Ann Microbiol (2011) 61:17–23
DOI 10.1007/s13213-010-0076-6

REVIEW

Culture independent analyses and wine fermentation: an overview of achievements 10 years after first application

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Received: 14 February 2010 / Accepted: 17 May 2010 / Published online: 13 June 2010
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Abstract Wine fermentations are complex microbial ecosystems, with both yeasts and bacteria taking part in the transformation process with their metabolic activities. Traditional microbiological methods do not allow a complete understanding of the microbial ecology of complex systems. This is due mainly to the capacity of certain microorganisms to grow on microbiological media preferentially with respect to others. Moreover, with these methods, stressed or damaged cells cannot be detected on the plates. In the last 10 years new methods based on the analysis of nucleic acids (DNA and RNA) extracted directly from the sample without the need for microbial cultivation have been developed. A method often used in this type of study is the polymerase chain reaction (PCR) coupled with denaturing gradient gel electrophoresis (DGGE). This paper aims to report the most important contributions of PCR-DGGE to the study of microbiological ecology during wine fermentation.

Keywords Wine fermentation · Culture-independent method · PCR-DGGE · Fermentation dynamic · Microbial ecology

This paper is part of the special issue "Wine Microbiology and safety: From the vineyard to the bottle (Microsafetywine), 19-20 November, 2009, Martina Franca (Italy)

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Introduction

Wine fermentations are complex microbial ecosystems, in which different species of yeasts, bacteria and moulds coexist. The main yeasts responsible for alcoholic fermentation belong to the genus *Saccharomyces* (in particular *S. cerevisiae*), while lactic acid bacteria (LAB) are responsible for malolactic fermentations (Fleet 1993). Since their metabolic activities drive the transformation of grape juice into wine, it is essential to understand the behaviour of these microorganisms during fermentation in order to obtain final products with the desired organoleptic characteristics.

Yeast and bacterial microbial dynamics have been studied since the 1970s (Barnett et al. 1972). Using classical methods, microbial count and diversity is determined by employing synthetic culture media containing agar. In some cases incubation in liquid media (broths) is also used to promote or select specific microorganisms for which isolation would be impossible without this step. The big drawback of classical microbiological culture-dependent methods is that it is impossible to describe the biodiversity in complex ecosystems precisely. When using enrichment methods and growth on microbiological media, the microbiota originally present in the sample is subject to important changes because of the capacity of certain species to take over the system and outgrow other microbial components (Pace 1997). For this reason, populations that are numerically less abundant, or in a stressed condition, are hard to recover and identify. Thus, using culture-dependent methods, there is a high risk of misidentification of the ecology of complex microbial ecosystems (Hugenholtz et al. 1998).

Since the end of the 1990s, molecular approaches have opened new frontiers in our understanding of microbial

ecology. These methods, generally termed culture-independent methods, are able to detect and identify microorganisms directly in the system without cultivation and isolation because they analyse their DNA or RNA. The novelty is represented by the extraction of nucleic acids directly in matrices that are subsequently analysed by methods able to highlight microbial diversity. Studying the DNA, it is possible to define how many, and which, microbial species are present in a specific sample, thereby giving a view of microbial diversity and ecology, while looking at the RNA allows us to understand the metabolically active portion of the population. This is very relevant in the case of food fermentations, like wine, where it is necessary to study the species responsible for metabolic transformations.

Analysis of the nucleic acids can be carried out by hybridisation with specific probes, by species-specific polymerase chain reaction (PCR) or by universal PCR and sequence-based separation and identification of PCR products. The disadvantage of species-specific PCR is that there is a limit to the number of species one can detect/identify in a sample. Moreover, one has to know which microorganisms to look for in a sample. Alternatively, with the use of universal primers, theoretically all the species of large groups are amplified. Sequence-based separation is then achieved by denaturing/temperature gradient gel electrophoresis (D/TGGE). D/TGGE was first developed for the study of the microbial ecology in environmental samples (Muyzer and Smalla 1998), but soon found application in food microbiology (Ercolini 2004).

The main advantages of direct approaches are: (1) no cultivation takes place and, therefore, the bias associated with the use of conventional microbiological media for enumeration/isolation is negated; (2) compared to the classic approach used so far in microbiology, which is based on isolation of strains from the food matrix and their identification either by physiological/phenotypical tests or by molecular methods, direct methods are less time-consuming and require less effort; and (3) they allow a parallel description of the populations of different microbial groups. On the other hand, these techniques generally require specialised personnel and relatively costly equipment. Furthermore, it has been determined that the detection limit for the most common method used in direct analyses, i.e. D/TGGE, is in the order of 10^3 colony forming units (cfu)/ml (Cocolin et al. 2000). As a consequence, microbial groups that are present and active, but at population levels lower than 10^3 cfu/ml will not be accounted for. It should be stressed that the definition of the detection limit in D/TGGE analysis is not always simple because it depends on the different affinity that the primers have towards the microbial species present in that ecosystem, which can change based on the specific group of microorganisms under consideration.

In PCR-D/TGGE, nucleic acids are subjected to amplification with universal primers, which are able to amplify, theoretically, all the DNA or RNA from bacteria and yeasts present in a specific ecosystem. After PCR, the amplicon consists of a mix of different amplification products, which are more diverse if the biodiversity in the sample is complex. The PCR product, consisting of different nucleotide sequences, is subsequently separated by electrophoresis through a polyacrylamide gel containing a chemical gradient of denaturants (urea and formamide). When DNA molecules encounter a point in the denaturant gradient able to partially open (denature) the double helix, their electrophoretic mobility changes, resulting in a complete stop at some point in the gel. Since denaturation is dependent on DNA sequence, different DNA molecules will have different electrophoretic mobility. Thus, this approach allows differentiation of microorganisms present in the same ecosystem, as long as they present amplification regions with different sequences. An interesting contribution to our knowledge of the microbial ecology of wine fermentations is provided by the application of PCR-DGGE to RNA extracted directly from the matrix and subjected to reverse transcription. DNA can persist in any given environment, sometimes long after a microorganism is dead. In contrast, RNA is degraded rapidly after cell death and, as a consequence, the application of RT-PCR-DGGE gives the fingerprint (or profiles) of populations that are alive and metabolically active. When RT-PCR-DGGE has been applied to wine fermentation, the results have compared fairly well with those obtained by PCR-DGGE (Mills et al. 2002), although in certain cases the RT-PCR-DGGE profiles were richer (Urso et al. 2008). For alcoholic and malolactic fermentations, studies have highlighted that, for yeasts, use of the D1–D2 loop of the 26S rRNA gene (Cocolin et al. 2000) and the V7–V8 region of the 16S rRNA gene for bacteria (Lopez et al. 2003) is optimal for the study of microbial ecology during wine production.

Lastly, a promising culture-independent method that unfortunately has not yet been exploited efficiently to study the microbial diversity in wine fermentation, is fluorescence in situ hybridisation (FISH). In this technique, a set of specific probes are used to target different microorganisms directly in the sample. The probes are labeled with different fluorophores, thereby allowing detection of several species simultaneously. Since FISH probes are generally designed to detect ribosomal RNA, only living cells are detected (Bottari et al. 2006). The only example available in the literature of the use of FISH in the field of wine fermentation refers to the study of Xufre et al. (2006), in which a set of FISH probes was developed to identify *S. cerevisiae* and non-*Saccharomyces* yeasts. The protocol was applied to isolated colonies obtained from two wine fermentation experiments, one industrial and one conducted

in the laboratory. It becomes obvious that the concept of FISH as a culture-independent method was not fully exploited in this study. Nevertheless, the authors clearly demonstrated the potential of this technique in wine fermentation.

PCR-DGGE in wine fermentations

Yeast ecology

The first study to use PCR-DGGE in the field of wine fermentation was published by Cocolin et al. (2000). In this study, the microbial dynamics of enological yeasts, such as *Saccharomyces cerevisiae*, *Metschikowia pulcherrima*, *Candida ethanolica* and *Kloekera apiculata* were studied during laboratory fermentations. An important aspect highlighted by this study was the possibility of monitoring yeast populations that were at least 0.01% with respect to the dominant *Saccharomyces*, defining in this way the sensitivity limit of the method. The protocol was then used to study yeast ecology during commercial fermentation of a sweet wine produced from grapes intentionally infected with *Botrytis cinerea*. Several non-*Saccharomyces* yeasts, such as *Metschnikowia* sp. and *Pichia anomala*, were identified (Cocolin et al. 2001). Moreover a constant signal from a *Candida* species was observed throughout the fermentation. Subsequent studies focussing on the same sweet wine fermentation highlighted that, for several yeast species, molecular signatures were present in the DGGE gels even if colonies were not visible on agar culture media (Mills et al. 2002). This aspect was particularly important for a *Candida* species, subsequently identified as *Candida zemplinina* (Sipiczki 2003). Also, the results of an RNA dot blot obtained in this study demonstrated that it was possible to detect metabolically active cells, which were not culturable, but were nevertheless present throughout the alcoholic fermentation. Following on from these first applications, PCR-DGGE has been applied extensively to enological studies. Cocolin et al. (2002) used it to monitor continuous fermentations, revealing the presence of non-*Saccharomyces* yeast only in the very first set up of the fermentor, while during the process *Saccharomyces* was the only one yeast detected in the gels.

The analysis of DNA and RNA with DGGE helped our understanding of the spoilage process by *Brettanomyces bruxellensis*, one of the most controversial enological yeasts, in 12 wines produced in the North of Italy (Cocolin et al. 2004). When the results of PCR-DGGE were compared with those of RT-PCR-DGGE, it became evident that *B. bruxellensis* was present as a living population in some samples, while in others the specific signature could be found only at the DNA level. Based on these results, it was speculated that wines in which only the DNA was found were subject to spoilage earlier than those in which RNA was also detected.

B. bruxellensis is also often found in wine ecology studies in France. Renouf et al. (2006a) used DGGE to follow the ecology of three wineries during alcoholic and malolactic fermentation. In all three cases, it was possible to detect the constant presence of *B. bruxellensis*, which is able to persist throughout the fermentation process and be present also in finished wines.

Prakitchaiwattana et al. (2004) evaluated the performance of DGGE for analysing yeasts associated with wine grapes as compared with traditional microbiological analysis. *Aerobasidium pullulans* was the main yeast species isolated at all maturation stages; however, it was not detected in DGGE when it was present below 10^3 cfu/g. DGGE highlighted the presence of *Metschnikowia* and *Hanseniaspora* species in matured and damaged grapes, but various species of yeasts also present at populations higher than 10^5 cfu/g were not detected. This latter study concluded that DGGE is less sensitive than agar cultures for determining grape yeast ecology; however, DGGE allows the detection of a greater diversity in a shorter time. These results suggest that both methods need to be performed in parallel in order to profile yeast ecology in wine grapes.

In the last few years, DGGE has been exploited to study fermentations of must obtained from healthy and *Botrytis*-affected grapes in Greece (Nisiotou et al. 2007) and to follow the dynamics of yeasts in a natural fermentation of Catalanesca grapes in the South of Italy (Di Maro et al. 2007). Nisiotou et al. (2007) used DGGE to analyse the DNA extracted from samples of must obtained from grapes affected or not by *B. cinerea*, and reported a diversification in yeast communities in the two samples. Several species, such as *C. zemplinina*, *Zygosaccharomyces bailii*, *Issatchenkia* spp. and *Kazachstania* sp. were detected in *Botrytis*-affected fermentations, which possessed in general a higher level of biodiversity than their healthy counterparts. It is interesting to highlight that, in *Botrytis*-affected musts, *S. cerevisiae* was not always able to predominate, since *Z. bailii* dominated in some cases. The prevalence of non-*Saccharomyces* yeasts, namely *Hanseniaspora* spp., *Issatchenkia* spp. and *Candida* spp., was also found in natural fermentation of Catalanesca grapes in the early stages, while the middle and end phase were dominated by *S. cerevisiae* (Di Maro et al. 2007). In both these studies, *A. pullulans* bands were often observed during fermentation, confirming the wide distribution of this species in grapes used for wine fermentation processes.

DGGE has been proposed as a method to determine the influence of enological practices on microbial populations dynamics. Cocolin and Mills (2003) studied the effect of

SO_2 addition in laboratory fermentations inoculated with a mixed culture of *S. cerevisiae*, *H. uvarum* and *C. zemplinina*, showing the absence of colonies belonging to the non-*Saccharomyces* immediately after the addition of sulfur dioxide, but a constant presence of DNA and RNA signals in DGGE analysis. This observation supports the hypothesis of the presence of viable but not culturable cells, that are not able to form colonies on agar medium, but are metabolically active during the fermentation. More recently, Andorrà et al. (2008, 2009) also studied the effect of SO_2 and temperature on microbial dynamics during wine fermentations. In both cases they demonstrated a good applicability of PCR-DGGE when yeast populations were present with similar counts. When *S. cerevisiae* started to take over the fermentation, the minor populations of *H. guillermondii* and *C. zemplinina* could not be detected unless they had counts above 10^4 cfu/ml, thereby precluding correct profiling of these yeasts. In order to overcome this problem, the authors proposed a quantitative PCR approach, able to quantify specifically the yeasts not observed in DGGE gels.

DGGE analysis represents an important investigational tool, particularly in complex microbial ecosystems. In the field of wine fermentations, sweet wine fermentations could be considered as such. The must used for the production of sweet wines comes from grapes that are allowed to dry on the plant or that are collected at maturation, but then stored in controlled temperature and humidity rooms, in order to allow loss of water. During the drying process, *B. cinerea* often participates in its noble form, to help dehydration of the berry by creating microfractures of the skin that enhance the possibility of water loss (Donèche 1993). It has been demonstrated that *Botrytis* can also affect species succession during wine fermentation (Fleet 1993). Sweet wines fermentations represent a unique microbial niche where important mould/yeast/bacterial interactions take place. Moreover, the high sugar concentration (usually 30–40% w/v) influences not only the physiology of *S. cerevisiae*, promoting the production of acetic acid through the up-regulation of the aldehyde dehydrogenases (Erasmus et al. 2003), but also the yeast dynamics during the fermentation process.

The ecology of yeasts in sweet wines fermentations was investigated in detail by Urso et al. (2008). In this latter paper the yeast ecology of Picolit wine fermentations in the Northeast of Italy, obtained from grapes naturally dried on the plant, was studied by DGGE. The beginning of the fermentation was dominated by moulds, at both DNA and RNA level but, after crushing, yeasts such as *Kloeckera apiculata* and *C. zemplinina*, followed by *S. cerevisiae*, were able to take over the fermentation in the grape juice. More interestingly, Rantsiou et al. (2009) reported a different picture of the yeast ecology during sweet wines fermentation of Erbaluce di Caluso grapes in the Northwest of Italy, when the DNA was analysed with respect to the RNA. In PCR-DGGE gels, constant populations of *S. cerevisiae* were observed, while in RT-PCR-DGGE analysis, additional signals belonging to *Torulaspora delbruekii* were detected throughout the fermentation. This aspect underlines the need to consider also RNA as an important target molecule for ecological studies, not only because it detects populations that are metabolically active, but also because it may give a better picture of the microbial diversity within a specific microbial ecosystem.

Lastly, DGGE analysis, apart from being able to profile different yeast species during wine fermentation, has also been applied also to distinguish strains of *Saccharomyces* sensu stricto. Manzano et al. (2004) developed a PCR-DGGE protocol in which *S. cerevisiae* could be separated from *S. paradoxus* and *S. bayanus/S. pastorianus* based on different DNA sequence of the internal transcribed spacers (ITS). A couple of primers, specific for *Saccharomyces* sensu stricto were developed, and a DGGE run could separate different species based on migration mobility. Moreover, several DGGE profiles for *S. cerevisiae* strains could also be observed, suggesting the possibility of exploiting the method presented to follow wine fermentations in which different strains of *S. cerevisiae* participate in the transformation process. The same approach was used by the same authors in order to determine if the *S. cerevisiae* starter culture added to a continuous industrial fermentation was able to predominate and conduct the process (Manzano et al. 2005). The PCR protocol was optimised to be specific for *S. cerevisiae* and *S. paradoxus*, resulting in different profiles in a TGGE gel. It was also demonstrated that at least three TGGE patterns could be obtained from a panel of 22 strains of *S. cerevisiae*, underlining the possibility of following a specific strain during fermentation, even in the presence of other strains of *S. cerevisiae* or *S. paradoxus*, due to the different migration patterns. The method proposed showed good applicability when used on DNA extracted directly from samples obtained from a continuous fermentation. Wines sampled at different timepoints from the beginning of the process always showed a single profile, identical to that of the starter culture added at the beginning of the fermentation, thereby proving the capacity of this yeast to persist for at least 40 days in the continuous reactor.

Bacterial ecology

While PCR-DGGE is now an approach frequently used to study yeast ecology, only a few papers have been published on bacterial ecology during wine making. One important problem that had to be solved was the non-specificity of the primers used to amplify bacteria. As demonstrated by Lopez et al. (2003), several “bacterial”-specific primers

were also able to amplify yeast, mould and plant DNA. A careful study of the sequences of ribosomal genes resulted in the definition of primers highly specific for bacteria that could be used for DGGE analysis. Moreover, other targets have been used for the same purpose. The β-subunit of the RNA polymerase (*rpoB*) was proposed as an alternative gene to use in DGGE (Dahllof et al. 2000). Specifically, Renouf et al. (2006b) used primers for *rpoB* to study bacterial ecology in different wineries. This latter paper found that differences in bacterial ecology were detected only at the beginning of the malolactic fermentations, while at the end only *Oenococcus oeni* was present.

As described for yeast ecology, the DGGE approach has been used to understand the effect of enological practices, more specifically the addition of SO_2, on bacterial dynamics. Andorrà et al. (2008) monitored LAB and acetic acid bacteria (AAB) dynamics during wine fermentation as affected by SO_2. While LAB were determined to be present at the threshold DGGE detection level (10^3–10^4 cfu/ml,) and only one band, belonging to *O. oeni*, was detected. For AAB, all the samples analysed showed two bands regardless of the fermentation conditions, underlining the fact that AAB were barely affected by yeast inoculation and SO_2 addition; these two bands were identified as *Acetobacter aceti* and *Gluconobacter hansenii*.

Choice of PCR-DGGE primers

When applying PCR-DGGE to study complex microbial populations, such as those present in wine fermentation, an important parameter that will influence the results obtained is the choice of the amplification target gene prior to DGGE.

The target gene has to have two basic characteristics: (1) it should be present in all members of the microbial group under consideration, and (2) it should have conserved regions, where universal primers can be designed, and variable regions, based on which separation is possible. Genes that fulfill these requirements are those that are involved in important and universal cell functions. Commonly, genes encoding rRNA fall within this category. In bacteria, various regions of the 16S rRNA coding gene have been used in PCR-DGGE, while in yeasts, the 26S rRNA coding gene is a common target.

One important advantage of the 16S and 26S rRNA coding genes is the fact that, for both genes, databases of sequences from a large number of representative species is available. This is important since it allows identification of DGGE bands by sequencing and comparison with the database. A drawback associated with the use of rRNA coding genes is the inherent sequence heterogeneity within the same species that is the result of multicopies of the genes with small differences in the sequence. The multicopies often result in multi-bands in the DGGE profiles, which complicate the analysis.

As mentioned above, the *rpoB* gene has been proposed as an alternative gene for use in PCR-DGGE. It is usually present as a single copy in each genome, thereby producing a single band in DGGE analysis. A limitation of using the *rpoB* gene is the restricted number of sequences available in the databases, which hinders the identification of unknown DGGE bands.

Several primers have been used in DGGE studies performed so far on wine fermentation (summarised in Table 1). The quality of information produced by PCR-DGGE is dependent on both the number and the resolution of amplicons in denaturing gradient gels. In fingerprinting the microbial communities of a food product, it is important that the method allows differentiation of the individual species

Table 1 Primers for polymerase chain reaction coupled with denaturing gradient gel electrophoresis (PCR-DGGE) used in wine fermentation ecology

Primers	Sequence (5′-3′)	Reference
Yeasts		
NL1[a]	GCCATATCAATAAGCGGAGGAAAAG	Cocolin et al. 2000
LS2	ATTCCCAAACAACTCGACTC	
U1*	GTGAAATTGTTGAAAGGGAA	Andorrà et al. 2008
U2	GACTCCTTGGTCCGTGTT	
403f	GTGAAATTGTTGAAAGGGAA	Di Maro et al. 2007
662r[a]	GACTCCTTGGTCCGTGTT	
Specific differentiation of *S. cerevisiae strains*		
Schaf[a]	GTAGTGAGTGATACTCTT	Manzano et al. 2005
Schar	AGAACATGTTGCCTAGAC	
Bacteria		
WBAC1[a]	GTCGTCAGCTCGTGTCGTGAGA	Lopez et al. 2003
WBAC2	CCCGGGAACGTATTCACCGCG	
*rpoB*1	ATTGACCACTTGGGTAACCGTCG	Renouf et al. 2006b
*rpoB*1o	ATCGATCACTTAGGCAATCGTCG	
*rpoB*2[a]	ACGATCACGGGTCAAACCACC	

[a] Primers with an added 5′ GC clamp to improve separation of the PCR amplicons (5′-CGC CCG CCG CGC GCG CGG CGG GCG GGG CGG GGG CAC CGC GCG-3′)

that are associated with a specific food. For this reason, the primers used and the conditions employed in PCR-DGGE need to be carefully considered and, if necessary, optimized, prior to application in real food samples (Cocolin et al. 2000).

Conclusions

The technological development and scientific advancements in the field of molecular biology have led to the creation of ever more precise and accurate analytical tools for the study of complex microbial systems. The application of such approaches has allowed better understanding of microbial processes that had previously been well characterized by traditional cultural methods but not completely elucidated. Culture-independent methods has led to a better comprehension of complex transformations. Importantly, the possible presence of viable but not culturable states, especially in the case of spoiling yeasts such as *B. bruxellensis*, should be evaluated carefully because of the direct connection of these microorganisms and their metabolic activities with the organoleptic quality of the final product.

The application of molecular methods to wine fermentations has led to a deeper knowledge of the interactions between different species of yeasts and between yeasts and bacteria, in such a way that possible interventions can be foreseen in order to drive the process and obtain wines with optimal organoleptic and sensory characteristics. Moreover, the description of new species, such as *C. zemplinina*, showing interesting features for wine fermentations, such as growth at low temperature, fructophylic behaviour and the production of glycerol (Sipiczki 2003), offers new possibilities, especially for fermentations that are particularly complex to control, even with the use of starter cultures, such as sweet wines fermentations. As described above, in this processes, *S. cerevisiae* is able to produce high quantities of acetic acid because it is subjected to osmotic stress. In order to enhance *S. cerevisiae* performance, a co-inoculation of *C. zemplinina* strains, able to consume only fructose, can be foreseen, thereby reducing the osmotic pressure in musts obtained from grape musts. It can be speculated that this practice should increase the fermentation speed and, at the same time, reduce the presence of high quantities of acetic acid, improving the sensory characteristics of the final wine.

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