RESOLUTION OIV-OENO 408-2011

MOLECULAR TOOLS FOR IDENTIFICATION OF SACCHAROMYCES CEREVISIAE WINE YEAST AND OTHER YEAST SPECIES RELATED TO WINEMAKING

THE GENERAL ASSEMBLY

In view of Article 2, paragraph 2, no. iv of the Agreement of 3 April 2001 establishing the International Organisation of Vine and Wine,

On proposal of the group of experts “Microbiology”

DECIDES to adopt the following Molecular tools for identification of Saccharomyces cerevisiae wine yeast and other yeast species related to winemaking

DECIDES to include the following Preambule when publicising the Molecular tools for identification of Saccharomyces cerevisiae wine yeast and other yeast species related to winemaking and the Molecular tools for identification of lactic acid bacteria [Oeno-MICRO 09-409] in grapes and wine

Molecular tools for identification of Saccharomyces cerevisiae wine yeast and other yeast species related to winemaking

The origin, development, changes and succession of various yeast species can be followed using specific molecular techniques allowing the differentiation and typing of yeast strains. Different techniques have recently been employed in studying the microbiology of wine making. These methods are sensitive and specific, addressing the different needs and they are simple, rapid, reproducible, efficient, reliable, and not expensive. The implementation of molecular methods for identification can be done without previous culture, or after enrichment, or also after isolation of microorganisms. It allows to better know the microbial system that develops at the surface of the grape or in wine. This guide aims to assist the laboratories performing microbiological analysis in their approach to the identification of yeast Saccharomyces cerevisiae and other yeast species [OENO-MICRO 09-408] related to winemaking and for the identification of lactic acid bacteria [OENO–MICRO 09-409] in grapes and wine. The techniques reported consist in the main application of the molecular techniques available. The detailed methods are referred to the bibliography.

Culture dependent and culture independent methods can be utilized for the identification and characterization of wine yeasts at different steps of winemaking,

Certified in conformity Porto, 24th June 2011
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

© OIV 2011

aging and storage.

CULTURE DEPENDENT METHODS

IDENTIFICATION OF YEAST STRAINS AT THE SPECIES LEVEL

rDNA PCR-RFLP (restriction fragments length polymorphism)

PRINCIPLE: This is a culture dependent method since it requires the extraction of DNA from a pure culture. It is based on the amplification of specific regions of the rDNA repeat units (such as the internal transcribed spacers, ITS1 and ITS2, and the embedded 5.8S rRNA gene, or the 26S rRNA gene). Thes regions have sequences highly conserved and sequences which show a high degree of genetic variability between strains of different species. In the majority of cases, the PCR amplified products from strains of the same species and of the same genus have identical molecular sizes, and species of the same genus have similar sizes. Even if of the same size, the sequence of these amplified regions differ according to species. The differences in sequences is revealed by restriction analysis by using different restriction endonucleases such as Cfo I, Hae III, Hinf I, DdeI, MboI. Restriction patterns differ with the species.

The DdeI enzyme is required for the differentiation between H. uvarum and H. Guilliermondii as reported by Esteve-Zarzoso et al. (1999) and Cadez et al., 2002, while the enzyme MboI is necessary to distinguish C. zemplinina from C. stellata (Sipicki, 2004).

In addition, other restriction enzymes should be used to differentiate species of the genus Saccharomyces and their hybrids (Gonzales et al., 2006)

RESULTS: This methodology has already been utilized to identify around 145 yeast species belonging to 26 different yeast genera and the restriction profiles of all the species identified are reported in Esteve-Zarzoso et al. (1999) and Zanol et al. (2010). ITS-5,8S data is available online (http://www.yeast-id.com).

REFERENCES:

1. Esteve-Zarzoso et al. (1999) Identification of yeasts by RFLP analysis of the 5.8 S rRNA gene and the two ribosomal internal transcribed spacers. Int. J. of Syst Bacteriolo. 49, 329-337.
2. Fernández-Espinar et al.(2000) RFLP analysis of the ribosomal transcribed spacers and the 5.8 S rRNA gene region of the genus Saccharomyces: a fast method for

Certified in conformity Porto, 24th June 2011
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

© OIV 2011

species identification and the differentiation of flor yeasts. Antoine Van Leeuwenhoek 78: 87-97.

3. De Llanos et al. (2004) Identification of species of genus Candida by RFLP analysis of the 5.8 S rRNA gene and the two ribosomal internal transcribed spacers. Antoine Van Leeuwenhoek 85: 175-185.
4. Baleiras Couto et al. (2005) Partial 26S rDNA restriction analysis as a tool to characterise non-Saccharomyces yeasts present during red wine fermentations. Int. J. Food Microbiol 102, 49-56.
5. Zanol G., Baleiras-Couto M.M., Duarte F.L. (2010) Restriction profiles of 26S rDNA as a molecular approach for wine yeasts identification. Ciência e Técnica Vitivinícola 25: 75-85.
6. Cadez N., Raspor P., de Cock A.W.A.M., Boekhout T., Smith M.Th. (2002) Molecular identification and genetic diversity within species of the genera Hanseniaspora and Kloeckera. FEMS Yeast Research 1, 279-289.
7. Sipiczki M. (2004) Species identification and comparative molecular and physiological analysis of Candida zemplinina and Candida stellata. J. Basic Microbiol. 44 (6), 471–479.
8. González S.S., Barrio E., Gafner J., Querol A. (2006) Natural hybrids from Saccharomyces cerevisiae, Saccharomyces bayanus and Saccharomyces kudriavzevii in wine fermentations. FEMS Yeast Res 6, 1221–1234.

Sequencing of D1/D2 region after PCR amplification

PRINCIPLE: This is a culture dependent method since it requires the extraction of DNA from a pure culture. It is based on the amplification of the D1/D2 region of 26S RNA gene and subsequently sequencing the amplicon obtained. The sequence of D1/D2 differs between different species more than 1%, and less than 1% between strains belonging to the same species.

RESULTS: The sequence of the D1/D2 region of 26S rRNA gene can distinguish between the generality of yeast species known. The sequence of the D1/D2 region of 26S rRNA gene allows classification and phylogenetic identification of unknown isolates, because the existing database is by far the largest of yeast genes.

REFERENCES:

1. Kurtzman and Robnett (1997) Identification of clinically important ascomycetous

Certified in conformity Porto, 24th June 2011
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

© OIV 2011

yeasts based on nucleotide divergence in the 5' end of the large-subunit (26S) ribosomal DNA gene. J Clin Microbiol. 35, 5 :1216-23.

2. Kurtzman and Robnett (1998) Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Antonie Van Leeuwenhoek. 73, 4,: 331-71.
3. Baleiras Couto et al. (2005) Partial 26S rDNA restriction analysis as a tool to characterise non-Saccharomyces yeasts present during red wine fermentations. Int. J. Food Microbiol 102, 49-56.

IDENTIFICATION OF WINE YEASTS AT THE STRAIN LEVEL

RFLP (restriction fragments length polymorphism) of mitochondrial DNA (mtDNA)

PRINCIPLE: Mitochondrial DNA (mtDNA) of S. cerevisiae is a small molecule of 65-80 Kb which grade of variability can be shown with by restriction analysis. The high degree of polymorphism of mtDNA allows to analyse the variability of wine specific S. cerevisiae strains. It's one of the most used methods for the characterization of wine isolates during alcoholic fermentations. Querol et al. (1992) and López et al. (2001) simplified the analysis method: extraction of total DNA from a pure culture-isolate and a restriction analysis with restriction enzymes HinfI or RsaI (Guillamón et al., 1994, Schuller et al, 2004; Lopes et al 2006) is only required.

RESULTS: This technique allows high throughput of strain identificfation in a short period of time. It can be used in wine industry because it's fast, secure and no PCR equipment is required.

REFERENCES:

1. Querol et al. (1992) A comparative study of different methods of yeast strain characterization. Syst. Appl. Microbiol. 15: 439-446.
2. Guillamón et al. (1994) Rapid characterization of four species of the Saccharomyces sensu stricto complex according to mitochondrial DNA patterns. Int. J. Bacteriol. 44: 708-714.
3. López et al. (2001) A simplified procedure to analyse mtDNA from industrial yeast. Int. J. Food Microbiology 68: 75-81.
4. Schuller D, Valero E., Dequin S., Casal (2004) Survey of molecular methods for the typing of wine yeast strains. FEMS Microbiology Letters 231, 19-26.

Certified in conformity Porto, 24th June 2011
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

© OIV 2011

5. Lopes C.A, Lavalle T.L, Querol A., Caballero A.C. (2006) Combined use of killer biotype and mtDNA-RFLP patterns in a Patagonian wine Saccharomyces cerevisiae diversity study. Antonie van Leeuwenhoek 89, 147–156.

Amplification of Delta (δ) sequences

PRINCIPLE: Delta sequences are elements of 0,3 Kb (334 bp) that flank Ty1 retrotransposons in S. cerevisiae. Between 35 and 55 copies of delta sequences have been found in the genome of yeast as a part of Ty1 retrotransposons or as isolated elements. However, these delta sequences are concentrated in genomic regions joining tRNA genes. The number and the location of these elements have certain intraspecific variability that Ness et al., (1993) used to design specific primers: δ_1 and δ_2 useful for S. cerevisiae strain differentiation. Legras and Karts (2003) optimized this technique by designing two new primers: δ_{12} and δ_{21} that are located near δ_1 and δ_2. The use of δ_{12} and δ_{21} or δ_{12} with δ_2 reveals a larger polymorphism that is reflected by more bands on the electrophoresis gel.

RESULTS: Legras and Karts (2003) distinguished 53 commercial strains using the combination of δ_{12} and δ_{21} or δ_{12} with δ_2. Schuller et al. (2004) were capable to differentiate a great number of wine yeast strains using δ_{12} and δ_2 primers.

REFERENCES:

1. Ness et al, (1993) Identification of yeast strains using the polymerase chain reaction. J. Sci. Food Agric. 62: 89-94.
2. Legras and Karst, (2003). Optimisation of interdelta for Saccharomyces cerevisiae strain characterization. FEMS Microbiol. Lett. 221 : 249-255.
3. Schuller et al., (2004). Survey of molecular methods for the typing of wine yeast strains. FEMS Microbiol. Lett. 231: 19-26.

Genotyping by microsatellites

PRINCIPLE: Microsatellites are short sequences composed of tandem repeats of one to ten nucleotides. These sequences are scattered throughout the yeast genome, both in coding and noncoding regions, but their rate is lower in coding regions. Microsatellite markers are polymorphic loci, their allelic diversity allows to type strains within the same species. Various loci are known and can be used for typing S. cerevisiae strains typing (Legras et al. 2005). The profiles obtained by a combination of

Certified in conformity Porto, 24th June 2011
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

© OIV 2011

at least six microsatellites allows the efficient differentiation of strains of S. cerevisiae.

RESULTS: Microsatellite markers are frequently used as genetic marker in studies of genetic mapping and genetic of population. The combination of six microsatellite loci is showed as a highly discriminant and reproducible technique and at the same time reveals geographic and technological relationships between strains. These polymorphic loci can be easily used to determine the profile of strains of S. cerevisiae during fermentation.

REFERENCES:

1. Schuller et al. (2004).Survey of molecular methods for the typing of wine yeast strains. FEMS Microbiol. Lett. 231: 19-26.
2. Legras et al. (2005) Selection of hypervariable microsatellite loci for the characterization of Saccharomyces cerevisiae strains. Int J Food Microbiol. 102: 73–83.
3. Schuller and Casal (2007) The genetic structure of fermentative vineyard-associated Saccharomyces cerevisiae populations revealed by microsatellite analysis. Antonie van Leeuwenhoek 91:137–150.

CULTURE INDEPENDENT METHODS

Quantitative PCR (qPCR)

SCOPE: Rapid detection and quantification of yeasts at the species level in must or wine.

PRINCIPLE: This method relies on the use of universal yeast primers designed on the variable D1/D2 domains of the 26S rRNA gene. This is one of the few gene sequences available for all known ascomycetous yeast species. Quantification is possible from the determination of the number of polymerisation cycles needed to overpass a signal threshold. Higher the concentration of target species' DNA in the sample, fewer cycles are needed to overpass the target threshold. Calibration curves permit the precise quantification.

RESULTS: This technique has been used for the enumeration of Candida stellata, Dekkera bruxellensis, Hanseniaspora uvarum, and Saccharomyces cerevisiae cells in mixed fermentations in both synthetic medium and wine. The assay is linear over 5 orders of magnitude, and the detection limit is approximately 102 CFU/ml. The

Certified in conformity Porto, 24th June 2011
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

© OIV 2011

presence of other, nontarget wine microorganisms (both yeast and bacteria) in the samples does not significantly affect the qPCR assay.

A rapid qPCR method was developed for detecting and quantifying several non-Saccha- romyces yeasts, using species-specific primers for C. zemplinina, T.delbrueckii, I. orientalis, and M. pulcherrima (Zott et al., 2010).

REFERENCES:

1. Hierro, Esteve-Zarzoso, Gonzalez, Mas and Guillamon et al. (2006) Real-Time Quantitative PCR (qPCR) and Reverse Transcription-QPCR for Detection and Enumeration of Total Yeasts in Wine. Appl. Environ. Microbiol. 72: 7148–7155.
2. Zott K, Claisse O, Lucas P, Coulon J, Lonvaud-Funel A, Masneuf-Pomarede I (2010) Characterization of the yeast ecosystem in grape must and wine using real-time PCR. Food Microbiology 27:559-567.

PCR-DGGE (Denaturing gradient gel electrophoresis)

SCOPE: identification of yeasts at the species level in must or wine

PRINCIPLE: This method relies on the amplification of yeast 26S rDNA by using universal primers U1 (linked with a GC clamp) and U2. Amplification fragments are separated according to their length and nucleotide composition in a denaturing polyacrylamide gel (gradient from 20 to 60% of urea and formamide). Amplification fragments of interest are excised directly from the gel and sequenced for microbial species identification, taking the sequence band of yeast 26S ADN as reference. One of the advantages is the possibility to identify also viable but not culturable yeast, but whose ADN is also amplified.

RESULTS: This technique has been used for the analysis of microbial populations in both grape and wine samples and has led to the identification of different yeast species (Candida diversa, Candida sorboxylosa, Candida stellata, Dekkera bruxellensis, Hanseniaspora occidentalis, Hanseniaspora uvarum, Issatchenkia hanoiensis, Issatchenkia occidentalis, Issatchenkia orientalis, Issatchenkia terricola, Kluyveromyces thermotolerans, Metschnikowia pulcherrima, Pichia kluyveri, Saccharomyces cerevisiae, Saccharomycodes ludwigii, Torulaspora delbrueckii, Zygosaccharomyces bailii). Yeast populations above 103 cells/mL are adequately detected and identified by PCR-DGGE. PCR-DGGE is not a quantitative tool.

REFERENCES:

1. Cocolin L, Heisey A, and Mills D.A. (et al. (2001). Direct Identification of the

Certified in conformity Porto, 24th June 2011
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

© OIV 2011

Indigenous Yeasts in Commercial Wine Fermentations. Am. J. Enol. Vitic. 52:1:: 49-53.

Certified in conformity Porto, 24th June 2011
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

© OIV 2011