenting must were reported to have an inducible β-glucosidase activity, but this enzyme was inhibited by glucose [55]. Other yeast strains such as *Candida molischiana* [56] and *C. wickerhamii* [57] also possess activities towards various β-glucosides and they were little influenced by the nature of aglycon [58]. β-Glucosidase from *C. molischiana* was immobilized to Duolite A-568 resin, showing similar physicochemical properties to those of free enzyme. The immobilized enzyme was found to be very stable under wine conditions and could be used repeatedly for several hydrolyses of bound aroma [59]. *Endomyces fibuliger* also produces extracellular β-glucosidase when grown in malt extract broth [60].

Screening 370 strains belonging to 20 species of yeasts, all of the strains of the species *Debaryomyces castelli, D. hansenii, D. polymorphus, Kloeckera apiculata* and *Hansenula anomala* showed β-glucosidase activity [19]. A strain of *D. hansenii* exhibited the highest exocellular activity and some wall-bound and intracellular activity and its synthesis, occurred during exponential growth, was enhanced by aerobic conditions and repressed by high glucose concentration. The optimum condition for this enzyme was pH 4.0-5.0 and 40ºC. This enzyme was immobilized using a one-step procedure on hydroxyapatite. The immobilized enzyme exhibited a lower activity than the purified free enzyme, but was much more stable than the enzyme in cell-free supernatant [61]. Their studies have shown the ability of several wine yeasts to hydrolyse terpenoids, norisoprenoids and benzenoids glycosides; among wine yeasts *Hanseniaspora uvarum* was able to hydrolyse both glycoconjugated forms of pyranic and furanic oxides of linalool [62]. Other authors have also shown the important role of non-*Saccharomyces* species in releasing glycosidic bound fraction of grape aroma components [3].

Finally, the situation regarding *S. cerevisiae* is more complex because this yeast is capable to modify the terpenic profile of the wine; so, it can produce citronellol from geraniol and nerol, the intensity of this transformation depends on the yeast strain used [63]. Other authors propose a more complex scheme: geraniol was transformed by these yeasts into geranyl acetate, citronellyl acetate and citronellol, while nerol was transformed into neryl acetate; in addition, geraniol was transformed into linalool and nerol was cyclized to α-terpineol at must pH [64].

Few data are available regarding glycosidase activities of oenological yeast strains and the technological properties of the enzymes. Low α-rhamnosidase, α-arabinosidase or β-apiosidase activities were detected in *S. cerevisiae* [65]. Nevertheless, data on β-glucosidase activity on *Saccharomyces* are contradictory. First results showed that these yeasts had a very low activity [66] but Delcroix *et al.* [65] found three enological strains showing high β-glucosidase activity. On the other hand, Darriet *et al.* [67] have shown that oxidases located in the periplasmic space of a strain of *S. cerevisiae* were able to hydrolyse monoterpene glucosides of Muscat grapes; they found also that the activity of this β-glucosidase was glucose independent. Mateo and Di Stefano [68] detected β-glucosidase activity in different *Saccharomyces* strains on the basis of its hydrolytic activity on *p*-nitrophenyl-β-D-glucoside (*p*NPG) and terpene glucosides of Muscat juice. This enzymatic activity is induced by the presence of bound β-glucose as carbon source in the medium and seems to be a characteristic of the yeast strain. This β-glucosidase is associated with the yeast cell wall, is quite glucose independent but is inhibited by ethanol. These results could open new pathways regarding other glycosidase activities in *S. cerevisiae*; α-rhamnosidase, α-arabinosidase or β-apiosidase activities could be induced in wine yeast by changing the composition of the medium including inductive compounds, as well as in filamentous fungi [69].

2.2. Proteases

Non-*Saccharomyces* strains are also used in vinification regarding their ability to produce several enzymes [70]. Our research has also been focused to the study of these enzymes, particularly proteases. Proteases are categorized on the basis of their catalytic mechanism, the amino acid residues present in the catalytic site and their three-dimensional structure. According to the NC-IUBMB, proteases can be categorized into four mechanistic classes which include the serine endopeptidases, cysteine endopeptidases, aspartic endopeptidases and metalloendopeptidases. Each type of protease has a specific ability to break a certain peptide bond and exhibits a characteristic set of functional amino acid residues arranged in a specific configuration to produce its catalytic site [71, 72]. The aspartic proteases secreted by non-*Saccharomyces* yeasts have a tertiary structure consisting of two approximately symmetric lobes with each lobe carrying an aspartic acid residue to form the catalytic site. In contrast to other types of proteases, the activity of the aspartic proteases is dependent on pH conditions [73].

The ability of yeasts to release proteases has been observed by many researchers because of their potential to degrade haze proteins in wine and to generate nutrient sources for microorganisms [74]. Protein haze is one of the most important changes for alcoholic beverages producers. This phenomenon occurs in juice with low polyphenol content as a result of coagulation of proteins in alcoholic beverage from unfavorable storage conditions, resulting in their aggregation. The denatured proteins can either

precipitate to form an amorphous sediment or deposit, or can flocculate producing a suspended unstable and unsightly haze in bottle [75]. The presence of haze reduces the commercial value of the product making it unacceptable for consumers because it may be perceived as microbial spoilage [76]. Typically in industry, the haze caused by proteins is removed from wine by bentonite fining but, under certain conditions, it may have an adverse effect on the quality of beverage because some colour, flavor and aroma compounds may be removed together with proteins [76]. Because of the drawbacks presented by this treatment, alternative methods to remove haze-causing proteins have been investigated, amongst these the application of proteolytic enzymes [77]. Dizy & Bisson [78] demonstrated that strains of *Hanseniaspora* produced the most proteolytic activity in juice and affected the protein profile of the finished product.

Besides the potential to aid in haze reduction, the extracellular proteolytic activity of non-*Saccharomyces* yeasts may also hold potential to increase the assimilable nitrogen sources for the grown of microorganisms during fermentation [79]. Insufficient initial assimilable nitrogen sources may lead to stuck or sluggish fermentations [80]. On the other hand, compounds contributing to the fermentation bouquet of beverages, such as esters, higher alcohols and volatile fatty acids arise as primary metabolites of yeast sugar and aminoacid metabolism [8].

3. Conclusions

Yeasts have been traditionally used for the production of wine. Their enzymes provide interesting wine organoleptic characteristics: glycosidases and proteases are crucial enzymes in these processes [5]. Monoterpenes, norisoprenoids, benzene derivatives and aliphatic components are involved in Muscat grape juice and wine. These compounds have been detected in glycosidically-bound form: therefore, the liberation could enhance wine aroma. In order to confirm our previous laboratory results, assays were also carried out in Muscat wine, and volatile compounds were analysed by GC/MS. Muscat wine (13% v/v initial alcohol) showed only a moderated overall terpene increase (1.1-1.3 folds) when treated with these strains. These results are conditioned by the effect of ethanol on glycolytic enzymes. Going into detail, the use of these strains offered an increase of the levels of ho-trienol, 2-phenylethanol and 2,6-dimethyl-3, 7-octadien-2, 6-diol in wine. The sum of ho-trienol, linalool and terpineol seems to play an important role in the aromatic definition of the wines of Loureiro and Alvarinho varieties [81]. 2-Phenylethanol also participates to confer fruity and floral notes to these wines, and its presence is related to the metabolic activity of the non-*Saccharomyces* yeasts [8]. Our findings are similar to the observations of Fernandez *et al.* [62] who shown the ability of several wine yeasts to hydrolyze terpenoids, norisoprenoids and benzenoids glycosides; among wine yeasts *H. uvarum* was able to hydrolyse both glycoconjugated forms of pyranic and furanic oxides of linalool. Our results open the possibility to the use of these strains to be used to improve the aromatic characteristics of the wines, in regard to liberation of terpenes. The production of wines with the addition of non-*Saccharomyces* strains has been traditionally related to high concentrations in vinyl-phenols (4-vinyl-phenol, 4-vinyl-guayacol) reaching concentrations up to 1 mg/L [42, 82]. The concentration of 4-vinyl-phenol in the tested wines was under 90 µg/L, which enables the use of our selected strains in winemaking.

By the other way, yeast protease may liberate amino acids and peptides from grape protein during fermentation which can benefit growth of microorganisms during or after alcoholic fermentation. Another aspect is that yeast cells may release nitrogen containing metabolites to the media. The composition of amino acids peptides and proteins in wine is based on grape related compounds transferred and transformed during the winemaking process and breakdown products through the protease activity from yeasts and compounds released by yeasts [83]. Results obtained in our laboratory in previous work allow to conclude that protease activity in *Pichia* and *Wickerhamomyces* isolates was very low [84], according with results obtained by other authors [34, 85]. These authors suggested that *Hanseniaspora* isolates could be interesting to obtain this enzymatic activity, but some contradictory data have been obtained. Many of these studies have been conducted with *H. uvarum* (*K. apiculata*) isolates and, on the basis of the results obtained in our work, exocellular protease this specie has a very low activity. On the other hand, assays made by these authors have used acidic pH buffers and we have shown that protease from *Hanseniaspora* yeasts is pH dependent, showing maximum values at pH 6.0.

The aspartic proteases secreted by non-*Saccharomyces* yeasts have a tertiary structure consisting of two approximately symmetric lobes with each lobe carrying an aspartic acid residue to form the catalytic site. Unlike the other types of proteases, the activity of the aspartic proteases is dependent on pH conditions [73, 86]. Aspartic endopeptidases (E3.4.23.x) are widely distributed in living organisms from vertebrates to fungi, plants and retroviruses. Most of these enzymes are composed of approximately 323 to 340 amino acid residues, with molecular weights ranging between 35.000 to 50.000 Daltons (Da) and isoelectric points (pI) ranging between 3 and 4.5 because of the high percentage of acidic amino acid residues (about 13%) in the proteins. They have optimum function at pH 3 to 4. They show substrate specificity towards extended peptide substrates and residues with large hydrophobic side chains on either side of the scissile bond [87].

Nevertheless, according to the MEROPS and Protein Data Bank (PDB), there are eight sub-families within the aspartic proteases. These subfamilies differ according to the specific residues in the active site, the position of the catalytic aspartic acid residues in the peptide chains, substrate specificity, the number of disulfide bridges in their structure

and the optimal pH at which the enzymes function, varying from acidic to neutral [88, 89].

Conflict of Interest

The authors confirm that this article content has no conflict of interest.

Acknowledgements

This work has been performed within the Program VLC/Campus, Microcluster IViSoCa and was supported by grants from INV-AE112-66049/UV, UV-AE-2006-0239 and INIA RM2007-00001.

REFERENCES

[1] Cray JA, Bell ANW, Bhaganna P, Mswaka AY, Timson DJ, Hallsworth, JE. The biology of habitat dominance; can microbes behave as weeds? Microb Biotechnol 2013; 6: 453–92.

[2] Gil JV, Mateo JJ, Jiménez M, Pastor A, Huerta T. Aroma compounds in wine as influences by apiculate yeasts. J Food Sci 1996; 61: 1247–9.

[3] Mendes Ferreira A, Clımaco MC, Mendes Faia A. The role of non–*Saccharomyces* species in releasing glycosidic bound fraction of grape aroma components–a preliminary study. J Appl Microbiol: 2001; 91: 67–71.

[4] Pretorius IS. The genetic analysis and tailoring of wine yeasts. Topics in current genetics, Vol. 2 (De Winde JH, ed.) 2003 pp. 99–141. Springer–Verlag, Berlin.

[5] Maicas S, Mateo JJ. Hydrolysis of terpenyl glycosides in grape juice and other fruit juices: a review. Appl Microbiol Biotechnol 2005; 67: 322–55.

[6] Pretorius IS, Van der Westhuizen TJ, Augustyn OPH. Yeast biodiversity in vineyards and wineries and its importance to the South African wine industry. S Afr J Enol Vitic 1999; 20: 61–74.

[7] Swiegers JH, Pretorius IS. Yeast modulation of wine flavour Adv Appl Microbiol 2005; 57: 131–75.

[8] Swiegers JH, Bartowsky EJ, Henschke PA, Pretorius IS Yeast and bacterial modulation of wine aroma and flavour Austr J Grape Wine Res 2005; 11: 139–73.

[9] Swiegers JH, Kievit RL, Siebert T, Lattey KA, Bramley BR, Francis IL, King ES, Pretorius IS. The influence of yeast on the aroma of Sauvignon blanc wine Food Microbiol 2009; 26: 204–11.

[10] Andorrà I, Berradre, M, Rozès, N, Mas A, Guillamón J M, Esteve–Zarzoso B. Effect of pure and mixed cultures of the main yeast species on grape must fermentation European J Food Res Technol 2010; 231: 215–24.

[11] Mateo JJ, Jiménez M, Huerta T, Pastor A, Contribution of different yeasts isolated from musts of Monastrell grapes to the aroma of wine Int J Food Microbiol 1991, 14: 153–60.

[12] Rodríguez ME, Lopes CA, Barbagelata RJ, Barda NB, Caballero AC. Influence of *Candida pulcherrima* Patagonian strain on alcoholic fermentation behaviour and wine aroma. Int J Food Microbiol 2010; 138: 19–25.

[13] Ciani M, Maccarelli F. Oenological properties of non–*Saccharomyces* yeasts associated with wine–making. World J Microbiol Biotechnol 1998; 14: 199–203.

[14] Romero AM, Mateo JJ, Maicas S. Characterization of an ethanol tolerant 1,4–β–xylosidase produced by *Pichia membranifaciens* Lett Appl Microbiol 2012; 55: 354–61.

[15] Fleet GH, Lafon–Lafourcade S, Ribereau–Gayon P. Evolution of yeasts and lactic acid bacteria during fermentation and storage of Bordeaux Wines. Appl Environ Microbiol 1984; 48: 1034–8.

[16] Clemente–Jimenez JF, Mingorance–Cazorla L, Martinez–Rodriguez S, Las Heras–Vazquez F J, Rodriguez–Vico F. Molecular characterization and oenological properties of wine yeasts isolated during spontaneous fermentation of six varieties of grape must Food Microbiol 2004; 21: 149–55.

[17] Lambrechts M G, Pretorius IS. Yeast and its importance to wine aroma S Afr J Enol Vitic 2000; 21: 97–129.

[18] Ubeda–Iranzo JF, Briones–Perez AI, Izquierdo–Canas PM. Study of the oenological characteristics and enzymatic activities of wine yeasts Food Microbiol 1998; 15: 399–406.

[19] Rosi I, Vinella M, Domizio P. Characterization of β–glucosidase activity in yeasts of oenological origin J Appl Bacteriol 1994; 77: 519–27.

[20] Spagna G, Barbagallo RN, Palmeri R, Restuccia C, Giudici P. Properties of endogenous β–glucosidase of a *Pichia anomala* strain isolated from Sicilian musts and wines Enzyme Microb Technol 2002; 31: 1036–41.

[21] Cordero-Otero R, Ubeda JF, Briones–Perez AI, Potgieter N, Villena MA, Pretorius IS, Van Rensburg P. Characterization of the β–glucosidase activity produced by enological strains of non–*Saccharomyces* yeast J Food Sci 2003; 68: 2564–9.

[22] Garcia A, Carcel C, Dulau L, Samson A, Aguera E, Agosin E, Gunata Z. Influence of a mixed culture with *Debaryomyces vanriji* and *Saccharomyces cerevisiae* on the volatiles of a Muscat wine J Food Sci 2002; 67: 1138–43 .

[23] Sadoudi M, Tourdot–Marechal R, Rousseaux S, Steyer D, Gallardo–Chacon JJ, Ballester J, Vichi S, Guerin–Schneider R, Caixach J, Alexandre H. Yeast–yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by single or co–culture of non–*Saccharomyces* and *Saccharomyces* yeasts Food Microbiol 2012; 32: 243–53.

[24] Yanai T, Sato M. Isolation and properties of β–glucosidase produced by *Debaryomyces hansenii* and its application in winemaking Am J Enol Vitic 1999; 50: 231–5.

[25] Arevalo–Villena M, Ubeda-Iranzo J, Briones Perez A. Enhancement of aroma in white wines using a beta–glucosidase preparation from *Debaryomyces pseudopolymorphus* (A–77) Food Biotechnol 2007; 21: 181–94.

[26] Scanes KT, Hohmann S, Prior BA. Glycerol production by

the yeast *Saccharomyces cerevisiae* and its relevance to wine: a review S Afr J Enol Vitic 1998; 19: 15–21.

[27] Nieuwoudt HH, Prior BA, Pretorius IS, Bauer FF. Glycerol in South African table wines: an assessment of its relationship to wine quality S Afr J Enol Vitic 2002; 23: 22–30.

[28] Comitini F, Gobbi M, Domizio P, Romani C, Lencioni L, Mannazzu I, Ciani M. Selected non–*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae* Food Microbiol 2011; 28: 873–82.

[29] Prior BA, Toh TH, Jolly N, Baccari CL, Mortimer RK. Impact of yeast breeding for elevated glycerol production on fermentation activity and metabolite formation in Chardonnay S Afr J Enol Vitic 2000; 21: 92–9.

[30] Henick–Kling T, Edinger W, Daniel P, Monk P. Selective effects of sulfur dioxide and yeast starter culture addition on indigenous yeast populations and sensory characteristics of wine J Appl Microbiol 1998; 84: 865–76.

[31] Romano P, Suzzi G. Origin and production of acetoin during wine yeast fermentation (mini review) Appl Environ Microbiol 1996; 62: 309–15.

[32] Zironi R, Romano P, Suzzi G, Battistutta F, Comi G. Volatile metabolites produced in wine by mixed and sequential cultures of *Hanseniaspora guilliermondii* or *Kloeckera apiculata* and *Saccharomyces cerevisiae* Biotechnol Lett 1993; 15: 235–8.

[33] Zott K, Thibon C, Bely M, Lonvaud–Funel A, Dubourdieu D, Masneuf–Pomarede I. The grape must non–*Saccharomyces* microbial community: impact on volatile thiol release Int J Food Microbiol 2011; 151: 210–5.

[34] Strauss MCA, Jolly NP, Lambrechts MG, van Rensburg P. Screening for the production of extracellular hydrolytic enzymes by non–*Saccharomyces* wine yeasts J Appl Microbiol 2001; 91: 182–90.

[35] Domizio P, Romani C, Lencioni L, Comitini F, Gobbi M, Mannazzu I, Ciani M. Outlining a future for non–*Saccharomyces* yeasts: selection of putative spoilage wine strains to be used in association with *Saccharomyces cerevisiae* for grape juice fermentation Int J Food Microbiol 2011; 147: 170–80.

[36] Vidal S, Francis L, Williams P, Kwiatkowski M, Gawel R, Cheynier W, Waters E. The mouth–feel properties of polysaccharides and anthocyanins in a wine like medium Food Chem 2004; 85: 519–25.

[37] Fleet G, Prakitchaiwattana C, Beh A, Heard G. The yeast ecology of wine grapes In: Ciani, M (Ed), Biodiversity and Biotechnology of Wine Yeasts Research Signpost, Kerala, India, 2002 pp 1–17.

[38] Mateo JJ, Jimenez M. Monoterpene in grape juice and wines J Chromatogr A 2000, 881, 557–567.

[39] Francis MJO, Allcock C. Geraniol β–D–glucoside: occurrence and synthesis in rose flowers Phytochemistry 1969; 8: 1339–47.

[40] Cordonnier R, Bayonove C. Mise en evidence dans la baie de raisin, variété Muscat d'Alexandrie, de monotèrpenes liés, révélables par une ou plusieurs encimes du fruti C R Acad Sci Paris 1974; 278: 3387– 90.

[41] Williams PJ, Strauss CR, Wilson B, Massy–Westropp RA. Use of C_{18} reversed–phase liquid chromatography for the isolation of monoterpene glycosides and nor–isoprenoid precursors from grape juice and wines J Chromatogr 1982; 235, 471–80.

[42] Gunata Z. Etude et exploitation par voie enzymatize des precurseurs d'arômes du raisin de nature glycosidique Revue des Oenologues 1993; 74: 22–7.

[43] Chairote G, Rodriguez F, Crouzet J. Characterization of additional volatile flavor components of apricot J Agric Food Chem 1981; 46: 1898–901

[44] Engel KH, Flath RA, Butter RG, Mon TR, Ramming DW, Teranishi R. Investigation of volatile constituents in nectarines 1 Analytical and sensory characterization of aroma components in some nectarine cultivars J Agric Food Chem 1988; 36: 549–53.

[45] Ogawa K, Yasuyuki I, Guo W, Watanabe N, Usui T, Dong S, Tong Q, Sakata K. Purification of a β–primeverosidase concerned with alcoholic aroma formation in tea leaves (*Cv Shuixian*) to be processed to oolong tea J Agric Food Chem 1997; 45: 877–82.

[46] Rapp A, Mandery H. Wine aroma Experientia 1986; 42: 873–84.

[47] Manitto P. Byosynthesis of natural products, Ellis Horwood Publisher, Chichester 1980.

[48] Hock R, Benda J,Schreier P. Formation of terpenes by yeasts during alcoholic fermentation Z Lebensm Unters Forsch 1984; 179: 450–2.

[49] Wilson B, Strauss CR, Williams PJ. The distribution of free and glycosidically bound monoterpenes among skin, juice and pulp fractions of some white grape varieties Am J Enol Vitic 1986; 37: 107–11.

[50] Gunata Z. Flavor enhancement in fruit juices and derived beverages by exogenous glycosidases and consequences of the use of enzyme preparations. In: Whitaker, J R (ed) Handbook of Food Enzymology Marcel Dekker, New York 2002 pp 303–330.

[51] Gunata Z, Bitteur S, Brillouet JM, Bayonove C, Cordonnier R. Sequential enzymic hydrolysis of potentially aromatic glycosides from grape *Carbohydr Res* 1988; 184: 139–49.

[52] Gunata Z, Blondeel C, Vallier MJ, Lepoutre JP, Sapis J C, Watanabe N. An endoglycosidase from grape berry skin of *cv* M Alexandrie, hydrolysing potentially aromatic dissacharide glycosides J Agric Food Chem 1998; 46: 2748–53.

[53] Gunata Z, Bayonove C, Tapiero C, Cordonnier R. Hydrolysis of grape monoterpenyl β–D–glucosides by various β–glucosidases J Agric Food Chem 1990; 38: 1232–6.

[54] Razungles A, Bayonove CL, Cordonnier RE, Baumes RL. Investigation on the carotenoids of the mature grape Vitis 1987; 26: 183–91.

[55] Grossmann M, Rapp A, Rieth W. Enzymatische freisetzung gebundener aromastoffe in wein Dtsch Lebensm Rdsch 1987; 83: 7–12.

[56] Gonde P, Ratomahenina R, Arnaud A, Galzy P. Purification and properties of the exocellular β–glucosidase of *Candida wickerhamii* (Zikes) Meyer and Yarrow capable of hydrolysing soluble cellodextrines Can J Biochem Cell Biol

1985; 63: 1160–6.

[57] Leclerc M, Arnaud A, Ratomahenina R, Galzy P, Nicolas M. The enzyme system in a strain of *Candida wickerhamii* Meyer and Yarrow participating in the hydrolysis of cellodextrins J Gen Appl Microbiol 1984, 30: 509–21.

[58] Gunata Z, Brillouet JM, Voirin S, Baumes B, Cordonnier R. Purification and some properties of an β –L–arabinofuranosidase from *Aspergillus niger* Action on grape monoterpenyl arabinofuranosyl glucosidases J Agric Food Chem 1990; 38: 772–6.

[59] Gueguen Y, Chemardin P, Pien S, Arnaud A, Galzy P. Enhancement of aromatic quality of Muscat wine by the use of immobilized β –glucosidase J Biotechnol 1997; 55: 151–6.

[60] Brimer L, Nout MJR, Tuncel, G. Glycosidase (amygdalase and linamarase) from *Endomyces fibuliger* (LU677): formation and crude enzyme properties Appl Microbiol Biotechnol 1998; 49: 182–8.

[61] Riccio P, Rossano R, Vinella M, Domizio P, Zito F, Sanseverino F, D'Elia A, Rosi I. Extraction and immobilization in one step of two β–glucosidases released from a yeast strain of *Debaryomyces hansenii* Enz Microb Technol 1999; 24: 123–9.

[62] Fernandez M, Di Stefano R, Briones A. Hydrolysis and transformation of terpene glycosides from Muscat must by different yeast species Food Microbiol 2003; 20: 35–41.

[63] Hernandez LF, Espinosa JC, Fernandez M, Briones A. β–Glucosidase activity in a *Saccharomyces cerevisiae* wine strain Int J Food Microbiol 2003; 80: 171–6.

[64] Di Stefano R, Magiorotto G, Gianotti S. Transformazioni di nerolo e geraniolo indotte dai lieviti Riv Vitic Enol 1992; 42: 43–9.

[65] Delcroix A, Gunata Z, Sapis JC, Salmon JM, Bayonove C. Glycosidase activities of three enological yeast strains during wine making Effect on the terpenol content of Muscat wine Am J Enol Vitic 1994; 45: 291–6.

[66] Gunata Z, Dugelay I, Sapis JC, Baumes R, Bayonove C. Action des glycosidases exogènes au cours de la vinification: liberation de l'arôme à partir des précurseurs glycosidiques J Int Sci Vigne Vin 1990; 24: 133–44.

[67] Darriet P, Boidron JN, Dubourdieu D. L'hydrolyse des hétérosides terpéniques du Muscat a Petit Grains par les enzymes périplasmiques de *Saccharomyces cerevisiae* Conn Vigne Vin 1988; 22: 189–95.

[68] Mateo JJ, Di Stefano R. Description of the β–glucosidase activity of wine yeasts Food Microbiol 1997; 14: 583–91.

[69] Dupin I, Gunata Z, Sapis JC, Bayonove C, M'Bairaroua O, Tapiero C. Production of a β–apiosidase by *Aspergillus niger* Partial purification, properties and effect on terpenyl apiosylglucosides from grape J Agric Food Chem 1992; 40: 1886–91.

[70] Mateo JJ, Peris L, Ibañez C, Maicas S. Characterization of glycolytic activities from non–*Saccharomyces* yeasts isolated from Bobal musts J Ind Microbiol Biotechnol 2011; 38: 347–54.

[71] Barrett AJ, Rawlings ND, Woessner JF. Handbook of Proteolytic Enzymes, second ed Academic Press, London. 2004

[72] Tyndall JDA, Nall T, Fairlie DP. Proteases universally recognize β–strands in their active sites Chemical Rev 2005; 105: 973–1000.

[73] Borelli C, Ruge E, Lee JH, Schaller M, Vogelsang A, Monod M, Korting HC, Huber R, Maskos K. X–ray structures of Sap1 and Sap5: Structural comparison of the secreted aspartic proteinases from *Candida albicans* Proteins: Structure, Functions and Bioinformatics 2008; 72: 1308–19.

[74] Lagace LS, Bisson LF. Survey of yeast acid proteases for effectiveness of wine haze reduction Am J Enol Vitic 1990; 41: 147–55.

[75] Pocock KF, Waters EJ. Protein haze in bottled white wines: How well do stability tests and bentonite fining trials predict haze formation during storage and transport? Austr J Grape Wine Res 2006; 12: 212–20.

[76] Waters EJ, Alexander G, Muhlack R, Pocock KF, Colby C, O'Neill BK, Høj PB, Jones P. Preventing protein haze in bottled white wine Austr J Grape Wine Res 2005; 11: 215–25.

[77] Rosi I, Costamagna L. Screening for extracellular acid protease(s) production by wine yeasts J Inst Brewing 1987; 93: 322–4.

[78] Dizy M, Bisson LF. Proteolytic activity of yeast stains during grape juice fermentation Am J Enol Vitic 2000; 51: 155–67.

[79] Cramer AC, Vlassides S, Block DE. Kinetic model for nitrogen–limited wine fermentations Biotechnol Bioeng 2002; 77: 49–60.

[80] Henschke PA, Jiranek V. Hydrogen sulfide formation during fermentation: effect of nitrogen composition in model grape must In: *International symposium on nitrogen in grapes and wine*, 1993 pp 172–184. American Society for Enology and Viticulture Seattle, Davis.

[81] Oliveira JM, Oliveira P, Baumes RL, Maia MO. Volatile and glycosidically bound composition of Loureiro and Alvarinho wines Food Sci Technol Int 2008; 14: 341–53.

[82] Sefton MA, Williams PJ. Generation of oxidation artifacts during the hydrolysis of norisoprenoid glycosides by fungal enzyme preparations *J Agric Food Chem* 1991; 39: 1994–7.

[83] Alexandre H, Heintz D, Chassagne D, Guilloux–Benatier M, Charpentier C, Feuillat M. Protease A activity and nitrogen fractions released during alcoholic fermentation and autolysis in enological conditions J Ind Microbiol Biotechnol 2001; 26: 235–40.

[84] Madrigal T, Maicas S, Mateo JJ. Glucose and ethanol tolerant enzymes produced by *Pichia* (*Wickerhamomyces*) isolates from enological ecosystems Am J Enol Vitic 2013; 64: 126–33.

[85] Charoenchai C, Fleet GH, Henschke PA, Todd BEN. Screening of non–*Saccharomyces* wine yeasts for the presence of extracellular hydrolytic enzymes Austr J Grape Wine Res 1997; 3: 2–8.

[86] Cascella M, Micheletti C, Rothlisberger U, Carloni P. Evolutionarity conserved functional mechanics across pepsin–like and retroviral aspartic proteases J Am Chem Soc 2005; 127: 3734–42.

[87] Rawlings ND, Barrett AJ, Bateman A. MEROPS: the peptidase database Nucl Acids Res 2009; 38, Database issue D227–D233.

[88] Rawlings ND, Bateman A. Pepsin homologues in bacteria BMC Genomics 2009; 10: 437–48.

[89] Rawlings. ND, Barrett AJ, Bateman A. Asparagine peptide lyases A seventh catalytic type of proteolytic enzymes J Biol Chem 2011; 286: 38321–8.