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The role of non-*Saccharomyces* yeasts in industrial winemaking

Summary The fermentation of grape juice into wine is a complex microbiological process, in which yeasts play a central role. Traditionally, identification and characterization of yeast species have been based on morphological and physiological characteristics. However, the application of molecular biology techniques represents an alternative to the traditional methods of yeast identification and are becoming an important tool in solving industrial problems. Although *Saccharomyces cerevisiae* is responsible for the alcoholic fermentation, the presence of non-*Saccharomyces* species could be important since they produce secondary metabolites, which can contribute to the final taste and flavor of wines.

Key words Wine · Yeast identification · Non-*Saccharomyces* wine yeasts · Enzymes · Aroma

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Introduction

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 more or less in succession throughout the fermentation process. Many studies in various countries have described the isolation and identification of yeasts from grape surfaces, and quantitative data on the ecology of grapes yeast have concluded that the isolation process of the total yeast population of the grapes is complex and dependent on many factors [for a detailed review see 22]. Fermentations are initiated by the growth of various species of *Candida, Debaryomyces, Hanseniaspora, Hansenula, Kloeckera, Metschnikowia, Pichia, Schizosaccharomyces, Torulaspora*, and *Zygosaccharomyces*. 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 [22]. During the first step of the fermentation low-fermentative yeasts produce some important reactions in must which improve the final flavor of wines. In this work we describe briefly that the agents responsible for these reactions are enzymes produced both inside and outside the cell.

Identification of non-*Saccharomyces* wine yeasts

The isolation and correct identification of non-*Saccharomyces* yeasts are important tools to understand the type of enzymatic reactions occurring during the early stages of fermentation. Yeasts are classified on the basis of their morphological, physiological, and biochemical properties [4, 36]. To improve conventional methods, various kits have been developed (Table 1). However, commercial kits were designed to meet the needs of clinical yeast diagnosis, and the databases are restricted to 40 to 60 yeast species of clinical importance. In general, it is necessary to conduct from 50 to 100 tests in order to reliably identify yeasts at the species level; one to two weeks are often required to obtain a result. Moreover, the interpretation of the data requires considerable expertise and is further complicated.

Table 1 Different kits and systems used to identify foodborne yeasts

System	Reference
API 20C	70
ATB 32 ID	60
AutoMicrobic	54
AutoMicrobic	19
Microring YT	68
MicroScan	39
MicroScan	70
Minitek	42
Quantum II	54
Quantum II	63
Uni-Yeast-Tek	64
Yeastsldent	54

More recently developed methods to identify yeasts are based on analysis of the total protein of the cell [28] and fatty acids by using gas chromatography [1]. However, the reproducibility of these techniques is questionable, because they are based on the physiological state of the yeasts. Recent progress in molecular biology has led to the development of new techniques for yeast identification. These include e.g. RFLP mitochondrial DNA, chromosomal DNA electrophoresis, ribosomal DNA restriction analysis, RAPDs (Table 2).

Table 2 Studies about wine yeast identification using molecular biology techniques

Methodology	Genus	Reference
δ elements	*Saccharomyces*	50
Intron splice site	*Saccharomyces*	16
Karyotype	*Saccharomyces*	7, 11, 27, 34, 45, 55, 67, 72, 79
	Hanseniaspora	66
	Zygosaccharomyces	72
Microsatellite	*Saccharomyces*	2
Nested PCR	*Brettanomyces*	35
Plasmids	*Saccharomyces*	53
	Zygosaccharomyces	53
RAPDs	*Saccharomyces*	2, 3, 57
	Metschnikowia	43
	Rhodotorula	57
	Zygosaccharomyces	3
	Candida	57
	Pichia	57
	Torulaspora	57
	Hansenula	57
RFLP-karyotype	*Candida*	78
	Kloeckera	78
	Schizosaccharomyces	78
RFLP-mtDNA	*Saccharomyces*	14, 26, 27, 34, 45, 55, 56
	Kluyveromyces	5
	Zygosaccharomyces	29
	Brettanomyces	35
RFLP-ITS/5.8S	*Candida*	14, Guillamón et al. (in press)
rRNA gene	*Hanseniaspora*	14, Guillamón et al. (in press)
	Saccharomyces	14, Guillamón et al. (in press)
	25 different genera	Esteve-Zarzoso et al. (in press)

In several hemiascomycetous yeasts (e.g. *Saccharomyces cerevisiae*, *Torulaspora delbrueckii* and *Candida glabrata*), rRNA genes are located in a single genomic region composed of 100–150 tandem repeats of a fragment of 9 kb. These fragments contain two transcriptional units, one of which (7 kb) is a cluster of the genes coding for the 18S, 5.8S and 25S rRNAs and two internal transcribed spacers, ITS1 and ITS2 [for a review see 37, 81]. The second unit, which is transcribed in the opposite direction, corresponds to the 5S rRNA. Previous results demonstrated that the complex ITS regions (non-coding and variable) and the 5.8S rRNA gene (coding and conserved) are useful to measure close genealogical relationships, since they exhibit far greater interspecific differences than the 18S and 25S rRNA genes. Moreover, because of the existence of conserved sequences within this region, the intraspecific variation, which is low, has been proved very useful for species identification [33, 47, 74, 83]. Using the restriction analysis of this region, thirty-three wine yeast species and 129 different food yeast species from 25 different genera (Guillamón et al. and Esteve-Zarzoso et al., in press) were identified. By applying this methodology, it is possible to find species never described before in winemaking, and furthermore to discover unknown enzymatic activities which increase the flavor of the wines.

Enzyme activities of non-*Saccharomyces* wine yeasts

The available aromas in the grape impart and define the characteristics and the final quality of the wine. Terpenic compounds account for most of these aromas. Grape processing liberates small quantities of aromatic terpenols; however, odourless precursors in the grape present a large, untapped reserve for wine aromas. The action of grape enzymes and *Saccharomyces* enzymes are insufficient to carry on this transformation completely. Various enzyme activities can improve the process of winemaking and enhance wine quality [9, 10, 80]. Pectinases increase juice extraction from grapes, improve wine clarification and facilitate wine filtration. The aroma and flavor properties of wine can be enhanced by glycosidases that hydrolyse non-volatile glycosidic precursors of the grape. To achieve these reactions, commercial preparations of the enzymes are purchased and added to must or wine. In most cases these enzymes are prepared from fungi [80].

Yeasts involved in winemaking could be important producers of these and other enzymes. *S. cerevisiae*, the principal wine yeast, is not recognised as a significant producer of extracellular proteases, lipases or proteolytic enzymes, although a few strains have been reported recently to degrade polygalacturonate [23, 46]. Various authors have reported glycosidase production by this species and the potential for these enzymes to impact on wine flavor [15, 17,

18]. Apart from *S. cerevisiae*, it is now recognised that the non-*Saccharomyces* species contribute to the enzymatic reactions occurring in the must during the early stages of vinification [31]. There is little information on the production of these enzymes by non-*Saccharomyces* wine yeasts (Table 3), although extracellular protease activity has been reported in some strains of *Kloeckera apiculata* [38], and glucosidase activity in strains of *Candida, Pichia* and *Hanseniaspora* [25, 30, 76].

Grape proteins influence the clarification and stabilisation of must and wine. The yeast proteases hydrolyse the peptide linkages between amino acid units of proteins, improving the clarification process. These enzymes also play a major role during the autolysis process in wines kept on yeast lees during ageing. However, due to the particular conditions found in wine, only a few proteases are active [44]. Another important aspect of yeast proteolytic activity is its potential for use in protein haze reduction [49]. The action of non-*Saccharomyces* strain proteases on the hydrolysis of wine proteins was investigated by Lagace and Bisson [38]. Recently Charoenchai et al. [13] reported the effect of nitrogen sources on the production of extracellular proteases by non-*Saccharomyces* wine yeasts. From 26 yeast strains, protease activity was observed in strains of *Candida pulcherrima, K. apiculata* and *Pichia anomala*.

Table 3 Main enzymatic activities described in non-*Saccharomyces* wine yeasts

Enzymatic activity	Genera	Reference
Protease	*Candida, Kloeckera, Pichia*	13, 38
β-glucosidase	*Candida, Debaryomyces, Hanseniaspora, Hansenula, Kloeckera, Kluyveromyces, Metschnikowia, Pichia, Saccharomycodes, Schizosaccharomyces, Zygosaccharomyces*	25, 30, 61, 62, 76
Esterase	*Brettanomyces, Debaryomyces, Rhodotorula*	6, 41, 69
Pectinase	*Candida, Cryptococcus, Kluyveromyces, Rhodotorula*	20, 46, 59, 77
Lipase	*Candida*	13

The role of pectinases in winemaking has been reviewed by Canal-Llaubères [9]. Some of the applications are mash treatment for juice extraction, juice clarification, wine filtration and also color extraction. The use of pectolytic enzymes for maceration may also increase the terpenol content of juice [51]. Although pectin esterase and polygalacturonase activities increase during grape ripening [24] and are produced by non-*Saccharomyces* yeasts present in must, the addition of fungal pectinase preparations is a common industrial practice. About non-*Saccharomyces* yeasts, pectinolytic activity has been reported in various species of *Candida, Cryptococcus, Kluyveromyces*, and *Rhodotorula* [20, 46, 59, 77]. However, pectinolytic activity was not found in any of the wine yeasts screened by Charoenchai et al. [13], suggesting little influence of wine yeasts on the pectin composition of the must or wine.

Difficulties in the clarification and filtration can also arise from the presence of high-molecular-weight β-glucans produced by *Botrytis cinerea* in infected grapes. Even low glucan concentrations may cause filtration problems and it is impossible to remove them by conventional treatments such as centrifugation and fining. This problem can be solved by the action of glucanases. The presence of β-(1,3)-D-glucanases has been reported in many yeast species [21]. These enzymes show endo- and exo-activities and they are constitutive glycoproteins [32, 65]. *S. cerevisiae* excretes several β-(1,3)-glucanases and the presence of a cell wall endo-β-(1,3)-glucanase activity in strains of dried yeasts used in winemaking has been demostrated by Canal-Llaubères [8].

Glycosidases such as β-glucosidase, β-xylosidase, β-apiosidase, α-rhamnosidase and α-arabinofuranosidase have been described as being involved in flavor releasing processes [for a review see 82]. However, many studies have only focused on β-glucosidases because of their wide occurrence in plants, fungi and yeasts [40]. The effect of β-glucosidases isolated from different yeast species on the hydrolysis of grape terpenyl-glycosides has been investigated. Großmann et al. [25] studied the β-glucosidase from *Hansenula* species found in must. This enzyme, although able to liberate aroma substances in wine, seems to be less effective in must. According to Dubourdieu et al. [18], the liberation of terpenols during fermentation can be explained by yeast β-glucosidase activity. Studies from Vasserot et al. [76] were focused on the β-glucosidase activities of other yeast strains such as *Hanseniaspora vineae*, and Günata et al. [30] studied *Candida* species. A recent, extensive review of 317 strains from 20 wine yeast species indicates that yeasts of the *Candida, Debaryomyces, Hanseniaspora, Kloeckera, Kluyveromyces, Metschnikowia, Pichia, Saccharomycodes, Schizosaccharomyces*, and *Zygosaccharomyces* genera carry out β-glucosidase activities [61]. Saha and Bothast [62] did a further screening of 48 yeast strains of the genera *Candida, Kluyveromyces, Debaryomyces* and *Pichia* for production of extracellular glucose tolerant β-glucosidase activity. All yeast strains tested produced extracellular β-glucosidase activity, but enzymes from only 15 yeasts showed very high glucose tolerance.

Other yeast enzymes such as esterases are also involved in the formation of aroma compounds. However, very little research has been devoted to this type of enzyme. Yeast esterases studied include those of the genus *Brettanomyces* [69] and the species *S. cerevisiae* [52, 71, 73] and *Rhodotorula mucilaginosa* [41]. Recently, the isolation and partial

characterization of an esterase from a *Debaryomyces hansenii* strain has been reported [6].

Lipases can degrade lipids originating from the grape or from autolytic reactions of yeasts, releasing free fatty acids into the juice or wine, which may potentially affect wine quality. Although properties of lipoxigenase and peroxide-cleaving enzymes from grapes have been well established [12, 48, 75], few data are available about lipase production by non-*Saccharomyces* yeasts. Ratledge and Tan [58] reviewed the production of extracellular lipases by yeasts, but data about wine yeasts were not presented. Several wine yeasts from the genus *Candida* have been described as able to hydrolyse tributyrin [13], but further research is needed to determine their possible application in winemaking.

The addition of exogenous enzymes to solve filtration problems (proteases, pectinases and glucanases) or to increase aroma (glycosidases) is a frequent practice in wineries. These enzymes are normally produced by bacteria or filamentous fungi; although commercial preparations of such enzymes are available, they are complex undefined mixtures of enzymes. Previous studies [13, 62] have revealed the potential of indigenous wine yeasts to produce extracellular enzymes of enological significance to modify grape juice and to improve sensory properties of wine.

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