

Chapter 6

Wine Fermentation

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Abstract The molecular biology revolution has brought forth significant new advances with application in microbiological analysis during wine production and storage. For example, traditional methods for microbial strain identification have been mostly supplanted in favor of ribosomal RNA-based methods for speciation of cultured yeast and bacterial populations in wine. Moreover culture-independent molecular methods now allow for more rapid profiling of complex populations, or quantification of targeted species, thereby enhancing the information available to the winemaker. Finally, the availability of microbial genome sequences provides a wealth of new opportunities to understand and exploit the microorganisms in wine, as well as identify the key genetic factors underlying wine flavor development or depreciation. In general, advances in molecular biology are fundamentally changing how scientists and winemakers assess the microbial ecology of winemaking, providing new insight into the wonderfully complex conversion of grape juice to wine.

1 Introduction

The conversion of grape juice to wine is a biotechnological tradition dating back to the dawn of civilization. Throughout the ages numerous winemaking strategies were developed resulting in the range of wine products, from champagne to port, available today. However, since the time of Pasteur (1873) the microbial contribution to the production of wine has become a subject of research and, often, debate. Wine composition and quality are functions of many different intrinsic and extrinsic variables, many of which are microbiologically mediated. A large diversity of microbes are inherent to winemaking including various yeasts, bacteria and fungi. Prominent in this process are *Saccharomyces* species (predominantly *S. cerevisiae*), which dominate the alcoholic fermentation, and the lactic acid bacteria (LAB), which carry out the malolactic conversion. Efforts to determine the population size and potential impact of different microbes on the winemaking process are critical to production of a flavorful product. Spoilage is considered growth of organisms that are unwanted at any particular place and time in the winemaking process (Sponholz 1993). Thus the same microorganism can be both beneficial and detrimental to the

winemaking process. For example, growth of *S. cerevisiae* is required during the alcoholic fermentation, but growth can be detrimental if it occurs in a finished, and bottled, wine.

Interestingly, both academics and winemakers have good reason to be interested in the microbial ecology of the winemaking process. From an academic perspective wine represents an ideal landscape in which to study basic concepts of microbial ecology. Several factors promote this view. First and foremost, wine is a liquid medium that allows samples to be properly mixed prior to analysis, thus ensuring a representative sampling. This contrasts with the situation for those studying microbial growth on solid surfaces (e.g., barrel stave, grape surface or vineyard soil) in which the microbial populations are heterogeneous and spatially distributed across the surfaces. As a consequence, representative sampling of microbes on surfaces becomes a more statistically challenging process than sampling of a liquid medium like must or wine. A second reason why wine is an attractive platform for microbial ecology is the diversity of microbes present which enables one to witness a range of microbial interactions from commensalisms and neutralisms to antagonisms.

From the winemaker perspective, close monitoring of the microbial changes occurring throughout the winemaking process is beneficial for several reasons: to promote and guide yeast during the alcoholic fermentation, to verify the growth of the bacteria during the malolactic conversion, and ultimately to ensure the stability of the wine before bottling and storage (Delfini and Formica 2001). The evolution of undesired microbes during different stages of winemaking can produce volatile acidity, off-flavors and polysaccharide hazes, all of which can diminish the quality and acceptability of the final product (Sponholz 1993). Even prior to the onset of fermentation, the grapes themselves can be infected with molds, yeasts and bacteria that can enter and alter the fermentation in a negative fashion. Improper wine storage and handling post-fermentation can encourage microbiological faults, which can negatively impact wine quality. As a result the winemaker must conduct basic physical, chemical, sensory and microbiological analyses of musts and wines to assure wine quality.

Whether it is for an investigation of basic ecological concepts or for the applied goal of predicting possible wine spoilage, one must have accurate and reproducible methods for enumeration of various microbial constituents at different stages in wine production. Both indirect and direct approaches can be used to view these populations. In this review, we will summarize both approaches and comment on the future use of newer molecular tools to view the microbial diversity inherent in wine fermentations.

1.1 A Brief Overview of the Winemaking Process

Given the process of winemaking is ancient, it is not surprising that a multitude of winemaking styles and wine products abound, each of which can influence the microbial presence. In this section we will describe the core aspects of winemaking

and the important microbiological changes inherent to that process. More detailed descriptions of the many strategies employed in winemaking can be found elsewhere (Boulton, et al. 1996; Ribereau-Gayon, et al. 2000). A general schematic of common steps in red and white winemaking is presented in Fig. 6.1.

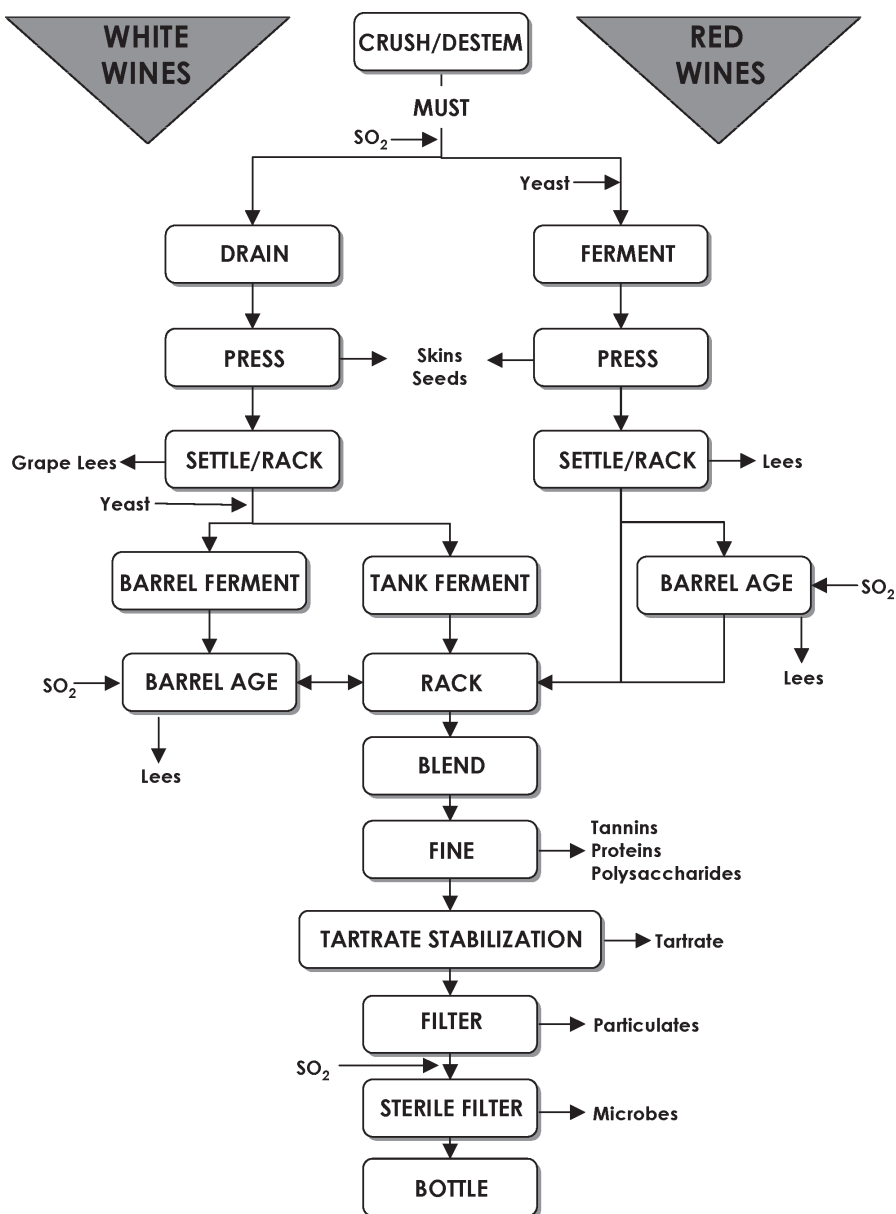


Fig. 6.1 General schematic for production of white and red wines

Winemaking begins with the collection and crushing of grapes. For white wines the grape juice is separated away from the skins and clarified via cold settling, filtration or centrifugation. The juice is then moved to a barrel or fermentation tank and the alcoholic fermentation is carried out by yeasts indigenous to the juice, or via inoculation of a selected *S. cerevisiae* starter culture. White wine fermentations are typically carried out for roughly one to two weeks at temperatures around 10 to 18°C. Upon consumption of available glucose and fructose, the main sugars in grape juice, the wine is considered “dry” and separated from the yeast and grape lees (sediment).

Red wines are produced slightly differently than white wines. After crushing the skins are left in the fermentation to allow for color extraction. Like white wines, the alcoholic fermentation commences either through the action of indigenous yeasts or via direct inoculation of a starter culture. During the fermentation the grape material tends to float to the top of the vat forming a “cap.” To better enable extraction of red pigments and to influence wine flavor, winemakers typically punch down the cap or pump juice from the bottom over the cap. After a suitable period of time, the wine is separated from the grape skins and the fermentation is completed in another vessel. As described for white wines, the red wine is now “dry” and devoid of the main juice sugars.

After the alcoholic fermentation, wines often are spontaneously, or purposely, taken through a malolactic fermentation in which the high level of malate in the juice is converted to lactate, mostly by indigenous or inoculated LAB. Unlike the alcoholic fermentation, the malolactic fermentation is a stylistic consideration by the winemaker, who, through use of antimicrobial additions (primarily sulfur dioxide) or filtration may choose to prevent this fermentation from initiating.

Once the wine has been taken through the alcoholic and, if desired, the malolactic fermentation, the wine is often stored in tanks or barrels to allow flavor development. The residence time for storage is primarily determined by the style of wine and winemaker choice. Often white wines are not stored for long periods of time while reds are frequently stored in oak barrels for several years. While the average wine contains approximately 13 percent ethanol, the alcohol by itself does not preclude future spoilage. Consequently winemakers must take great care to prevent exposure of the wine to oxygen, which can encourage microbial growth, as well as judiciously use antimicrobials (again, primarily sulfur dioxide) to prevent microbial spoilage.

1.2 Microorganisms in the Winery Environment

The initial environment that affects the microbial makeup of a wine fermentation is that of the vineyard. Although a drastically different environment than juice or wine, the types of microbes present on grapes will have an impact on the ensuing ecology in the wine fermentation, particularly in the early stages. Microorganisms appear to colonize around the grape stomata where small amounts of exudate are secreted

(Ribereau-Gayon, et al. 2000). The apiculate yeasts, *Hanseniaspora* and *Kloeckera*, its asexual anamorph, are the most prevalent vineyard yeasts and typically represent over half the yeast flora on grapes (Pretorius, et al. 1999). Other yeast genera present on berries include: *Metschnikowia*, *Candida*, *Cryptococcus*, *Rhodotorula*, *Pichia*, *Zygosaccharomyces* and *Torulopsis* (Barnett, et al. 1972; Rosini, et al. 1982; Moore, et al. 1988). Also present in the vineyard are numerous other yeasts, some of which have an impact on wine: *Sporobolomyces*, *Kluyveromyces*, and *Hansenula* (Davenport 1974). *Saccharomyces* species are relatively scarce among healthy berries (Vaughan-Martini and Martini 1995; Mortimer and Polsinelli 1999). On damaged berries, *Saccharomyces* is present at significant but low levels (10^5 to 10^6 CFU per berry), compared to total microbial population levels of 10^7 to 10^8 CFU per berry (Mortimer and Polsinelli 1999). Mortimer (1999) suggested honey bees, wasps, and fruit flies as likely vectors for carrying and spreading *Saccharomyces* and other yeasts among damaged grapes.

Filamentous fungi also colonize the grape surface. Mold and mildew damage can influence the grape and wine microbial ecology in several ways. Of much interest is the mold *Botrytis cinerea*, known as noble rot or gray mold rot, depending on the degree of infection. Noble rot appears to occur on healthy berries where fungal hyphae penetrate cracks surrounding the stomatal opening or fissures in the cuticle; gray mold rot, on the other hand, infests damaged berries (Ribereau-Gayon, et al. 1980). The former mode of infection is associated with the high quality of sweet wines of limited production, such as Auslese/Beerenauslese/Trockenbeerenauslese, Tokay, Sauternes and others from California, South Africa and Japan (Dittrich 1991). Dehydration caused by increased porosity of the grape skin results in higher sugar concentration (as much as twice as high) in the resulting must. *Botrytis* sp. infection metabolizes sugars and malic and tartaric acids, reducing the total sugar content somewhat and raising the pH of the wine (Hofmann 1968). Moreover, *Botrytis* sp. infection encourages the proliferation of acetic acid bacteria (AAB) (Joyeux, et al. 1984) and yeasts (Le-Roux, et al. 1973) on grapes which, in turn, can affect the chemical composition and microbial makeup of the must/juice. Such damaged berries, whether resulting from mold attack, precipitation-induced swelling, hail or other pests are considered "very rich depositories" of microorganisms (Mortimer and Polsinelli 1999).

A distinct turning point occurs between the vineyard and the winery. As soon as the grapes are handled they become exposed to a new pool of organisms. The transfer of molds, yeasts and bacteria from equipment and surfaces represents the potential introduction of "resident" winery microbes to the grapes (Peynaud and Dimerco 1959) and, conversely, new sources of substrate are made available to existing microbes on the grape. The microbial populations present on equipment surfaces will vary according to the extent of sanitation employed on everything from picking knives, mechanical harvesters and grape bins, to crushers, tanks, hoses and pumps, to the walls and floors. Various species from the genera *Saccharomyces*, *Candida*, *Pichia*, and *Brettanomyces* were associated with winery equipment and surfaces (Peynaud and Dimerco 1959). Variables—with respect to time, temperature, and handling—of grape transport between the vineyard and the

winery likely affect the ecology of the grapes. Since some damage to the grapes occurs, especially with machine-harvested fruit, activity of microorganisms in the resulting juice probably occurs.

As a result of grape microflora and grape processing, the resultant juice, fermenting must and wine are complex microbial ecologies hosting a diverse collection of yeast and bacteria. The dominant yeast responsible for alcoholic fermentation—the bioconversion of six-carbon sugars to ethanol—is *S. cerevisiae*. However, juice and wine may also contain a wide array of yeast genera including *Metschnikowia*, *Dekkera* (anamorph *Brettanomyces*), *Pichia*, *Candida*, *Hanseniaspora* (anamorph *Kloeckera*), *Kluyveromyces*, *Issatchenkia*, *Torulaspora*, *Debaryomyces*, *Saccharomycodes*, *Zygosaccharomyces* and *Schizosaccharomyces* among others (Fleet 2003; Loureiro and Malfeito-Ferreira 2003). Depending on the acidity and the nutrient, oxygen and ethanol concentrations in the juice or wine, the active bacteria present typically include both LAB and AAB (Osborne and Edwards 2005). Other bacteria such as bacilli, clostridia, actinomyces or streptomyces have been identified in the wine environment, however, these represent relatively rare occurrences.

Once the grapes have been thoroughly crushed (or pressed), a new stage begins in the selection of yeast and bacterial species. The change in environment results in a change in selective pressures. Furthermore, enological practices alter the conditions of the juice. Most notably, sulfur dioxide is widely utilized in winemaking to control populations of microorganisms. Most wine-related bacteria and many yeasts are sensitive to molecular SO_2 . Molecular SO_2 is present in solution in pH-dependent equilibrium with bisulfite (HSO_3^-) and sulfite (SO_3^{2-}) ions. The proportion of the molecular SO_2 species is higher at lower pH; for instance, at pH 3.0, the proportion of molecular SO_2 in solution is 10 times higher than at pH 4.0 (Boulton, et al. 1996). *Saccharomyces* species are relatively resistant to SO_2 , as are the apiculate yeasts (Stratford, et al. 1987). The bacteriolytic enzyme lysozyme is another antimicrobial additive increasingly used to reduce bacterial populations (Fuglsang, et al. 1995), however, its use on a large production scale is limited by cost (Pretorius 2000).

1.3 Yeast Ecology during Fermentation

As fermentations proceed, metabolites and other products generated by yeasts can impact the performance of other organisms. The products of fermentation, CO_2 and ethanol, are prime examples. The evolution of CO_2 can inhibit yeast growth at 15 g/L (Dittrich 1991), but it has the primary effect of excluding O_2 from the fermenting medium. This action, especially in combination with alcohol, prevents the growth of aerobic organisms generally associated with spoilage. Different yeast species and strains have varying sensitivities to ethanol. *S. cerevisiae* species, as would be expected, have high ethanol tolerance—it can generally ferment to 15 percent to 16 percent ethanol (Gao and Fleet 1988; Dittrich 1991). Gao and Fleet (1988) showed

that some strains of *C. stellata* had relatively high ethanol tolerance, as well, while *Kloeckera* sp. exhibited fairly low tolerance. Higher temperatures appear to decrease ethanol tolerance. Some non-*Saccharomyces* yeasts found in wine are capable of producing ethanol – namely *Torulaspora delbrueckii*, *Saccharomyces ludwigii* at moderate to high levels, *C. stellata* at low to moderate levels, and the apiculate yeasts at low levels – with production limits a likely indication of ethanol tolerance (Ciani and Maccarelli 1998). The resistance of *S. cerevisiae* to its metabolic products, especially ethanol, work to select it as a dominant organism in fermentations.

In addition to ethanol, yeasts produce other metabolites that broadly inhibit cell growth. Medium chain fatty acids, octanoic and decanoic acids, act by interfering with plasma membrane integrity (Alexandre and Charpentier 1998; Bisson 1999). Wine yeast also produce killer factors which can impact the survival of other yeasts within the same environment (van Vuuren and Jacobs 1992). Finally different strains of *S. cerevisiae* have been shown to produce anywhere from 25 to 100 ppm of SO₂ (Thornton 1991).

Obviously a major factor affecting microbial composition in wine fermentations is the practice of inoculation with commercial or otherwise selected strains of *S. cerevisiae*. Inoculation can be particularly effective in combination with SO₂ in reducing non-*Saccharomyces* populations and promoting the growth of *S. cerevisiae* (Constanti, et al. 1998; Egli, et al. 1998).

1.4 The Lactic Acid Bacteria

The LAB involved in wine are comprised of acid and ethanol-tolerant strains primarily from four genera *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Oenococcus* (formerly *Lc. oeni*) (Sponholz 1993; Lonvaud-Funel 1999; Osborne and Edwards 2005). These microbes are commonly found on grapes and in the winery environment. Newly fermented wines contain low populations of LAB, usually less than 10³ CFU per mL (Davis, et al. 1985), however, damage to the grapes increases this number by several orders of magnitude. Three main factors that dictate the extent of LAB growth in wine are pH, ethanol and antimicrobial additions such as SO₂ or lysozyme. These latter additions purposely reduce LAB concentrations to enable proper growth of *S. cerevisiae* and/or to microbially stabilize the wine. Wine pH also strongly influences which LAB species will be present. Higher pH wines (above pH 3.5) often harbor species of *Lactobacillus* and *Pediococcus*, both during and after fermentation, while lower pH wines (< 3.5) typically only contain *O. oeni* (Fleet 1998; Osborne and Edwards 2005).

Ethanol production from the dominant *S. cerevisiae* population also serves to reduce all LAB populations in the first few weeks of the alcoholic fermentation. However, as the wine is stored, the ability of ethanol-tolerant LAB to emerge increases. Growth substrates can be available at this stage as a consequence of yeast cell lysis and release of nutrients into the wine (Lonvaud-Funel 1999).

1.5 The Acetic Acid Bacteria

AAB are Gram-negative obligate aerobes that produce acetic acid, acetaldehyde and ethyl acetate from both glucose and ethanol (Ribereau-Gayon, et al. 2000). AAB are spoilage organisms commonly found in wine, beer and cider (Kerstens, et al. 2006). Those found in wine belong to the genera *Acetobacter*, *Gluconobacter* and *Gluconacetobacter* (Osborne and Edwards 2005). Previously lumped together under the genus *Acetobacter*, *Gluconobacter* was given its own genus because of its poor growth in beer and its ability to produce large amounts of gluconic acid from glucose (Adams 1998).

Damaged grapes often contain significant populations of *Gluconobacter* (Fleet 1998; Du Toit and Lambrechts 2002). In addition, production of gluconic acid and other carbonyl-containing compounds by *Gluconobacter* sp. can bind to SO₂, thus lowering the overall efficacy of sulfite additions (Barbe, et al. 2001). Therefore, from a winery's perspective, *Gluconobacter* sp. growth poses the biggest threat to juices and *Acetobacter* sp. to finished wines (Du Toit and Lambrechts 2002). Because they are aerobic bacteria, minimizing air contact of static wine is an effective means of controlling AAB. However, static populations of AAB will start multiplying again with exposure to air—as found in the pumping and transferring of wine (Sponholz 1993). Bartowsky and co-workers found *A. pasteurianus* to be a major culprit in the production of acetic acid in bottles of red wine that had been stored upright (Bartowsky, et al. 2003).

2 Culture-independent Studies on Wine Microbial Ecology

Most approaches to identify and enumerate microbes in wine involve enrichment techniques (Boulton, et al. 1996; Fugelsang 1997). Such methods are considered “indirect” because one is not enumerating the original cells in the sample, but their progeny, as enriched in a specific medium. Various texts (Boulton, et al. 1996; Fugelsang 1997) describe both general and selective growth media for plating yeasts and bacteria from wine. Unfortunately, plating and enrichment procedures are time consuming as colonies for some wine-related microbes take up to a week or more to appear on a plate. Additionally, once colonies do appear on a plate, definitive identification of the microbe requires further testing. More importantly, culture-based techniques typically underestimate the size and diversity of a population as sublethally injured or viable, but non-culturable (VBNC) cells, common in wine, may fail to grow on plates (Kell, et al. 1998; Millet and Lonvaud-Funel 2000). Understanding this difference between a true total cell count and a culturable population is important as VBNC or injured cells are still metabolically active.

At present a true VBNC state is only associated with a bacterial response to adverse environmental conditions such as starvation, changes in pH or the presence of antimicrobials (Barer and Harwood 1999). Alternatively, the non-culturable cells

may be sublethally injured by the chemicals used in wine such as sorbate, sulfite or ethanol, thus losing the ability to grow on standard culture media (Stevenson and Graumlich 1978; Fleet 1990; Davidson 1997; Fleet and Mian 1998). In either case, VBNC or sublethally injured organisms may still play a role in wine spoilage. Thus the examination of these states in wine-related microbes may help explain some of the known spoilage problems where correlations between spoilage and plating of specific microorganisms are difficult to derive. One such example is the production of the phenolic taints by *Brettanomyces* sp. (teleomorph *Dekkera*). *Brettanomyces* sp. have been shown to produce 4-ethylphenol from phenolic acids, at higher levels than other microorganisms present in the wine fermentations (Chatonnet, et al. 1995). However, it has been difficult to correlate the amount of 4-ethylphenol to the population size of *Brettanomyces* sp. in the wine. In some cases, when *Brettanomyces* sp. are not detected at significant levels, the compounds are still detected at high levels (Chatonnet, et al. 1995; Rodrigues, et al. 2001; Fugelsang and Zoecklein 2003). Another example is provided by Coton, et al. (1998) in studies of *O. oeni*. They inoculated *O. oeni* strain 9204 into a red wine after MLF and an addition of sulfite. After one month no colonies were detected, however, *O. oeni*-derived histidine decarboxylase activity was still detectable.

While the VBNC state or sublethal injury may provide an explanation for cases such as wine spoilage by *Brettanomyces*, few studies have examined the possible VBNC state in wine-related microbes. While the ability of bacteria to enter the VBNC state has been documented in many different environments, a number of studies also suggest that a VBNC state may exist in yeasts. First, Rodrigues and Kroll (1985), using the direct epifluorescent filter technique (DEFT) on *S. cerevisiae*, found that cell counts using acridine orange dye correlated well with plating results of non-stressed cells, but heat-treated samples gave higher counts compared to plating results. This observation, while not explained as a VBNC state, is similar to the higher direct viable counts of stressed cells seen by Xu, et al. (1982) that lead to the VBNC theory.

Regardless of whether non-culturable cells in wine are truly VBNC or simply sublethally injured, the fact that these cells continue to influence wine flavor and palatability argues the need for use of culture independent or “direct” analysis methods to assess the true population.

Various direct approaches have been developed for enumerating microbes in wine. The technique employed most often is simple microscopy from which wine-makers can readily differentiate yeast from bacteria, as well as determine microbial concentrations directly in wine with the use of counting chambers (Amerine and Kunkee 1968). Microscopic analysis, combined with use of simple stains such as methylene blue (Borzani and Vairo 1958), enables colorimetric differentiation of live versus dead yeast cells.

Another direct technique used by the wine industry to score microbial populations employs the enzyme luciferase to assay for microbial-borne ATP (de Boer and Beumer 1999; Gracias and McKillip 2004). This general approach has been adapted to various commercial systems for use in wineries. While this approach is useful in specific applications, such as to assess winery surfaces after cleaning, the

method also can detect ATP from other, non-microbial, biological sources and, thus, is problematic for use directly in wine fermentations.

Fluorescent dyes have also been employed to directly assess yeast viability in wine. Use of flow cytometry enables information on cell size and shape to be obtained by light scattering. In addition cell viability can be assessed directly using fluorescent dyes to view the metabolic state of yeast and bacteria in wine (Malacrino, et al. 2001; Boyd, et al. 2003; Chaney, et al. 2006; Herrero, et al. 2006). Flow cytometry can also be useful if, instead of stains, DNA probes or antibodies specific for a particular microbial species are used (Graca da Silveira, et al. 2002). This would allow not only the enumeration of targeted microbes but, coupled with a live/dead marker, also the percentage of living cells versus dead.

One particularly successful direct analysis application is the use of direct epifluorescence technique (DEFT) in which microbial-based cleavage of a fluorescent substrate enables direct counting of viable cells through a fluorescent microscope. Using this method Millet and Lonvaud-Funel (2000) first demonstrated significant non-culturable populations of both bacteria and yeast in aging wine, detecting at least 100-fold higher viable cell numbers using DEFT by comparison to that obtained by plating. They also demonstrated that both *A. aceti* and *P. damnosus* inoculated into wine maintained a higher viable cell population determined by DEFT, compared to plating. Interestingly, most of the non-culturable *A. aceti* population recovered the ability to grow on plates after aeration (Millet and Lonvaud-Funel 2000). In a later study, du Toit, et al (2005) used DEFT to demonstrate that *A. aceti* could survive for up to 71 days under anaerobic conditions in sulfited wine. These AAB exhibited a 100-fold difference between plating and DEFT counts and, upon addition of oxygen, the *A. aceti* populations became culturable. The yeasts *C. stellata*, *S. cerevisiae*, *Z. bailii* and *Rhodotorula mucilaginosa* were also found to enter the non-culturable state after addition of SO₂ in botrytized wine (Divol and Lonvaud-Funel 2005). Only *Z. bailii* and *R. mucilaginosa* were resuscitated in laboratory conditions. The fermentative yeasts *C. stellata* and *S. cerevisiae* were not able to recover, likely due to the presence of ethanol and a high osmotic environment (Divol and Lonvaud-Funel 2005). While these studies suggest that VBNC populations may exist in wine and that the VBNC state most likely plays a role in wine spoilage, more studies are needed to better understand the physiology of organisms in the VBNC state and their ability to effect wine during maturation.

2.1 Nucleic Acid-based Approaches

Numerous nucleic acid-based assays have been developed for directly characterizing microbes in wine. The first such techniques used probes generated to whole bacterial genomes to reveal specific LAB populations in wines (Sohier and Lonvaud-Funel 1998). More recently an array of probes used in fluorescence *in situ* hybridization (FISH) have been developed for direct analysis of LAB from wine (Blasco, et al. 2003). This includes specific probes for common wine species *O. oeni*, *P. damnosus*,

P. parvulus, *P. pentosaceus*, *Lb. plantarum*, *Lb. casei/paracasei*, *Lb. brevis*, *Lb. hilgardii* and most *Leuconostoc* species among others (Blasco, et al. 2003). The FISH approach was tested *in situ* on actual wine samples directly identifying *O. oeni* in 20 wines that had undergone the malolactic fermentation. While use of this method directly on wine has been rare, the general FISH approach is commonly used to characterize other environments. Moreover, the flexibility of the probe design to target whole taxa or select species enables useful representation of the possible populations present.

Other molecular survey techniques have been developed to profile total fungal or bacterial populations in natural environments (Head, et al. 1998). The more common of these methods employ amplification of ribosomal RNA genes by PCR followed by cloning (Head, et al. 1998), terminal restriction fragment length polymorphism (TRFLP) (Marsh 1999) or denaturing gradient gel electrophoresis (PCR-DGGE) (Muyzer and Smalla 1998).

2.2 Studies Employing PCR-DGGE

Of the popular survey methods, PCR-DGGE has been used the most to characterize both yeast and bacteria in the wine environment. PCR-DGGE was first applied to wine yeasts by Cocolin, et al (2000) who developed primers that amplified a portion of the D1-D2 loop of the yeast rRNA large subunit gene. That work demonstrated that the population shifts of different wine-related yeasts, such as *S. cerevisiae*, *M. pulcherrima*, *C. ethanolica*, and *K. apiculata*, in laboratory-based mixed-culture fermentations could be easily followed using PCR-DGGE. Importantly, this work revealed that PCR-DGGE could identify yeast populations that were at least 0.01 percent or higher of the dominant *Saccharomyces* sp. population, thereby defining the limits of detection for this method. This approach was then applied to follow yeast populations within a commercial sweet wine fermentation revealing the temporal presence of fungal species (*B. cinerea*) and several non-*Saccharomyces* yeasts, including *Metschnikowia* sp. and *Pi. anomala*, in the early stages of the fermentations along with the emergence and persistence of a dominant *S. cerevisiae* population (Cocolin, et al. 2001). This work also revealed the persistence of a *Candida* sp. DGGE signature throughout a complete wine fermentation (some 104 days later!). Additional studies on this commercial sweet wine fermentation revealed that PCR-DGGE signals for many non-*Saccharomyces* yeast populations could persist well into the fermentation and long after these yeasts could be identified on culture media (Mills, et al. 2002). This was particularly evident for the *Candida* sp. population (see Fig. 6.2), a species later determined to be *C. zemplinina* (Sipiczki 2003). DGGE signatures from both RNA and DNA templates directly purified from wine revealed *C. zemplinina* signatures persisted throughout the fermentation even when direct plating exhibited a relatively low number of cells. Direct RNA dot blot analyses using *C. zemplinina* specific probes revealed the size of that population at the end of the fermentation to be relatively

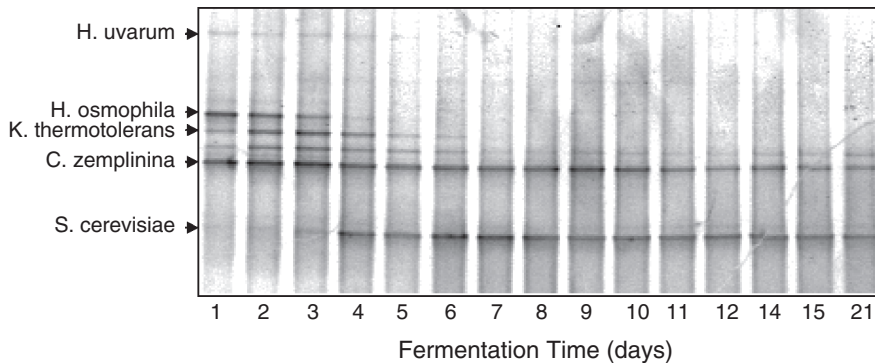


Fig. 6.2 Example of PCR-DGGE profiling of yeast constituents of a botrytized-wine fermentation. Individual bands were excised and sequenced to identify genera and species. Note that bands representing *C. zemplinina* and *S. cerevisiae* populations are evident throughout the fermentation

high ($>10^6$ cells per mL) when only 100–1000 CFU per mL could be detected by plating. These results provided some of the first evidence that metabolically active, yet non-culturable, yeasts persist in wine fermentations. Interestingly, when grown in isolation on grape juice, the *C. zemplinina* EJ1 isolate was shown to be exclusively fructophilic, a result which suggests a resource neutralism established between that strain and the dominant glucophilic *S. cerevisiae* culture present in these commercial fermentations.

Since the initial application of PCR-DGGE on wine several other groups have adopted this approach to directly profile yeasts in commercial fermentations. Cocolin, et al (2002) used PCR-DGGE to monitor a continuous wine fermentation, demonstrating a temporal occurrence of *M. pulcherrima* during the early stages of the fermentation and a stable presence of the inoculated *S. cerevisiae* culture throughout. Recently Renouf, et al (2006a) employed PCR-DGGE to follow wine production through alcoholic and malolactic fermentations at three wineries in France. In all three cases a relatively stable population of *Brettanomyces bruxelensis*, considered by many to be a spoilage yeast, was present after the alcoholic fermentation and appeared better able to prosper in the harsh environment of finished wine (e.g., low sugar and high ethanol).

While PCR-DGGE is a common approach to profile bacteria in other niches, the use of this approach in wine was complicated by inherent problems associated with primer specificity. Several groups have demonstrated how common primer sets used for bacterial PCR-DGGE mis-amplify eukaryotic DNAs (yeasts, molds or plants) (Lopez, et al. 2003; Dent, et al. 2004). To resolve this problem new 16S rRNA gene-based primers were developed (Lopez, et al. 2003), or alternative alleles such as the *rpoB* gene, were profiled (Dahllof, et al. 2000). The latter provided an elegant approach since it obviated both the problems inherent in mis-amplification of non-bacterial DNAs, but also reduced the problems inherent to the heterogeneity among multiple copies of the 16S rDNA within the same bacteria. A limitation with this approach is the relative lack of *rpoB* gene sequences in public databases

restricts the ability to concretely identify DGGE bands upon re-sequencing. Renouf, et al. (2006b) recently employed *rpoB*-targeted primers to follow bacterial populations in wine from three wineries using PCR-DGGE. This work revealed significant differences in initial bacterial populations at each winery, likely a reflection of different winemaking practices and differential use of antimicrobial additions. In addition *O. oeni* was repeatedly and consistently observed throughout all the winery fermentations, even in fresh must samples. Finally, this approach revealed a secondary *Pediococcus* population at one winery after racking and sulfating of the wine, suggesting emergence of a spoilage population.

PCR-DGGE approaches have been applied less frequently to characterize the yeast or bacterial populations on wine grapes. Prakitchaiwattana, et al. (2004) found mostly the *Aureobasidium pullulans*, a ubiquitous environmental yeast, on undamaged grapes and *Metschnikowia* and *Hanseniaspora* sp., as well as *Au. pullulans*, on damaged grapes. The authors noted that PCR-DGGE was not as sensitive as plating and could not detect yeasts at population levels lower than 10⁴ CFU per gram (of grapes), however, they did note that a greater diversity of fungal species from grapes could be witnessed by DGGE. Recently Renouf and Lonvaud-Funel (2007) developed a modified enrichment method, followed by PCR-DGGE and other molecular methods, to demonstrate that the grapes may be a source for the spoilage yeast *Br. bruxellensis* (teleomorph *Dekkera bruxellensis*). This remarkable finding provides a clue as to a non-winery source for this problematic yeast.

2.3 Direct PCR Approaches

While the molecular profiling methods discussed above have provided a window from which to view all of the individual constituents of wine fermentation, both culturable and non-culturable, other PCR approaches directly assay for select population members. Endpoint PCR assays have been developed for several wine yeast and bacteria. Lopez, et al (2003) used a multiplex PCR approach amplifying different segments of the yeast *S. cerevisiae* COX1 gene to differentiate different starter strains. The authors then demonstrated they could employ the multiplex PCR directly on wine fermentation samples to assess implantation of a dominant starter culture. Ibeas, et al. (1996) developed a nested PCR method using primers designed to a putative RAD4 gene which readily detected *D. bruxellensis* and synonymous strains. The assay was employed directly on sherry wine samples to reveal the presence of *D. bruxellensis* in wine suspected to contain *Dekkera* sp. contamination. Cocolin, et al. (2004) used a similar approach designing specific primers to the *Br. bruxellensis* and *Br. anomalus* 26S rRNA gene to directly confirm *Brettanomyces* sp. contamination within wine using a single PCR reaction. The authors also showed that *Br. bruxellensis* and *Br. anomalus* were further resolved by a restriction digestion of the resultant amplicon. Cocolin, et al. (2003) also developed 26S rRNA gene PCR primers for specific amplification of *H. uvarum* and *C. zemplinina*. In that work the authors revealed a persistence of both RNA and DNA signatures for

H. uvarum and *C. zemplinina* in sulfited wine, even though no growth of either species was witnessed on plating media. While some researchers suggest that detection of RNA, even ribosomal RNA, is a signal of a metabolically active state—mostly due to the fact that RNA degrades more rapidly than DNA—the actual metabolic state of these cells remained unknown and the persistence of RNA signatures from dead cells in wine has yet to be examined. Thus the detection of *H. uvarum* and *C. zemplinina* RNA signatures in wine more than 20 days after no cognate colonies could be enriched on plates provides a useful example of how PCR approaches need to be viewed with caution since both live and dead cells may be detected.

Several groups have developed direct PCR methods to identify the specific bacteria in wine. Zapparoli, et al. (1998) and Bartowsky and Henschke (1999) independently developed direct PCR assays to identify the malolactic bacterium *O. oeni* using primers specific for the malate decarboxylase gene (*mleA*) or the 16S rRNA gene, respectively. In both cases the threshold for detection in wine was around 10^3 to 10^4 cells per mL. Others have used direct PCR to detect bacterial genes associated with a specific taint. Le Jeune, et al. (1995) developed PCR primers that amplify the gene encoding histidine decarboxylase (HDC), the cause of the biogenic amine histamine, from several LAB. Coton, et al. (1998) then used this assay to survey 118 wines from Southwestern France and found nearly half of the wines surveyed possessed an amplifiable HDC allele. Gindreau, et al. (2001) used a similar strategy to detect exopolysaccharide-producing strains of *P. damnosus* with a detection limit in wine of 100 CFU per mL.

2.4 Real-time or Quantitative PCR (QPCR) Approaches

A more recent technique that has found wide application in wine fermentations is QPCR. In QPCR the logarithmic amplification of a DNA target sequence is linked to the fluorescence of a reporter molecule. Several different reporter formats exist for QPCR (Hanna, et al., 2005), however, a common reporter used for detection of wine-related organisms is the dye SYBR Green (Vitzthum and Bernhagen 2002). This fluorescent dye binds double stranded DNA molecules and has an excitation wavelength of about 250_{nm} and an emission wavelength around 497_{nm}. This fluorescence, which is read after each round of DNA amplification, may either be compared to an external standard curve known as absolute quantification or it may be compared to an internal or external control sample in a method known as relative quantification (Livak and Schmittgen 2001).

Relative quantification is primarily used to follow gene expression. While the use of relative quantification to analyze gene expression in wine-related microbes provides valuable insights into their biology, the use of absolute quantification is by far the most common type of QPCR employed in wine ecology. To date, QPCR detection methods have been developed for direct enumeration of wine-related microorganisms including *O. oeni* (Pinzani, et al. 2004), LAB (Neeley, et al. 2005), AAB (Gonzalez, et al. 2006), total yeasts (Hierro, et al. 2006), *D. bruxellensis* (Phister and Mills 2003;

Delaherche, et al. 2004), *S. cerevisiae* (Martorell, et al. 2005) and *Zygosaccharomyces* species (Casey and Dobson 2004; Rawsthorne and Phister 2006).

With QPCR, specific bacteria or non-*Saccharomyces* yeasts in wine fermentations can be enumerated in the presence of high levels of *Saccharomyces* sp. For example, Gonzalez, et al. (2006) detected populations of AAB as low as 10 CFU per mL in the presence of overwhelming amounts of *Saccharomyces* (10^7 CFU per mL). By comparison, other survey methods such as PCR-DGGE, or even microscopy, generally require at least 1,000 to 10,000 organisms per mL (Cocolin, et al. 2000). QPCR provides a rapid method of detection, compared to conventional plating methods. Organisms such as *D. bruxellensis* can be detected and enumerated in as little as one to two hours, which is a substantial improvement on the five to 10 days required for conventional analysis by plates (Phister and Mills 2003). This time difference provides winemakers the opportunity to intervene long before spoilage is an issue.

The targets for QPCR assays vary between organisms; the most common is an rRNA gene in each organism, as this may be the only sequence information available for many wine-related organisms (Neeley, et al. 2005; Gonzalez, et al. 2006; Rawsthorne and Phister 2006). However, other sequences have been targeted, such as the gene encoding of the malolactic enzyme from *O. oeni* (Pinzani, et al. 2004), or even bands isolated by RAPD-PCR from *S. cerevisiae* (Martorell, et al. 2005). In a most prescient example, Delaherche, et al. (2004) used QPCR to solely enumerate exopolysaccharide-producing, or “ropy,” strains of *Pediococcus* in wine. Production of exopolysaccharide by pediococci in wine can result in a viscous, unpalatable spoilage. By targeting the *dps* gene within a pediococcal exopolysaccharide cluster (Walling, et al., 2005), the authors were able to directly enumerate only those strains that had potential for ropy spoilage. This is a promising addition to the QPCR approach because in many cases the pediococci present might not harbor the specific genes responsible for a taint. In such a situation QPCR-based enumeration of microbes at the species level will not determine the potential risk for a certain taint.

At present the promise of QPCR approaches as a means to more fully describe wine microbial ecology is yet unrealized. While a large number of QPCR detection assays have been developed, few have used these approaches in larger scale ecological studies. Regardless, since the approach is now commonly used in a number of service laboratories for microbial screening of wines, one would predict that direct QPCR-based survey data on different wineries' microbiota will be forthcoming.

3 Culture-dependent Studies on Wine Microbial Ecology

Given its prominence in various countries, it is not surprising that wine fermentations and winery environments are some of the most studied microbial ecologies. Indeed, the microbial diversity present in wine production, described in a previous section, was obtained chiefly through enrichment studies. Unlike the use of culture-independent approaches that revealed metabolically active, but

non-culturable, populations in wine (Millet and Lonvaud-Funel 2000), the main benefit of new molecular identification methods of enriched isolates is to further delineate the species (or subspecies) of yeast and bacteria present in different wine settings. This has provided new resolution to the types of yeasts and bacteria present but, unlike the culture-independent methods, has not dramatically changed the overall view of the ecology. This section will focus on select molecular identification methods used in post-enrichment analyses of wine-related yeast and bacteria that have advanced our understanding of wine microbial ecology. In general, these molecular methods fall into two categories: those that seek to identify genus and species (and/or subspecies), and those that seek to differentiate strains of the same species. For general information on standard enrichment methods used for wine-related yeasts or bacteria readers are directed elsewhere (Boulton, et al. 1996; Deák and Beuchat 1996; Fugelsang 1997; Boundy-Mills 2006).

3.1 Species and Subspecies Identification and Differentiation

3.1.1 rRNA Gene Sequence Analysis

Clearly no advance in microbial identification has had a more significant impact on rapid identification of enrichment isolates than ribosomal RNA gene analysis (Olsen, et al. 1994). Numerous methods have been developed to profile and catalog differences among rRNA genes in both yeast and bacteria (Towner and Cockayne 1993; Fernandez-Espinar, et al. 2006). Perhaps the most significant for identification of genus and species is the direct sequencing of the 16S rRNA gene in bacteria (Cole, et al. 2005) and the 26S rRNA gene (Kurtzman and Robnett 1998), and, to a lesser extent, the 18S rRNA gene (Valente, et al. 1999) in yeast through comparison to existing databases. Indeed, these methods combined with advances in colony PCR methodology (Hofmann and Brian 1991; Ward 1992) have enabled rapid identification of species from microbial isolates enriched from wine. A common approach is to segregate isolates on the basis of colony morphology and identify genus and species by rRNA gene-sequencing from only select morphotypes. A drawback to sequencing methods is the cost of sequencing which, while decreasing, is still prohibitive for large-scale ecological studies.

In addition to the rRNA gene sequences themselves, the spacer regions between the rRNA genes in yeast (Montrocher, et al. 1998; Egli and Henick-Kling 2001; Belloch, et al. 2002) and bacteria (Le Jeune and Lonvaud-Funel 1997) have been used to further differentiate both yeast and bacteria in wine, primarily assisting in subspecies differentiation.

3.1.2 rRNA Gene RFLP Approaches

An economical method to identify genus and species of yeasts enriched from wine fermentations is the ITS-restriction fragment length polymorphism (RFLP) method

(Guillamon, et al. 1998; Esteve-Zarzoso, et al. 1999). To facilitate identification of yeasts these authors developed a web-accessible database of yeast ITS RFLP patterns enabling direct comparisons of newly generated ITS RFLP patterns (<http://www.yeast-id.com/english/index.html>). While this approach is not as discriminatory as 26S rRNA gene sequence (Arias, et al. 2002), the method has been used to profile yeast species evolution in numerous wine settings (Fernandez, et al. 1999; Granchi, et al. 1999; Pramateftaki, et al. 2000; Esteve-Zarzoso, et al. 2001; Jemec, et al. 2001; Raspor, et al. 2002).

Similar rRNA gene RFLP approaches have been used to identify wine-related bacteria. RFLP of amplified 16S rRNA gene has been employed for identification of wine-related LAB (Rodas, et al. 2003) and AAB (Poblet, et al. 2000). Additional species level discrimination has been achieved by RFLP of the 16S-23S intergenic spacer region (Ruiz, et al. 2000). Since these rRNA gene RFLP approaches require a database of RFLP patterns with which to identify new strains, they are infrequently used by comparison to partial sequencing of 16S rRNA gene as a means to speciate wine-related bacteria.

3.1.3 PCR-DGGE

Others have employed PCR-DGGE or PCR-TGGE of rRNA gene segments to differentiate individual wine yeast isolates (Hernan-Gomez, et al. 2000; Manzano, et al. 2004; Manzano, et al. 2005; Manzano, et al. 2006). Given the discriminatory power of T/DGGE this method works well as long as standards are run for each potential species that one might observe in that environmental niche. This approach is also useful to monitor primary enrichment cultures from wine or grape substrates. Bae and co-workers (Bae, et al. 2006) used this approach to reveal the LAB on grape surfaces that were enriched via different media. In general, however, T/DGGE approaches for identification purposes are technically problematic since control strains must be present in the gel and band co-migration with known standards is not clear confirmation of identify.

3.1.4 Probes

A less popular approach to identify wine microbes enriched on various media is use of specific nucleotide probes targeting ribosomal RNA genes. Stender, et al. (2001) used peptide nucleic acid probes to identify the spoilage yeast, *D. bruxellensis*, on enrichment plates. Recently Xufre, et al. (2006) developed 26S rRNA gene probes for identification of numerous wine-related yeast including *S. cerevisiae*, *C. stellata*, *H. uvarum*, *H. guilliermondii*, *K. thermotolerans*, *K. marxianus*, *T. delbrueckii*, *Pi. membranaefaciens* and *Pi. anomala*. While these latter probes were fluorescently labeled for use in direct *in situ* hybridization applications, the authors have only demonstrated their utility in identifying yeast colonies post-enrichment. Others have used more specific genomic probes to differentiate colonies of closely related LAB species (Sohier, et al. 1999).

3.1.5 PCR Screens for Taint-related Genes within Isolates

Linkage of specific genes within wine microbes to specific taints has led to a series of post-enrichment screens to understand the ecological distribution of these alleles. For the most part this effort has focused on wine LAB as few taint-related genes have been cloned and characterized from spoilage yeasts or AAB. In the recent past genes responsible for ropiness (Gindreau, et al. 2001), acrolein taint (Claisse and Lonvaud-Funel 2001) and biogenic amine production (Le Jeune, et al. 1995; Landete, et al. 2005; Costantini, et al. 2006), have become targets for PCR screens.

3.2 *Methods for Intraspecific Differentiation*

While rRNA gene analysis has fostered a monumental advance in wine microbiology by enabling rapid speciation of isolates, the tools for differentiating between different strains of the same species have advanced as well. This, in turn, has enabled a much more exquisite dissection of individual constituents at the strain level within the winery and local environs. Two general approaches have been taken to differentiate subspecies and strains of wine-related bacteria or yeast. The first employs whole or sub-genomic analysis through pulse field gel electrophoresis (PFGE), various genomic RFLP methods or newer array approaches to directly examine the isolate genomic makeup. The second approach employs whole genome PCR sampling techniques that result in strain-specific fingerprints from which differentiation of strains is possible.

3.2.1 Wine Yeast

Until recently the two main “gold standard” methods for differentiation of wine yeast strains have been whole genome PFGE (karyotyping [Carle and Olson 1985]) or RFLP of the mitochondrial genome (mito-RFLP [Lee, et al. 1985]). Because of its easier application, more researchers have employed the mito-RFLP method to differentiate yeast strains within wine fermentations revealing similar successions of different *S. cerevisiae* strains throughout various wine fermentations (Querol, et al. 1994; Sabate, et al. 1998).

Other groups have developed whole genome PCR sampling approaches that amplify targeted or arbitrary segments of the yeast genome resulting in strain-specific fingerprints. In general whole genome PCR sampling techniques are popular because of the ease of use, however, the reproducibility of these approaches – which vary among laboratories, personnel and thermocyclers – is often problematic. One approach employs primers to amplify repeated regions in the genome such as delta elements of the Ty transposon (Ness, et al. 1993), intron splice sites (de Barros Lopes, et al. 1998), minisatellites (Mannazzu, et al. 2002; Marinangeli, et al. 2004) or micro-satellite markers (Hennequin, et al. 2001). Another approach uses primers

that do not target known repeated sequences, but work by priming at arbitrary sites to generate a specific fingerprint enabling differentiation of yeast strains (random amplified polymorphic DNA [RAPD]) (Baleiras Couto, et al. 1996; Torriani, et al. 1999; Xufre, et al. 2000; Bujdoso, et al. 2001), REP (Hierro, et al. 2004) and ERIC (Hierro, et al. 2004). A modification of these approaches, amplified fragment length polymorphism (AFLP), employs a chromosomal shearing step prior to adapter ligation and PCR using adapter-based primers. De Barros Lopes, et al. (1999) used this approach to differentiate strains of *S. cerevisiae* and *D. bruxellensis*.

In the last 15 years these methods have tremendously enabled strain discrimination emerging from plating studies on wine and facilitated a multitude of studies. Most have examined *S. cerevisiae* populations in different wine settings. Schutz and Gafner (1993) employed karyotyping to demonstrate the diversity of *S. cerevisiae* strains in spontaneous wine fermentations, compared to inoculated fermentations. The spontaneous fermentations were shown to contain several different strains of *S. cerevisiae* that competed within the fermentation, while the inoculated fermentation was dominated by the inoculated strain (Schutz and Gafner 1994). Around the same time, Querol and co-workers (1992, 1994) employed the mito-RFLP approach to characterize spontaneous and inoculated fermentations and noted similar results. Many subsequent studies have since revealed a multitude of *S. cerevisiae* strains present in spontaneous and inoculated fermentations in various regions or oenological conditions (Gutierrez, et al. 1997; Epifanio, et al. 1999; Esteve-Zarzoso, et al. 2001; Granchi, et al. 2003; Torija, et al. 2003; Demuyter, et al. 2004; Blanco, et al. 2006; Lopes, et al. 2006). Interestingly *S. cerevisiae* strains that dominated fermentations in one year, either through inoculation or emerging indigenously, have been shown to dominate the same winery in the following year (Constanti, et al. 1997; Sabate, et al. 1998).

Perhaps the most promising advance to intraspecific discrimination of wine yeasts has been achieved through use of whole genome sequences. Full genome sequences are available for *S. cerevisiae* (Goffeau, et al. 1996), *Schizosaccharomyces pombe* (Wood, et al. 2002), *K. lactis* (Dujon, et al. 2004) and *Db. hansenii* (Dujon, et al. 2004). In addition several genome sequencing projects for wine-related yeasts or fungi are currently underway including *D. bruxellensis*, *K. thermotolerans*, *S. bayanus* and *B. cinerea* (see <http://www.ncbi.nlm.nih.gov>). An increasingly common approach to examine strain evolution and differentiation is to use comparative genomic hybridization (CGH) with whole or partial genomic arrays. In this fashion chromosomal loci that are shared or missing among strains are documented with the level of discrimination dictated by the level of genome coverage present on the array. Moreover, both small nucleotide polymorphisms and/or larger gene deletions can be witnessed. More importantly, the biological implications of these polymorphisms can be inferred by the encoded genetic content. As a consequence, array-based differentiation is fundamentally more informative than the fingerprinting methods described above, given that the witnessed differences are linked to an *in silico* prediction of the underlying metabolism. To date, relatively few groups have used CGH to characterize wine yeasts. Winzeler and co-workers (2003) characterized

14 different *S. cerevisiae* strains, including several wine-related isolates from Tuscany. They noted a bias for polymorphisms, both gene deletions and single nucleotide changes, in subtelomeric regions. Moreover a genealogical relationship of the 14 strains was developed on the basis of 11,115 probes clearly demonstrated the phylogenetic clustering of the “wild” wine-related strains and separation from the other laboratory strains. Dunn and co-workers (2005) used a similar approach to characterize four different commercial *S. cerevisiae* strains used in winemaking. The four strains showed similar differences from the sequenced *S. cerevisiae* strain S288C (a laboratory strain) and revealed a moderate level of inter-strain differences mostly in transporter genes. These differences were documented functionally in that strains with lower numbers of metallothionien alleles (CUP1) were shown to be sensitive to the fungicide, sulfomethuron methyl.

3.2.2 Wine Bacteria

Similar to the situation with wine yeast, a prominent and reproducible method for bacterial strain differentiation is through use of PFGE, in this case to separate genomic RFLP patterns generated by using rare cutter restriction enzymes (Kelly, et al. 1993). Often whole genome PCR sampling approaches, such as RAPDs, are used in concert with PFGE and rRNA gene typing to provide a polyphasic description of isolates (Rodas, et al. 2005).

A major focus of the wine bacterial analysis has been the malolactic starter culture, *O. oeni*. Studies using a variety of molecular approaches to discriminate isolates have suggested a homogeneous nature to the species (Kelly, et al. 1993; Viti, et al. 1996; Zavaleta, et al. 1997; Sato, et al. 2001). Using PFGE methods, Tenreiro and co-workers (1994) proposed two major lineages for *O. oeni*. However, a subsequent analysis suggested the two groupings were less divergent than originally believed (Ze-Ze, et al. 2000). Recently De Rivas, et al. (2004) used multi-locus sequence typing (MLST) of five genes (*gyrB*, *ddl*, *mleA*, *pgm* and *recP*) to examine allelic diversity and population structure of various oenococcal isolates. Interestingly, MLST was able to differentiate 18 strains that could only be differentiated into two groups by ribotyping. This allelic diversity suggests a higher level of genetic heterogeneity among oenococcal isolates than had been previously suggested by other molecular typing methods. De Rivas, et al. (2004) also concluded that recombination has played a major role in generating genetic diversity in *O. oeni*. Interestingly, the same authors recently characterized 16 *Lb. plantarum* strains using MLST and arrived at much the same general conclusion: the strains possessed a high level of sequence heterozygosity that suggested frequent recombination (de las Rivas, et al. 2006).

The recent publication of whole genome sequences for various wine-related LAB (Kleerebezem, et al. 2003; Makarova, et al. 2006) and AAB (Prust, et al. 2005), has laid the foundation for future intra-specific discrimination. To date, however, only one CGH study has been published on a wine-related species, *Lb. plantarum* (Molenaar, et al. 2005), albeit none of the strains in that work came from

the wine environment. Regardless, most of the variability noted centered on genes involved in sugar utilization, in addition to bacteriocin, exopolysaccharide and prophage encoded differences. From this work, the *Lb. plantarum* clade was delineated into two distinguishable clusters, a conclusion confirming previous molecular differentiation studies.

4 Future Directions for Wine Fermentation Ecology

As advances in molecular methods improve it is clear that the ability to discriminate specific strains, as well as to define their impact on wine production, will improve. One advance that is readily accessible, and decreasing in cost, is the use of high throughput QPCR assays. As this technology becomes more accessible and moves into the average winery laboratory, the ability to rapidly profile and enumerate the microbial contents of winery fermentations could become a normal part of wine-making. With such information in hand winemakers could more readily (and rapidly) spot problem microbes, or even problem alleles, within their fermentations. If such data were collected in a winery each year, winemakers could look to historical data on the previous year's fermentations to help investigate specific anomalies. From a more academic perspective the high throughput QPCR assays are ideal for doing "epidemiological" surveys to identify the reservoirs of spoilage microbes such as *Br. bruxellensis*, or even problem alleles, such as histidine decarboxylases from LAB. The latter approach is currently underway in several laboratories and will provide insight into the ecological reservoirs of genes associated with specific wine taints.

Similar to QPCR, a greater accessibility of microarrays will strongly influence ecological research in wine. Arrays have been created which contain specific 16S rRNA gene sequences to allow discrimination of large number of microbial clades within a single hybridization event (Wilson, et al. 2002). Such arrays allow simultaneous detection and discrimination of diverse sets of microbes and, with high throughput methods, would enable comprehensive microbial ecological analysis (Zhou 2003; Gentry, et al. 2006). To date, however, no such arrays containing specific 26S rRNA gene sequences exist for assessing yeast diversity. Regardless, as the cost of the arrays decrease and utility of the technology advances, these tools will undoubtedly provide wine researchers with methods to advance our understanding of the microbial changes inherent in wine production.

It is hard to discuss the future directions of wine microbiological research without commenting on the tremendous advances in DNA sequencing technology. The exponential increase in publicly available microbial genome sequences will continue to induce wine researchers to adapt to this new bioinformatic landscape. While *S. cerevisiae* was the first eukaryote sequenced over 10 years ago (Goffeau, et al. 1996), we are still in the early stages of genomic analysis of the many different microorganisms that impact wine fermentations. Many more genome sequences are needed. In particular access to sequences for a large number of *S. cerevisiae* and

O. oeni strains currently employed as starter cultures would advance our understanding of the encoded metabolic diversity present within these important species, and help optimize their application in wine production. In addition, genome sequence for a number of other yeasts (e.g., *H. uvarum*, and *C. stellata*), AAB (e.g., *A. aceti*, *A. pasteurianus*, *G. hanseii*) and LAB (e.g., *Lb. hilgardii*, *Lb. buchneri*, *P. damnosus*, *P. parvulus*) species would enable new insights into these important species. Inherent in that new information will be a profoundly better ability to differentiate additional strains and a new understanding of their role in wine production and wine flavor development.

Given that different wine production schemes and regional styles foster different microbial consortia, it is not unreasonable to assume a sort of microbial “terroir” which, in part, endows specific wineries and winemaking styles with a particular flavor profile. However, fully viewing the underlying microbial changes inherent in such environments is problematic. The advent of inexpensive DNA sequencing technologies has now fostered a new approach, metagenomic sequencing, in which all DNA present in a particular environmental niche are cloned and sequenced. Such approaches are now frequently used to profile both the microbial diversity, and also the encoded metabolic capacity from environments (Tyson, et al. 2004). From such an approach it is easy to imagine how a robust description of the microbial community in a specific wine fermentation could be revealed. Moreover the resultant sequence description would reveal the underlying genetic potential within such a fermentation, enabling *in silico* metabolic reconstructions and comparisons (e.g., temporal changes within a fermentation or comparison between fermentations), all without considering microbial cell wall boundaries. In essence the aggregate microbiota of the wine fermentation itself could be considered a “super-organism” with defined metabolic capacity. It is from these types of approaches that *in silico* metabolic models can be generated from complex ecosystems. With such models in hand, future researchers—and winemakers—will have an abundance of information to ensure more flavorful and consistent fermentations in the years to come.

References

- Adams, M. R. 1998. Vinegar, p. 1–44. In Wood, B. J. B. (ed.), *Microbiology of Fermented Foods*, 2 ed., Blackie Academic & Professional, London, UK.
- Alexandre, H., and C. Charpentier. 1998. Biochemical aspects of stuck and sluggish fermentation in grape must. *J. Ind. Microbiol. Biotechnol.* 20:20–27.
- Amerine, M. A., and R. E. Kunkee. 1968. Microbiology of winemaking. *Ann. Rev. Microbiol.* 22:323–58.
- Arias, C. R., J. K. Burns, L. M. Friedrich, R. M. Goodrich, and M. E. Parish. 2002. Yeast species associated with orange juice: evaluation of different identification methods. *Appl. Environ. Microbiol.* 68:1955–61.
- Bae, S., G. H. Fleet, and G. M. Heard. 2006. Lactic acid bacteria associated with wine grapes from several Australian vineyards. *J. Appl. Microbiol.* 100:712–27.
- Baleiras Couto, M. M., B. Eijmsa, H. Hofstra, J. H. Huis in't Veld, and J. M. van der Vossen. 1996. Evaluation of molecular typing techniques to assign genetic diversity among *Saccharomyces cerevisiae* strains. *Appl. Environ. Microbiol.* 62:41–6.

- Barbe, J. C., G. De Revel, A. Joyeux, A. Bertrand, and A. Lonvaud-Funel. 2001. Role of botrytized grape microorganisms in SO₂ binding phenomena. *J. Appl. Microbiol.* 90:34–42.
- Barer, M. R., and C. R. Harwood. 1999. Bacterial viability and culturability. *Adv. Microb. Physiol.* 41:93–137.
- Barnett, J. A., M. A. Delaney, E. Jones, A. B. Magson, and B. Winch. 1972. The numbers of yeasts associated with wine grapes of Bordeaux. *Arch. Microbiol.* 83:52–55.
- Bartowsky, E. J., and P. A. Henschke. 1999. Use of a polymerase chain reaction for specific detection of the malolactic fermentation bacterium *Oenococcus oeni* (formerly *Leuconostoc oenos*) in grape juice and wine sample. *Aust. J. Grape Wine Res.* 5:39–44.
- Bartowsky, E. J., D. Xia, R. L. Gibson, G. H. Fleet, and P. A. Henschke. 2003. Spoilage of bottled red wine by acetic acid bacteria. *Lett. Appl. Microbiol.* 36:307–14.
- Belloch, C., T. Fernandez-Espinar, A. Querol, M. Dolores Garcia, and E. Barrio. 2002. An analysis of inter- and intraspecific genetic variabilities in the *Kluyveromyces marxianus* group of yeast species for the reconsideration of the *K. lactis* taxon. *Yeast* 19:257–68.
- Bisson, L. F. 1999. Stuck and sluggish fermentations. *Amer. J. Enol. Vitic.* 50:107–119.
- Blanco, P., A. Ramilo, M. Cerdeira, and I. Orriols. 2006. Genetic diversity of wine *Saccharomyces cerevisiae* strains in an experimental winery from Galicia (NW Spain). *Ant. van Leeuwenhoek* 89:351–7.
- Blasco, L., S. Ferrer, and I. Pardo. 2003. Development of specific fluorescent oligonucleotide probes for *in situ* identification of wine lactic acid bacteria. *FEMS Microbiol. Lett.* 225:115–23.
- Borzani, W., and M. L. Vairo. 1958. Quantitative adsorption of methylene blue by dead yeast cells. *J. Bacteriol.* 76:251–5.
- Boulton, R. B., V. L. Singleton, L. F. Bisson, and R. E. Kunkee. 1996. Principles and practices of winemaking. Chapman & Hall, New York.
- Boundy-Mills, K. 2006. Methods for investigating yeast biodiversity. In Rosa, C. A., Peter, G. (eds.), *Biodiversity and Ecophysiology of Yeasts*, Springer Berlin.
- Boyd, A. R., T. S. Gunasekera, P. V. Attfield, K. Simic, S. F. Vincent, and D. A. Veal. 2003. A flow-cytometric method for determination of yeast viability and cell number in a brewery. *FEMS Yeast Res.* 3:11–6.
- Bujdoso, G., C. M. Egli, and T. Henick-Kling. 2001. Characterization of *Hanseniaspora* (Kloeckera) strains isolated in Finger Lakes wineries using physiological and molecular techniques. *Food Technol. Biotechnol.* 39:83–91.
- Carle, G. F., and M. V. Olson. 1985. An electrophoretic karyotype for yeast. *Proc. Natl. Acad. Sci.* 82:3756–60.
- Casey, G. D., and A. D. Dobson. 2004. Potential of using real-time PCR-based detection of spoilage yeast in fruit juice—a preliminary study. *Int. J. Food Microbiol.* 91:327–35.
- Chaney, D., S. Rodriguez, K. Fugelsang, and R. Thornton. 2006. Managing high-density commercial scale wine fermentations. *J. Appl. Microbiol.* 100:689–98.
- Chatonnet, P., D. Dubourdieu, and J. N. Boidron. 1995. The influence of *Brettanomyces/Dekkera* sp. yeasts and lactic acid bacteria on the ethylphenol content of red wines. *Amer. J. Enol. Vitic.* 46:463–468.
- Ciani, M., and F. Maccarelli. 1998. Oenological properties of non-*Saccharomyces* yeasts associated with winemaking. *World J. Microbiol. Biotechnol.* 14:199–203.
- Claissé, O., and A. Lonvaud-Funel. 2001. Primers and a specific DNA probe for detecting lactic acid bacteria producing 3-hydroxypropionaldehyde from glycerol in spoiled ciders. *J. Food Prot.* 64:833–7.
- Cocolin, L., L. F. Bisson, and D. A. Mills. 2000. Direct profiling of the yeast dynamics in wine fermentations. *FEMS Microbiol. Lett.* 189:81–7.
- Cocolin, L., A. Heisey, and D. A. Mills. 2001. Direct identification of the indigenous yeasts in commercial wine fermentations. *Amer. J. Enol. Vitic.* 52:49–53.
- Cocolin, L., M. Manzano, S. Rebecca, and G. Comi. 2002. Monitoring of yeast population changes during continuous wine fermentation by molecular methods. *Amer. J. Enol. Vitic.* 53:24–27.

- Cocolin, L., and D. A. Mills. 2003. Wine yeast inhibition by sulfur dioxide: a comparison of culture-dependent and -independent methods. *Amer. J. Enol. Vitic.* 54:125–130.
- Cocolin, L., K. Rantsiou, L. Iacumin, R. Zironi, and G. Comi. 2004. Molecular detection and identification of *Brettanomyces/Dekkera bruxellensis* and *Brettanomyces/Dekkera anomalous* in spoiled wines. *Appl. Environ. Microbiol.* 70:1347–55.
- Cole, J. R., B. Chai, R. J. Farris, Q. Wang, S. A. Kulam, D. M. McGarrell, G. M. Garrity, and J. M. Tiedje. 2005. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Res.* 33:D294–6.
- Constanti, M., M. Poblet, L. Arola, A. Mas, and J. M. Guillamon. 1997. Analysis of yeast populations during alcoholic fermentation in a newly established winery. *Amer. J. Enol. Vitic.* 48:339–344.
- Constanti, M., C. Reguant, M. Poblet, F. Zamora, A. Mas, and J. M. Guillamon. 1998. Molecular analysis of yeast population dynamics: Effect of sulphur dioxide and inoculum on must fermentation. *Int. J. Food Microbiol.* 41:169–175.
- Costantini, A., M. Cersosimo, V. Del Prete, and E. Garcia-Moruno. 2006. Production of biogenic amines by lactic acid bacteria: screening by PCR, thin-layer chromatography, and high-performance liquid chromatography of strains isolated from wine and must. *J. Food Prot.* 69:391–6.
- Coton, E., G. Rollan, A. Bertrand, and A. Lonvaud-Funel. 1998. Histamine-producing lactic acid bacteria in wines: Early detection, frequency, and distribution. *Amer. J. Enol. Vitic.* 49:199–204.
- Dahllof, I., H. Baillie, and S. Kjelleberg. 2000. *rpoB*-based microbial community analysis avoids limitations inherent in 16S rRNA gene intraspecies heterogeneity. *Appl. Environ. Microbiol.* 66:3376–80.
- Davenport, R. R. 1974. Microecology of yeasts and yeast-like organisms associated with an English vineyard. *Vitis* 13:123–130.
- Davidson, P. M. 1997. Chemical preservatives and natural antimicrobial compounds, In Doyle, M., Beuchat, L. R., and Montville, T. (eds.), *Food Microbiology Fundamentals and Frontiers*. ASM Press, Washington DC, p. 520–556.
- Davis, C. R., D. Wibowo, R. Eschenbruch, T. H. Lee, and G. H. Fleet. 1985. Practical implications of malolactic fermentation: A review. *Amer. J. Enol. Vitic.* 36:290–301.
- de Barros Lopes, M., S. Rainieri, P. A. Henschke, and P. Langridge. 1999. AFLP fingerprinting for analysis of yeast genetic variation. *Int. J. Syst. Bacteriol.* 49:915–24.
- de Barros Lopes, M., A. Soden, A. L. Martens, P. A. Henschke, and P. Langridge. 1998. Differentiation and species identification of yeasts using PCR. *Int. J. Syst. Bacteriol.* 48:279–86.
- de Boer, E., and R. R. Beumer. 1999. Methodology for detection and typing of food-borne microorganisms. *Int. J. Food Microbiol.* 50:119–30.
- de Las Rivas, B., A. Marcobal, and R. Munoz. 2004. Allelic diversity and population structure in *Oenococcus oeni* as determined from sequence analysis of housekeeping genes. *Appl. Environ. Microbiol.* 70:7210–9.
- de Las Rivas, B., A. Marcobal, and R. Munoz. 2006. Development of a multi-locus sequence typing method for analysis of *Lactobacillus plantarum* strains. *Microbiology* 152:85–93.
- Deák, T., and L. R. Beuchat. 1996. *Handbook of food spoilage yeasts*. CRC Press, Boca Raton.
- Delaherche, A., O. Claisse, and A. Lonvaud-Funel. 2004. Detection and quantification of *Brettanomyces bruxellensis* and ‘ropy’ *Pediococcus damnosus* strains in wine by real-time polymerase chain reaction. *J. Appl. Microbiol.* 97:910–5.
- Delfini, C., and J. V. Formica. 2001. *Wine microbiology : Science and technology*. Marcel Dekker, New York.
- Demuyter, C., M. Lollier, J. L. Legras, and C. Le Jeune. 2004. Predominance of *Saccharomyces uvarum* during spontaneous alcoholic fermentation, for three consecutive years, in an Alsatian winery. *J. Appl. Microbiol.* 97:1140–8.
- Dent, K. C., J. R. Stephen, and W. E. Finch-Savage. 2004. Molecular profiling of microbial communities associated with seeds of *Beta vulgaris* subsp. *vulgaris* (sugar beet). *J. Microbiol. Methods.* 56:17–26.

- Dittrich, H. H. 1991. Wine and Brandy, In Rehm, H.-J. and Reed, G. (eds.), *Biotechnology: A multi-volume comprehensive treatise*, Weinheim New York, p. 463–506.
- Divol, B., and A. Lonvaud-Funel. 2005. Evidence for viable but non-culturable yeasts in botrytis-affected wine. *J. Appl. Microbiol.* 99:85–93.
- du Toit, W. J., and M. G. Lambrechts. 2002. The enumeration and identification of acetic acid bacteria from South African red wine fermentations. *Int. J. Food Microbiol.* 74:57–64.
- du Toit, W. J., I. S. Pretorius, and A. Lonvaud-Funel. 2005. The effect of sulphur dioxide and oxygen on the viability and culturability of a strain of *Acetobacter pasteurianus* and a strain of *Brettanomyces bruxellensis* isolated from wine. *J. Appl. Microbiol.* 98:862–71.
- Dujon, B., D. Sherman, G. Fischer, P. Durrens, S. Casaregola, I. Lafontaine, J. de Montigny, C. Marck, C. Neugeglise, E. Talla, N. Goffard, L. Frangeul, M. Aigle, V. Anthouard, A. Babour, V. Barbe, S. Barnay, S. Blanchin, J.-M. Beckerich, E. Beyne, C. Bleykasten, A. Boisrame, J. Boyer, L. Cattolico, F. Confaniolieri, A. de Daruvar, L. Despons, E. Fabre, C. Fairhead, H. Ferry-Dumazet, A. Groppi, F. Hantraye, C. Hennequin, N. Jauniaux, P. Joyet, R. Kachouri, A. Kerrest, R. Koszul, M. Lemaire, I. Lesur, L. Ma, H. Muller, J.-M. Nicaud, M. Nikolski, S. Oztas, O. Ozier-Kalogeropoulos, S. Pellenz, S. Potier, G.-F. Richard, M.-L. Straub, A. Suleau, D. Swennen, F. Tekaia, M. Wesolowski-Louvel, E. Westhof, B. Wirth, M. Zeniou-Meyer, I. Zivanovic, M. Bolotin-Fukuhara, A. Thierry, C. Bouchier, B. Caudron, C. Scarpelli, C. Gaillardin, J. Weissenbach, P. Wincker, and J.-L. Souciet. 2004. Genome evolution in yeasts. *Nature* 430:35–44.
- Dunn, B., R. P. Levine, and G. Sherlock. 2005. Microarray karyotyping of commercial wine yeast strains reveals shared, as well as unique, genomic signatures. *BMC Genomics* 6:53.
- Egli, C. M., W. D. Efinger, C. M. Mittrakul, and T. Henick-Kling. 1998. Dynamics of indigenous and inoculated yeast populations and their effect on the sensory character of Riesling and Chardonnay wines. *J. Appl. Microbiol.* 85:779–789.
- Egli, C. M., and T. Henick-Kling. 2001. Identification of *Brettanomyces/Dekkera* species based on polymorphism in the rRNA internal transcribed spacer region. *Amer. J. Enol. Vitic.* 52:241–247.
- Epifanio, S., A. R. Gutierrez, M. P. Santamaria, and R. Lopez. 1999. The influence of enological practices on the selection of wild yeast strains in spontaneous fermentation. *Amer. J. Enol. Vitic.* 50:219–224.
- Esteve-Zarzoso, B., C. Belloch, F. Uruburu, and A. Querol. 1999. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *Int. J. Syst. Bacteriol.* 49:329–337.
- Esteve-Zarzoso, B., M. J. Peris-Toran, E. Garcia-Maiquez, F. Uruburu, and A. Querol. 2001. Yeast population dynamics during the fermentation and biological aging of sherry wines. *Appl. Environ. Microbiol.* 67:2056–61.
- Fernandez-Espinar, M. T., P. Martorell, R. de Llanos, and A. Querol. 2006. Molecular methods to identify and characterize yeasts in foods and beverages, In Querol, A. and Fleet, G. H. (eds.), *Yeasts in Food and Beverages*. Springer, Berlin Heidelberg, p. 55–82.
- Fernandez, M. T., J. F. Ubeda, and A. I. Briones. 1999. Comparative study of non-*Saccharomyces* microflora of musts in fermentation, by physiological and molecular methods. *FEMS Microbiol. Lett.* 173:223–229.
- Fleet, G. H. 1990. Food spoilage yeasts. In Spencer, J. and Spencer, D. (ed.), *Yeast Technology*. Springer-Verlag, New York, p. 124–166.
- Fleet, G. H. 1998. The microbiology of alcoholic beverages. In *Microbiology of Fermented Foods*, 2nd ed. Blackie Academic & Professional, New York, p. 217–262.
- Fleet, G. H. 2003. Yeast interactions and wine flavor. *Int. J. Food Microbiol.* 86:11–22.
- Fleet, G. H., and M. A. Mian. 1998. Induction and repair of sublethal injury in food spoilage yeasts. *J. Food Mycol.* 1:85–93.
- Fugelsang, K. 1997. *Wine Microbiology*. Chapman and Hall, New York, NY.
- Fugelsang, K. C., and B. W. Zoecklein. 2003. Population dynamics and effects of *Brettanomyces bruxellensis* strains on pinot noir (*Vitis vinifera* L.) wines. *Amer. J. Enol. Vitic.* 54:294–300.

- Fuglsang, C. C., C. Johansen, S. Christgau, and J. Adler Nissen. 1995. Antimicrobial enzymes: Applications and future potential in the food industry. *Trends Food Sci. Technol.* 6:390–396.
- Gao, C., and G. H. Fleet. 1988. The effects of temperature and pH on the ethanol tolerance of the wine yeasts, *Saccharomyces cerevisiae*, *Candida stellata* and *Kloeckera apiculata*. *J. Appl. Bacteriol.* 65:405–410.
- Gentry, T. J., G. S. Wickham, C. W. Schadt, Z. He, and J. Zhou. 2006. Microarray applications in microbial ecology research. *Microb. Ecol.* 52:159–75.
- Gindreau, E., E. Walling, and A. Lonvaud-Funel. 2001. Direct polymerase chain reaction detection of rosy *Pediococcus damnosus* strains in wine. *J. Appl. Microbiol.* 90:535–542.
- Goffeau, A., B. G. Barrell, H. Bussey, R. W. Davis, B. Dujon, H. Feldmann, F. Galibert, J. D. Hoheisel, C. Jacq, M. Johnston, E. J. Louis, H. W. Mewes, Y. Murakami, P. Philippsen, H. Tettelin, and S. G. Oliver. 1996. Life with 6,000 genes. *Science* 274: 563–7.
- Gonzalez, A., N. Hierro, M. Poblet, A. Mas, and J. M. Guillamon. 2006. Enumeration and detection of acetic acid bacteria by real-time PCR and nested PCR. *FEMS Microbiol. Lett.* 254:123–8.
- Graca da Silveira, M., M. Vitoria San Romao, M. C. Loureiro-Dias, F. M. Rombouts, and T. Abec. 2002. Flow cytometric assessment of membrane integrity of ethanol-stressed *Oenococcus oeni* cells. *Appl. Environ. Microbiol.* 68:6087–93.
- Gracias, K. S., and J. L. McKillip. 2004. A review of conventional detection and enumeration methods for pathogenic bacteria in food. *Can. J. Microbiol.* 50:883–90.
- Granchi, L., M. Bosco, A. Messini, and M. Vincenzini. 1999. Rapid detection and quantification of yeast species during spontaneous wine fermentation by PCR-RFLP analysis of the rDNA ITS region. *J. Appl. Microbiol.* 87:949–956.
- Granchi, L., D. Ganucci, C. Viti, L. Giovannetti, and M. Vincenzini. 2003. *Saccharomyces cerevisiae* biodiversity in spontaneous commercial fermentations of grape musts with 'adequate' and 'inadequate' assimilable-nitrogen content. *Lett. Appl. Microbiol.* 36:54–8.
- Guillamon, J. M., J. Sabate, E. Barrio, J. Cano, and A. Querol. 1998. Rapid identification of wine yeast species based on RFLP analysis of the ribosomal internal transcribed spacer (ITS) region. *Arch. Microbiol.* 169:387–92.
- Gutierrez, A. R., R. Lopez, M. P. Santamaria, and M. J. Sevilla. 1997. Ecology of inoculated and spontaneous fermentations in Rioja (Spain) musts, examined by mitochondrial DNA restriction analysis. *Int. J. Food Microbiol.* 36:241–5.
- Hanna, S. E., C. J. Connor, and H. H. Wang. 2005. Real-time polymerase chain reaction for the food microbiologist: Technologies, applications, and limitations. *J. Food Sci.* 70:49–53.
- Head, I. M., J. R. Saunders, and R. W. Pickup. 1998. Microbial evolution, diversity, and ecology: A decade of ribosomal RNA analysis of uncultivated microorganisms. *Microb. Ecol.* 35:1–21.
- Hennequin, C., A. Thierry, G. F. Richard, G. Lecointre, H. V. Nguyen, C. Gaillardin, and B. Dujon. 2001. Microsatellite typing as a new tool for identification of *Saccharomyces cerevisiae* strains. *J. Clin. Microbiol.* 39:551–9.
- Hernan-Gomez, S., J. C. Espinosa, and J. F. Ubeda. 2000. Characterization of wine yeasts by temperature gradient gel electrophoresis (TGGE). *FEMS Microbiol. Lett.* 193:45–50.
- Herrero, M., C. Quiros, L. A. Garcia, and M. Diaz. 2006. Use of flow cytometry to follow the physiological states of microorganisms in cider fermentation processes. *Appl. Environ. Microbiol.* 72:6725–33.
- Hierro, N., B. Esteve-Zarzoso, A. Gonzalez, A. Mas, and J. M. Guillamon. 2006. Real-time quantitative PCR (QPCR) and reverse transcription-QPCR for detection and enumeration of total yeasts in wine. *Appl. Environ. Microbiol.* 72:7148–55.
- Hierro, N., A. Gonzalez, A. Mas, and J. M. Guillamon. 2004. New PCR-based methods for yeast identification. *J. Appl. Microbiol.* 97:792–801.
- Hofmann. 1968. Biochemical changes caused by *Botrytis cinerea* and *Rhizopus nigricans* in grape must. *S. African J. Agric. Sci.* 11:335–348.
- Hofmann, M. A., and D. A. Brian. 1991. Sequencing PCR DNA amplified directly from a bacterial colony. *Biotechniques* 11:30–1.

- Ibeas, J. I., I. Lozano, F. Perdignes, and J. Jimenez. 1996. Detection of *Dekkera-Brettanomyces* strains in sherry by a nested PCR method. *Appl. Environ. Microbiol.* 62:998–1003.
- Jemec, K. P., N. Cadez, T. Zagorc, V. Bubic, A. Zupec, and P. Raspor. 2001. Yeast population dynamics in five spontaneous fermentations of Malvasia must. *Food Microbiol.* 18:247–259.
- Joyeux, A., S. Lafonlaurcade, and P. Ribereaugayon. 1984. Evolution of acetic acid bacteria during fermentation and storage of wine. *Appl. Environ. Microbiol.* 48:153–156.
- Kell, D. B., A. S. Kaprelyants, D. H. Weichart, C. R. Harwood, and M. R. Barer. 1998. Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. *Ant. van Leeuwenhoek* 73:169–87.
- Kelly, W. J., C. M. Huang, and R. V. Asmundson. 1993. Comparison of *Leuconostoc oenos* Strains by Pulsed-Field Gel Electrophoresis. *Appl. Environ. Microbiol.* 59:3969–3972.
- Kelly, W. J., C. M. Huang, and R. V. Asmundson. 1993. Comparison of *Leuconostoc oenos* strains by pulsed-field gel electrophoresis. *Appl. Environ. Microbiol.* 59:3969–3972.
- Kerstens, K., P. Lisdianti, K. Komagata, and J. Swings. 2006. The Family Acetobacteraceae: The Genera *Acetobacter*, *Acidomonas*, *Asaia*, *Gluconacetobacter*, *Gluconobacter*, and *Kozakia*. In Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K., Stackebrandt, E. (eds.), *The Prokaryotes*, Vol. 5. Springer, New York, p. 163–200.
- Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Turchini, S. A. Peters, H. M. Sandbrink, M. W. Fiers, W. Stiekema, R. M. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen. 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proc. Natl. Acad. Sci.* 100:1990–5.
- Kurtzman, C. P., and C. J. Robnett. 1998. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Ant. van Leeuwenhoek* 73:331–71.
- Landete, J. M., S. Ferrer, and I. Pardo. 2005. Which lactic acid bacteria are responsible for histamine production in wine? *J. Appl. Microbiol.* 99:580–6.
- Le Jeune, C., A. Lonvaud-Funel, B. ten Brink, H. Hofstra, J. M. van der Vossen. 1995. Development of a detection system for histidine decarboxylating lactic acid bacteria based on DNA probes, PCR and activity test. *J. Appl. Bacteriol.* 78:316–26.
- Le Jeune, C., and A. Lonvaud-Funel. 1997. Sequence of DNA 16S/23S spacer region of *Leuconostoc oenos* (*Oenococcus oeni*): application to strain differentiation. *Res. Microbiol.* 148:79–86.
- Le-Roux, G., R. Eschenbruch, and S.-I. De-Bruin. 1973. The microbiology of South African winemaking. III. The microflora of healthy and *Botrytis cinerea* infected grapes. *Phytophylactica* 5:51–54.
- Lee, S. Y., F. B. Knudsen, and R. O. Poyton. 1985. Differentiation of brewery yeast strains by restriction endonuclease analysis of their mitochondrial DNA. *J. Inst. Brew.* 91:169–173.
- Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25:402–8.
- Lonvaud-Funel, A. 1999. Lactic acid bacteria in the quality improvement and depreciation of wine. *Ant. van Leeuwenhoek* 76:317–31.
- Lopes, C. A., T. L. Lavalle, A. Querol, and A. C. Caballero. 2006. Combined use of killer biotype and mtDNA-RFLP patterns in a Patagonian wine *Saccharomyces cerevisiae* diversity study. *Ant. van Leeuwenhoek* 89:147–56.
- Lopez, I., F. Ruiz-Larrea, L. Cocolin, E. Orr, T. Phister, M. Marshall, J. VanderGheynst, and D. A. Mills. 2003. Design and evaluation of PCR primers for analysis of bacterial populations in wine by denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* 69:6801–7.
- Lopez, V., M. T. Fernandez-Espinar, E. Barrio, D. Ramon, and A. Querol. 2003. A new PCR-based method for monitoring inoculated wine fermentations. *Int. J. Food Microbiol.* 81:63–71.
- Loureiro, V., and M. Malfeito-Ferreira. 2003. Spoilage yeasts in the wine industry. *Int. J. Food Microbiol.* 86:23–50.
- Makarova, K., A. Slesarev, Y. Wolf, A. Sorokin, B. Mirkin, E. Koonin, A. Pavlov, N. Pavlova, V. Karamychev, N. Polouchine, V. Shakhova, I. Grigoriev, Y. Lou, D. Rohksar, S. Lucas,

- K. Huang, D. M. Goodstein, T. Hawkins, V. Plengvidhya, D. Welker, J. Hughes, Y. Goh, A. Benson, K. Baldwin, J. H. Lee, I. Diaz-Muniz, B. Dosti, V. Smeianov, W. Wechter, R. Barabote, G. Lorca, E. Altermann, R. Barrangou, B. Ganesan, Y. Xie, H. Rawsthorne, D. Tamir, C. Parker, F. Breidt, J. Broadbent, R. Hutkins, D. O'Sullivan, J. Steele, G. Unlu, M. Saier, T. Klaenhammer, P. Richardson, S. Kozyavkin, B. Weimer, and D. Mills. 2006. Comparative genomics of the lactic acid bacteria. *Proc. Natl. Acad. Sci.* 103:15611–6.
- Malacrino, P., G. Zapparoli, S. Torriani, and F. Dellaglio. 2001. Rapid detection of viable yeasts and bacteria in wine by flow cytometry. *J. Microbiol. Methods* 45:127–34.
- Mannazzu, I., E. Simonetti, P. Marinangeli, E. Guerra, M. Budroni, M. Thangavelu, F. Clementi. 2002. SED1 gene length and sequence polymorphisms in feral strains of *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* 68:5437–5444.
- Manzano, M., L. Cocolin, L. Iacumin, C. Cantoni, and G. Comi. 2005. A PCR-TGGE (Temperature Gradient Gel Electrophoresis) technique to assess differentiation among enological *Saccharomyces cerevisiae* strains. *Int. J. Food Microbiol.* 101:333–9.
- Manzano, M., L. Cocolin, B. Longo, and G. Comi. 2004. PCR-DGGE differentiation of strains of *Saccharomyces sensu stricto*. *Ant. van Leeuwenhoek* 85:23–7.
- Manzano, M., D. Medrala, C. Giusto, I. Bartolomeoli, R. Urso, and G. Comi. 2006. Classical and molecular analyses to characterize commercial dry yeasts used in wine fermentations. *J. Appl. Microbiol.* 100:599–607.
- Marsh, T. L. 1999. Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. *Curr. Opin. Microbiol.* 2:323–7.
- Marinangeli, P., D. Angelozzi, M. Ciani, F. Clementi and I. Mannazzu. 2004. Minisatellites in *Saccharomyces cerevisiae* genes encoding cell wall proteins: A new way towards wine strain characterisation. *FEMS Yeast Res.* 4: 427–435.
- Martorell, P., A. Querol, and M. T. Fernandez-Espinar. 2005. Rapid identification and enumeration of *Saccharomyces cerevisiae* cells in wine by real-time PCR. *Appl. Environ. Microbiol.* 71:6823–30.
- Millet, V., and A. Lonvaud-Funel. 2000. The viable but non-culturable state of wine microorganisms during storage. *Lett. Appl. Microbiol.* 30:136–41.
- Mills, D. A., E. A. Johannsen, and L. Cocolin. 2002. Yeast diversity and persistence in botrytis-affected wine fermentations. *Appl. Environ. Microbiol.* 68:4884–93.
- Molenaar, D., F. Bringel, F. H. Schuren, W. M. de Vos, R. J. Siezen, and M. Kleerebezem. 2005. Exploring *Lactobacillus plantarum* genome diversity by using microarrays. *J. Bacteriol.* 187:6119–27.
- Montrocher, R., M. C. Verner, J. Briolay, C. Gautier, and R. Marmesse. 1998. Phylogenetic analysis of the *Saccharomyces cerevisiae* group based on polymorphisms of rDNA spacer sequences. *Int. J. Syst. Bacteriol.* 48:295–303.
- Moore, K. J., M. G. Johnson, and J. R. Morris. 1988. Indigenous yeast microflora on Arkansas white riesling (*Vitis vinifera*) grapes and in model must systems. *J. Food Sci.* 53:1725–28.
- Mortimer, R., and M. Polsinelli. 1999. On the origins of wine yeast. *Res. Microbiol.* 150:199–204.
- Muyzer, G., and K. Smalla. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Ant. van Leeuwenhoek* 73:127–41.
- Neeley, E. T., T. G. Phister, and D. A. Mills. 2005. Differential real-time PCR assay for enumeration of lactic acid bacteria in wine. *Appl. Environ. Microbiol.* 71:8954–7.
- Ness, F., F. Lavalley, D. Dubourdieu, M. Aigle, and L. Dulau. 1993. Identification of yeast strains using the polymerase chain reaction. *J. Sci. Food Agric.* 62:89–94.
- Olsen, G. J., C. R. Woese, and R. Overbeek. 1994. The winds of (evolutionary) change: breathing new life into microbiology. *J. Bacteriol.* 176:1–6.
- Osborne, J. P., and C. G. Edwards. 2005. Bacteria in winemaking. In S. Taylor (ed.), *Advances in Food and Nutrition Research*, 50:139–177.
- Pasteur, L. 1873. *Études sur le vin, ses maladies, causes qui les provoquent, procédés nouveaux pour le conserver et pour le vieillir*, 2. éd., F. Savy, Paris.

- Peynaud, E., and S. Domerco. 1959. A review of microbiological problems in winemaking in France. *Amer. J. Enol. Vitic.* 10:69–77.
- Phister, T. G., and D. A. Mills. 2003. Real-time PCR assay for detection and enumeration of *Dekkera bruxellensis* in wine. *Appl. Environ. Microbiol.* 69:7430–4.
- Pinzani, P., L. Bonciani, M. Pazzagli, C. Orlando, S. Guerrini, and L. Granchi. 2004. Rapid detection of *Oenococcus oeni* in wine by real-time quantitative PCR. *Lett. Appl. Microbiol.* 38:118–24.
- Poblet, M., N. Rozes, J. M. Guillamon, and A. Mas. 2000. Identification of acetic acid bacteria by restriction fragment length polymorphism analysis of a PCR-amplified fragment of the gene coding for 16S rRNA. *Lett. Appl. Microbiol.* 31:63–7.
- Prakitchaiwattana, C. J., G. H. Fleet, and G. M. Heard. 2004. Application and evaluation of denaturing gradient gel electrophoresis to analyze the yeast ecology of wine grapes. *FEMS Yeast Res.* 4:865–77.
- Pramateftaki, P. V., P. Lanaridis, and M. A. Typas. 2000. Molecular identification of wine yeasts at species or strain level: a case study with strains from two vine-growing areas of Greece. *J. Appl. Microbiol.* 89:236–48.
- Pretorius, I. S. 2000. Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* 16:675–729.
- Pretorius, I. S., T. J. van der Westhuizen, and O. P. H. Augustyn. 1999. Yeast biodiversity in vineyards and wineries and its importance to the South African wine industry. a review. *S. African J. Enol. Vitic.* 20:61–74.
- Prust, C., M. Hoffmeister, H. Liesegang, A. Wiezer, W. F. Fricke, A. Ehrenreich, G. Gottschalk, and U. Deppenmeier. 2005. Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*. *Nature Biotechnol.* 23:195–200.
- Querol, A., E. Barrio, T. Huerta, and D. Ramon. 1992. Molecular monitoring of wine fermentations conducted by active dry yeast strains. *Appl. Environ. Microbiol.* 58:2948–2953.
- Querol, A., E. Barrio, and D. Ramon. 1994. Population dynamics of natural *Saccharomyces* strains during wine fermentation. *Int. J. Food Microbiol.* 21:315–23.
- Raspor, P., F. Cus, K. P. Jemec, T. Zagorc, N. Cadez, and J. Nemanic. 2002. Yeast population dynamics in spontaneous and inoculated alcoholic fermentations of Zametovka must. *Food Tech. Biotech.* 40:95–102.
- Rawsthorne, H., and T. G. Phister. 2006. A real-time PCR assay for the enumeration and detection of *Zygosaccharomyces bailii* from wine and fruit juices. *Int. J. Food Microbiol.* 112:1–7.
- Renouf, V., M. Falcou, C. Miot-Sertier, M. C. Perello, G. De Revel, and A. Lonvaud-Funel. 2006a. Interactions between *Brettanomyces bruxellensis* and other yeast species during the initial stages of winemaking. *J. Appl. Microbiol.* 100:1208–19.
- Renouf, V., O. Claisse, C. Miot-Sertier, and A. Lonvaud-Funel. 2006b. Lactic acid bacteria evolution during winemaking: use of *rpoB* gene as a target for PCR-DGGE analysis. *Food Microbiol.* 23:136–45.
- Renouf, V., and A. Lonvaud-Funel. 2007. Development of an enrichment medium to detect *Dekkera/Brettanomyces bruxellensis*, a spoilage wine yeast, on the surface of grape berries. *Microbiol Res.* 162:154–167.
- Ribereau-Gayon, J., P. Ribereau-Gayon, and G. Seguin. 1980. *Botrytis cinerea* in enology. In Coley-Smith, J.R., Jarvis, W.R. (ed.), *The Biology of Botrytis*. Academic Press, New York, p. 251–274.
- Ribereau-Gayon, P., D. Dubourdieu, B. Doneche, and A. Lonvaud. 2000. *Handbook of Enology Volume 1: The Microbiology of Wine and Vinifications*. John Wiley and Sons, LTD, New York, NY.
- Rodas, A. M., S. Ferrer, and I. Pardo. 2003. 16S-ARDRA, a tool for identification of lactic acid bacteria isolated from grape must and wine. *Syst. Appl. Microbiol.* 26:412–22.
- Rodas, A. M., S. Ferrer, and I. Pardo. 2005. Polyphasic study of wine *Lactobacillus* strains: taxonomic implications. *Int. J. Syst. Evol. Microbiol.* 55:197–207.
- Rodrigues, N., G. Goncalves, S. Pereira-da-Silva, M. Malfeito-Ferreira, and V. Loureiro. 2001. Development and use of a new medium to detect yeasts of the genera *Dekkera/Brettanomyces*. *J. Appl. Microbiol.* 90:588–99.

- Rodrigues, U. M., and R. G. Kroll. 1985. The direct epifluorescent filter technique (DEFT): increased selectivity, sensitivity and rapidity. *J. Appl. Bacteriol.* 59:493–9.
- Rosini, G., F. Federici, and A. Martini. 1982. Yeast flora of grape berries during ripening. *Microb. Ecol.* 8:83–89.
- Ruiz, A., M. Poblet, A. Mas, and J. M. Guillamon. 2000. Identification of acetic acid bacteria by RFLP of PCR-amplified 16S rDNA and 16S-23S rDNA intergenic spacer. *Int. J. Syst. Evol. Microbiol.* 50:1981–7.
- Sabate, J., J. Cano, A. Querol, and J. M. Guillamon. 1998. Diversity of *Saccharomyces* strains in wine fermentations: analysis for two consecutive years. *Lett. Appl. Microbiol.* 26:452–5.
- Sato, H., F. Yanagida, T. Shinohara, M. Suzuki, K.-i. Suzuki, and K. Yokotsuka. 2001. Intraspecific diversity of *Oenococcus oeni* isolated during red winemaking in Japan. *FEMS Microbiol. Lett.* 202:109–114.
- Schutz, M., and J. Gafner. 1993. Analysis of yeast diversity during spontaneous and induced alcoholic fermentations. *J. Appl. Bacteriol.* 75:551–558.
- Schutz, M., and J. Gafner. 1994. Dynamics of the yeast strain population during spontaneous alcoholic fermentation determined by CHEF gel electrophoresis. *Lett. Appl. Microbiol.* 19:253–257.
- Sipiczki, M. 2003. *Candida zemplanina* sp. nov., an osmotolerant and psychrotolerant yeast that ferments sweet botrytized wines. *Int. J. Syst. Evol. Microbiol.* 53:2079–83.
- Sohier, D., J. Coulon, and A. Lonvaud-Funel. 1999. Molecular identification of *Lactobacillus hilgardii* and genetic relatedness with *Lactobacillus brevis*. *Int. J. Syst. Bacteriol.* 49:1075–81.
- Sohier, D., and A. Lonvaud-Funel. 1998. Rapid and sensitive *in situ* hybridization method for detecting and identifying lactic acid bacteria in wine. *Food Microbiol.* 15:391–397.
- Sponholz, W. 1993. Spoilage by wine microorganisms. In Fleet, G. H. (ed.), *Wine Microbiology and Biotechnology*. Harwood Academic Publishers, Switzerland, p. 395–420.
- Stender, H., C. Kurtzman, J. J. Hyldig-Nielsen, D. Sorensen, A. Broome, K. Oliveira, H. Perry-O'Keefe, A. Sage, B. Young, and J. Coull. 2001. Identification of *Dekkera bruxellensis* (*Brettanomyces*) from wine by fluorescence *in situ* hybridization using peptide nucleic acid probes. *Appl. Environ. Microbiol.* 67:938–41.
- Stevenson, K. E., and T. R. Graulich. 1978. Injury and recovery of yeasts and molds. *Adv. Appl. Microbiol.* 23:203–17.
- Stratford, M., P. Morgan, and A. H. Rose. 1987. Sulfur dioxide resistance in *Saccharomyces cerevisiae* and *Saccharomyces ludwigii*. *J. Gen. Microbiol.* 133:2173–2179.
- Tenreiro, R., M. A. Santos, H. Paveia, and G. Vieira. 1994. Inter-strain relationships among wine leuconostocs and their divergence from other *Leuconostoc* species, as revealed by low frequency restriction fragment analysis of genomic DNA. *J. Appl. Bacteriol.* 77:271–80.
- Thornton, R. J. 1991. Wine yeast research in New-Zealand and Australia. *Crit. Rev. Biotechnol.* 11:327–345.
- Torija, M. J., N. Rozes, M. Poblet, J. M. Guillamon, and A. Mas. 2003. Effects of fermentation temperature on the strain population of *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.* 80:47–53.
- Torriani, S., G. Zapparoli, and G. Suzzi. 1999. Genetic and phenotypic diversity of *Saccharomyces sensu stricto* strains isolated from Amarone wine. *Ant. van Leeuwenhoek* 75:207–15.
- Towner, K. J., and A. Cockayne. 1993. *Molecular methods for microbial identification and typing*. Chapman & Hall, New York.
- Tyson, G. W., J. Chapman, P. Hugenholtz, E. E. Allen, R. J. Ram, P. M. Richardson, V. V. Solov'yev, E. M. Rubin, D. S. Rokhsar, and J. F. Banfield. 2004. Community structure and metabolism through reconstruction of microbial genomes from the environment. *Nature* 428:37–43.
- Valente, P., J. P. Ramos, and O. Leoncini. 1999. Sequencing as a tool in yeast molecular taxonomy. *Can. J. Microbiol.* 45:949–58.
- van Vuuren, H. J. J., and C. J. Jacobs. 1992. Killer yeasts in the wine industry - a review. *Amer. J. Enol. Vitic.* 43:119–128.

- Vaughan-Martini, A., and A. Martini. 1995. Facts, myths and legends on the prime industrial microorganism. *J. Ind. Microbiol.* 14:514–22.
- Viti, C., L. Giovannetti, L. Granchi, and S. Ventura. 1996. Species attribution and strain typing of *Oenococcus oeni* (formerly *Leuconostoc oenos*) with restriction endonuclease fingerprints. *Res. Microbiol.* 147:651–660.
- Vitzthum, F., and J. Bernhagen. 2002. SYBR Green I: an ultrasensitive fluorescent dye for double-stranded DNA quantification in solution and other applications. *Recent Res. Devel. Anal. Biochem.* 2:65–93.
- Walling, E., E. Gindreau, and A. Lonvaud-Funel. 2005. A putative glucan synthase gene *dps* detected in exopolysaccharide-producing *Pediococcus damnosus* and *Oenococcus oeni* strains isolated from wine and cider. *Int. J. Food Microbiol.* 98:53–62.
- Ward, A. C. 1992. Rapid analysis of yeast transformants using colony-PCR. *Biotechniques* 13:350.
- Wilson, K. H., W. J. Wilson, J. L. Radosevich, T. Z. DeSantis, V. S. Viswanathan, T. A. Kuczmarski, and G. L. Andersen. 2002. High-density microarray of small-subunit ribosomal DNA probes. *Appl. Environ. Microbiol.* 68:2535–2541.
- Winzeler, E. A., C. I. Castillo-Davis, G. Oshiro, D. Liang, D. R. Richards, Y. Zhou, and D. L. Hartl. 2003. Genetic diversity in yeast assessed with whole-genome oligonucleotide arrays. *Genetics* 163:79–89.
- Wood, V., R. Gwilliam, M. A. Rajandream, M. Lyne, R. Lyne, A. Stewart, J. Sgouros, N. Peat, J. Hayles, S. Baker, D. Basham, S. Bowman, K. Brooks, D. Brown, S. Brown, T. Chillingworth, C. Churcher, M. Collins, R. Connor, A. Cronin, P. Davis, T. Feltwell, A. Fraser, S. Gentles, A. Goble, N. Hamlin, D. Harris, J. Hidalgo, G. Hodgson, S. Holroyd, T. Hornsby, S. Howarth, E. J. Huckle, S. Hunt, K. Jagels, K. James, L. Jones, M. Jones, S. Leather, S. McDonald, J. McLean, P. Mooney, S. Moule, K. Mungall, L. Murphy, D. Niblett, C. Odell, K. Oliver, S. O'Neil, D. Pearson, M. A. Quail, E. Rabinowitsch, K. Rutherford, S. Rutter, D. Saunders, K. Seeger, S. Sharp, J. Skelton, M. Simmonds, R. Squares, S. Squares, K. Stevens, K. Taylor, R. G. Taylor, A. Tivey, S. Walsh, T. Warren, S. Whitehead, J. Woodward, G. Volckaert, R. Aert, J. Robben, B. Grymonprez, I. Weltjens, E. Vanstreels, M. Rieger, M. Schafer, S. Muller-Auer, C. Gabel, M. Fuchs, C. Fritz, E. Holzer, D. Moestl, H. Hilbert, K. Borzym, I. Langer, A. Beck, H. Lehrach, R. Reinhardt, T. M. Pohl, P. Eger, W. Zimmermann, H. Wedler, R. Wambutt, B. Purnelle, A. Goffeau, E. Cadieu, S. Dreano, S. Gloux, V. Lelaure, et al. 2002. The genome sequence of *Schizosaccharomyces pombe*. *Nature* 415:871–880.
- Xu, H., N. Roberts, F. Singleton, R. Attwell, D. Grimes, and R. Colwell. 1982. Survival and viability of non-culturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. *Microbial Ecol.* 8:313–323.
- Xufre, A., H. Albergaria, J. Inacio, I. Spencer-Martins, and F. Girio. 2006. Application of fluorescence *in situ* hybridization (FISH) to the analysis of yeast population dynamics in winery and laboratory grape must fermentations. *Int. J. Food Microbiol.* 108:376–84.
- Xufre, A., F. Simoes, F. Girio, A. Clemente, and M. T. Amaral-Collaco. 2000. Use of RAPD analysis for differentiation among six enological *Saccharomyces* spp. strains. *Food Tech. Biotechnol.* 38:53–58.
- Zapparoli, G., S. Torriani, P. Pesente, and F. Dellaglio. 1998. Design and evaluation of malolactic enzyme gene targeted primers for rapid identification and detection of *Oenococcus oeni* in wine. *Lett. Appl. Microbiol.* 27:243–6.
- Zavaleta, A. I., A. J. Martinez Murcia, and F. Rodriguez Valera. 1997. Intraspecific genetic diversity of *Oenococcus oeni* as derived from DNA fingerprinting and sequence analyses. *Appl. Environ. Microbiol.* 63:1261–1267.
- Ze-Ze, L., R. Tenreiro, and H. Paveia. 2000. The *Oenococcus oeni* genome: physical and genetic mapping of strain GM and comparison with the genome of a 'divergent' strain, PSU-1. *Microbiology* 146:3195–204.
- Zhou, J. 2003. Microarrays for bacterial detection and microbial community analysis. *Curr. Opin. Microbiol.* 6:288–94.

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