RESOLUTION OIV-OENO 409-2012

BIOLOGY MOLECULAR TOOLS FOR IDENTIFICATION OF GRAPE AND WINE LACTIC ACID BACTERIA

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 biology molecular tools for identification of grape and wine lactic acid bacteria.

BIOLOGY MOLECULAR TOOLS FOR IDENTIFICATION OF GRAPE AND WINE LACTIC ACID BACTERIA

Generality: During last decades different molecular methods have been developed for the identification of lactic acid bacteria (LAB) of oenological interest with the aim to reduce the time of analysis usually required to perform phenotypic assays. Moreover, molecular methods being not influenced by physiological and environmental conditions allow more reliable identification results.

They can be used for identification of lactic acid bacteria in grape must and at different steps of winemaking, aging and storage. Tools comprise culture dependent and culture independent methods. Molecular methods can identify to the species and to the strain level. Furthermore, bacteria strain can be differentiated from each other, and in addition, strains carrying specific genes can be detected whatever the species. The table summarizes the main utilizations of molecular methods for grape and wine lactic acid bacteria identification.

	Identification ofSpecies level	**Identification Strain level**	**Specific function**
Culture dependent (preliminary isolation of bacteria on plates and cultivation)	Hybridization (1): dot-blot, on colonies,		Hybridization (2): in dot-blot, on colonies, in situ ("FISH") and fluorescence microscopy

Certified in conformity Izmir, 22nd June 2012
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

	PCR based methods: -amplification of a consensus region and sequencing (3) -Species specific PCR(4) -RFLP or ARDRA: amplification and restriction (6)	PCR based methods: -RAPD: random amplification	PCR based methods: specific PCR(5)
		PFGE: restriction of genomic DNA by rare cutting enzymes and pulse field gel electrophoresis	
Culture independent methods (direct analysis of the sample) **1 Search for a species or a given function**	"FISH" microscopy *in situ* hybridization(1) PCR (4)		Hybridization (2): *in situ* ("FISH") PCR (5)
2 identification of bacteria without a priori	PCR-DGGE and sequencing (6)		

(1) Probe specific for the species

(2) the probe is a gene or a fragment of gene or a region that codes for a specific protein (enzyme)

(3) Primers amplify a consensus region (16S rDNA, rpoB)

(4) Primers are specific for the species

(5) Primers are specific for a gene or a region that codes for a specific protein (enzyme)

(6) Consensus amplicons are separated in denaturing gel electrophoresis, and then sequenced. The sequence is specific to each species.

CULTURE DEPENDENT METHODS

1. Preliminary

The methods of analysis are applied to pure cultures or colonies where the DNA is extracted or made accessible to the probes.

1.1. Isolation of clones and obtaining pure cultures

Most of the bacteria grown in wine can be isolated by traditional microbiological techniques, such as in favourable nutrient agar media. Depending on their

Certified in conformity Izmir, 22nd June 2012
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

concentration, a serial dilution of the wine sample in sterile physiological water (0.9% NaCl) is needed, before growth on specific nutrient agar media. Grape and wine lactic acid bacteria, are grown on the media described for enumeration (CII/MICRO/01/206). The bacterial population obtained can then be identified.

Clones are isolated on the plate and re-cultured in the same liquid medium, or again cultured on plate to purify the isolated strain. A single clone is then returned to liquid culture to provide the pure culture biomass that will be analyzed. In some cases, the colony is directly used for identification, eg for PCR (Polymerase chain reaction). All the colonies on the area of the media is analysed in colony hybridization

1.2. DNA extraction

DNA has to be extracted from the biomass harvested after culture. It is made accessible by in situ cell lysis for hybridization, on colonies or for microscopic analysis ("FISH"). Direct PCR on colonies does not need an extraction step: DNA is made available for amplification in the first cycle of temperature increase in the amplification protocol.

2. To the species level or for a specific function

2.1. Hybridization ["spot" (dot-blot), or colonies] with DNA probes specific to a species or to a region of the genome

Scope:

Identification of wine lactic acid bacteria to the species level, or a specific function (enzymatic activity).

Principle:

The common principle of the methods based on DNA-DNA hybridisation is that complementary nucleic acid fragments can hybridize under specific conditions depending mostly on the temperature and ionic strength of the buffer. After single-stranded denaturation, the DNA extracted from the bacteria to be identified, named the "target" can re-associate in hybridization conditions with a known single-strand of DNA, named "the probe" if the sequences are identical or close enough. Therefore, the choice of the probe is an vital decision, and it is this which determines the taxonomic level of the study. The probe can be all or a part of the genome that gives the specificity. Most of the time, 16S rDNA probes are used for the identification of species. But sometimes "cross hybridization" occurs and it is necessary to find other sequences. The same principle is applied to the identification of bacteria that carry

Certified in conformity Izmir, 22nd June 2012
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

specific genes, or regions implied in a given function. The probes are genes or parts of genes coded for the corresponding proteins. The best known applications are identification of spoilage bacteria.

Depending on the application, there are several hybridization procedures

i. methods using DNA extracted from the strain culture to be identified.

The target DNA is extracted from the culture to be identified and deposited and fixed on a membrane. The DNA probe, consisting in a reference labelled DNA, is left in contact with the membrane which is placed under the temperature conditions of hybridization. After the hybridization reaction and washings intended to eliminate the remaining non-hybridized probe, the membrane is treated to reveal the hybrids (target DNA/probe DNA).

ii. methods applied on the whole cells: on colonies.

The membrane is spread on the surface of the nutrient agar over the colonies that have developed, and then withdrawn. It retains the colonies that were transferred by simple contact. Then the membrane is treated for the lysis of cells (so that the DNA is accessible) and hybridization with the probe. After hybridization, we get picture of the plate where the colonies that are revealed belong to the same species as the reference probe. Using several specific DNA probes, this technique allows the identification of the lactic acid bacteria species of the sample after successive hybridizations/ dehybridizations of the same membrane.

References:

1. Lonvaud-Funel, A., Fremaux, C., Biteau, N., Joyeux A. (1991a). Speciation of lactic acid bacteria from wines by hybridisation with DNA probes. Food Microbiol., 8, 215-222.
2. Lonvaud-Funel, A., Joyeux, A., Ledoux, O. (1991b). Specific enumeration of lactic acid bacteria in fermenting grape must and wine by hybridization with non isotopic DNA probes. J. Appl. Bacteriol., 71, 501–508.
3. Sohier, D., Coulon, J., Lonvaud-Funel, A. (1999). Molecular identification of Lactobacillus hilgardii and genetic relatedness with Lactobacillus brevis. Int. J. Syst. Bacteriol., 49, 1075-1081.

iii. Restriction Polymorphism (RFLP) ribotyping:

Certified in conformity Izmir, 22nd June 2012
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

The restriction fragments obtained after the total digestion of the DNA extracted from the culture are separated by electrophoresis on agarose gels and transferred to nylon or nitrocellulose membranes for hybridization with a ribosomal DNA probe (Southern). The probe is previously marked.

After hybridization and visualization the pattern is the picture of the fragments generated by restriction on the entire length of the genomic region complementary to the probe.

The use of ribosomal (or possibly other genes) for the probe allows the identification of the strain of a species or subspecies by comparison of the size of the fragments in the ribosomal region (ribotyping) or possibly another particular region in the genome.

2.2. PCR (Polymerase chain reaction) based methods:

Scope:

Identification at species level of lactic acid bacteria previously isolated from must and wines by plate culture. PCR can be adapted to several levels of identification, genus, species, or strain. It only depends on the choice of the primers (short-sequence oligonucleotide). The principle is that the primer finds the complementary region to pair-up on the DNA template that is analyzed. Polymerisation is carried out by duplicating the template DNA segment from the end of the primer a great number of times which leads to the amplification product.

2.2.1. Amplification of consensus regions and sequencing: subunit of 16S ribosomal RNA, and rpoB

Principle:

Amplification product sequencing of the 16S rRNA-coding gene is the basic method. This approach is based on the obligatory presence of rRNA genes in bacteria. The nucleotide sequence of this gene is used for phylogenetic classification and for the identification of unknown isolates, given that the available database for this gene is the most extensive among bacterial genes. The nucleotide sequence of the region between the 16S and 23S rRNA-coding genes, termed the internal transcribed spacer (ITS), can be used for identification, but its sequence is less preserved. In this case PCR uses primers directed at universally conserved regions within the bordering 16S and 23S rRNA-coding genes, on either side of the ITS.

Another interesting sequence is rpoB that codes for a sub-unit of the RNA polymerase. It has been shown to be more suitable for identification since, (at least in the present knowledge). Indeed, there is only one copy of this gene in the bacteria, whereas there are several copies of the ribosomic operon, the sequence of which may differ within the same species. However its use is less common because the database is relatively

Certified in conformity Izmir, 22nd June 2012
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

less documented. For the main wine lactic acid bacteria a database has been established. Primers have been developed for wine lactic acid bacteria.

General protocol:

This method requires the extraction of DNA from the pure culture that is to be identified. The extracted DNA is used as a template in the PCR reaction. After separation by electrophoresis, the amplification products are purified using convenient kits, then sequenced. The resulting 16S rRNA-coding or rpoB-coding gene sequences are compared with those in the available database.

References:

1. Du Plessis, E.M.; Dicks L.M.T., Pretorius I.S., Lambrechts M.G., & du Toit M. (2004). Identification of lactic acid bacteria isolated from South African brandy base wines. Int. J. Food Microbiol. 91, 19-29
2. Sato, H., Yanagida, F., Shinohara, T., Suzuki, M., Suzuki, K., Yokotsuka, K. (2001). Intraspecific diversity Oenococcus oeni. FEMS Microbiol. Lett. 202, 109-114
3. Renouf, V., O. Claisse, and A. Lonvaud-Funel. 2006. rpoB gene: A target for identification of LAB cocci by PCR-DGGEand melting curves analyses in real time PCR. Microbiol. Met. 67:162-170.

2.2.2. Species specific PCR

PCR is adapted to the direct identification of species, because specific primers can be found which only amplify with a given species. Various primer pairs have been described for the identification of several species of lactic acid bacteria. In oenology the most necessary identification is the Oenococcus oeni species. The primers are based on regions of the malolactic enzyme that differ according to the species.

References:

1. Zapparoli G, Torriani S, Pesente P, Dellaglio F., 1998.Design and evaluation of malolactic enzyme gene targeted primers for rapid identification and detection of Oenococcus oeni in wine. Lett Appl Microbiol. 27:243-246.

2.2.3. Restriction Fragment Length Polymorphism (RFLP)

Scope:

Identification of species of lactic acid bacteria isolated from wines

Certified in conformity Izmir, 22nd June 2012
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

Principle:

Based on the same principle as ribotyping (see paragraph iii), the PCR amplification of the ribosomal region (or any other region), followed by restriction of the amplification product by appropriate enzymes gave the same information. However it is much easier and fast than the restriction followed by the hybridization.

The restriction of the 16S rRNA-coding or the RpoB-coding gene with appropriate restriction enzyme endonucleases, and the separation of fragments by agarose gel electrophoresis result in a characteristic pattern of DNA fragments that is specific of the particular species.

Results:

Discrimination of O. oeni strains from other species by RFLP analysis of 16 S rRNA.

Identification of lactic acid bacteria species by RFLP of rpoB gene.

References:

1. Claisse, O., V. Renouf, and A. Lonvaud-Funel. 2007. Differentiation of wine lactic acid bacteria species based on RFLP analysis of a partial sequence of rpoB gene. Journal of microbiological methods 69:387-390.
2. Zavaleta, A.I.; Martínez-Murcía, A.J.; Rodríguez-Valera, F. 16S-23S rDNA intergenic sequences indicate that Leuconostoc oenos is phylognetically homogeneous. Microbiology, 1996, 142, 2105-2114.
3. Sato, H.; Yanagida, F.; Shinohara, T.; Yokosutka, K. Restriction fragment length polymorphism analysis of 16S rRNA genes in lactic acid bacteria isolated from red wines. J. Biosc. Bioengin. 2000, 3, 335-337.

3. Identification at the strain level:

Scope: Identification of wine lactic acid bacteria at strain level.

3.1. PCR based methods: Randomly Amplified DNA (RAPD)

Principle:

RAPD is based on PCR with arbitrary sequence primers that are capable of hybridisation on various points of the genome. A single arbitrary oligonucleotide primer of about 10 nucleotides long is used for the amplification of random segments of genomic DNA and it generates a characteristic pattern that includes short DNA products of various lengths. It is also possible to use a combination of two or more

Certified in conformity Izmir, 22nd June 2012
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

oligonucleotides (multiplex RAPD) in a single PCR to generate more reliable RAPD profiles for typing strains. The RAPD profiles are typical of the strains and their reliability and discriminatory faculties are dependent on the primers' sequences.

Results:

This method enables to obtain a typical genetic fingerprint of the strain. It can be used to monitor the successful implantation of malolactic starter cultures during the vinification process. The main disadvantage stems from the lack of repeatability and reproducibility.

References:

1. Bartowsky, E.J., McCarthy, J.M., Henscheke, P.A. (2003). Differentiation of Australian wine isolates of Oenococcus oeni using random amplified polymorphic DNA (RAPD). Aus. J. of Grape and Wine Res., 9, 122-126
2. Du Plessis, E.M., Dicks, L.M.T. (1995). Evaluation of random amplified polymorphic DNA (RAPD)-PCR as a method to differentiate Lactobacillus acidophilus, Lactobacillus crispatus, Lactobacillus amylovorans, Lactobacillus gallinarum, Lactobacillus gasseri, and Lactobacillus johnsonii. Curr. Microbiol., 31, 114-118.
3. Reguant, C., Bordons, A. (2003). Typification of Oenococcus oeni strains by multiplex RAPD-PCR and study of population dynamics during malolactic fermentation. J. Appl. Microbiol., 95, 344-353.
4. Rodas, A.M., Ferrer, S., Pardo, I. (2005). Polyphasic study of wine Lactobacillus strains: taxonomic implications. Int. J. Sys. Evol. Microbiol., 55, 197-207.
5. Zavaleta, A.I., Martinez-Murcia, A.J., Rodriguez-Valera, F. (1997). Intraspecific genetic diversity of Oenococcus oeni as derived from DNA fingerprinting and sequence analyses. Appl. Environ. Microbiol., 63(4), 1261-1267.

3.2. Restriction genome pattern analysis by Pulsed Field Gel Electrophoresis (PFGE)

Principle:

Genomic DNA is digested with restriction enzymes with rare cutting sites. The entire length of the genome is fragmented into a number of fragments suitable both for an easy reading and clear differentiation between the strains. Bacterial cells from fresh cultures are recovered by centrifugation and immobilised in agarose blocks. The cell lysis and the DBA genomic restriction are conducted in the blocks in order that DNA is

Certified in conformity Izmir, 22nd June 2012
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

only specifically cut by the enzyme and not randomly by mechanical effects. The gel blocks are the loaded onto agarose gel and the fragments separated by PFGE electrophoresis. Under the pulse electric field the large fragments generated by the rare cutting enzymes are separated.

The restriction endonucleases ApaI and NotI are the most often used and well-suited for revealing polymorphism between O. oeni strains. Also, the enzymes SfiI, and SmaI have been used to differentiate the intraspecific level in diverse wine Lactobacillus species.

Results:

This is the most reliable technique for identifying at the strain level. It is reproducible and, if required, the use of a second enzyme generally dispels any doubt after a first restriction. It can be used for the control of purity of cultures during starter production. It is the most appropriate for the evaluation of the starter survival and establishment after inoculation for malolactic fermentation. The main disadvantage is that this technique is time-consuming due to the necessary preliminary step of culture before restriction and it requires special and costly equipment.

References:

1. Gindreau, E., Joyeux, A., De Revel, G., Claisse, O., Lonvaud-Funel, A. (1997). Evaluation de l´établissement des levains malolactiques au sein de la microflore bactérienne indigene. J. Int. Sci. Vigne Vin 31, 197-202.
2. Pardo I., Rodas A.; Ferrer S. (1998). Study on populations dynamics of Oenococcus oeni in wine by using RFLP-PFGE. Les entretiens scientifiques Lallemand nº 6. pp. 93-96.
3. Rodas, A.M., Ferrer, S., Pardo, I. (2005). Polyphasic study of wine Lactobacillus strains: taxonomic implications. Int. J. Sys. Evol. Microbiol., 55, 197-207.
4. Zapparoli, G., Reguant, C., Bordons, A., Torrioni, S., Dellaglio, F. (2000). Genomic DNA fingerprinting of Oenococcus oeni strains by pulsed-field electrophoresis and randomly amplified polymorphic DNA-PCR. Current Microbiol., 40, 351-355.

CULTURE “INDEPENDENT” METHODS:

Certified in conformity Izmir, 22nd June 2012
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

1. Preliminary

The culture-independent methods allow the determination of the diversity of bacterial populations occurring during winemaking without the need to isolate and culture. They also detect the possible viable but non-culturable (VBNC) bacterial cells.

These techniques involve:

- PCR: after the total DNA extraction from grape juice or wine samples, amplification with the Polymerase Chain Reaction (PCR) technique is performed using specific universal primers amplifying 16S DNA or specific to the species or strain;
- In situ hybridization: the use of specific probes to hybridize the bacteria from the sample directly onto DNA slide. Observation requires a microscope.

1.1. DNA extraction from a grape juice or a wine sample

DNA extraction and amplification methods have been established to work on samples of wines and musts.. Several DNA extraction procedures have been reported (Baleiras-Couto and Eiras-Dias, 2005; Jara et al., 2008; Pinzani et al., 2004, Savazzini and Martinelli, 2006; Marques et al., 2010). Generally various commercial kits are used according to the manufacturer's recommendations or to some modified protocols.

Renouf et al., 2006 described the following DNA extraction protocol which was adapted to wine sample:

10 ml of wine are centrifuged at 10,000 g at room temperature for 10 min. The pellet is washed in 1ml of Tris 10mM EDTA 1mM (TE) buffer. After a second centrifugation (10,000 g for 5 min), the supernatant is discarded and the pellet resuspended in 300 µl of 0.5 mM EDTA pH 8. 300 µl of glass beads are added (Ø 0.1 mm) and samples are mixed at maximum speed for 10 min. Then, 300 µl of lysis solution and 200 µl of protein precipitation solution are added and mixed for 20 s. Precipitation of cellular fragments is performed by holding the tube on ice for 5 min. After another centrifugation (10,000 g for 3 min), the supernatant containing the DNA is transferred in a new micro-centrifuge tube and 60 µL of a PolyVinyl-Pyrrolidone (PVP) 10% solution is added. Vortex at high speed for 10 s allows wine polyphenols precipitations, which inhibit amplification reaction. After centrifugation (10,000 g for 2 min), the supernatant is transferred to a clean 1.5 ml micro-centrifuge tube containing 300 µl of isopropanol at room temperature. The tube is gently mixed by inversion until a visible

Certified in conformity Izmir, 22nd June 2012
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

mass of DNA could be seen. After centrifugation (10,000 g for 15 min), 300 µl of room temperature 70% ethanol are added to the pellet before an ultimate stage of centrifugation (10,000 g for 2 min). Ethanol is carefully sucked up and the tube is dried. In total, 50 µl of pour preparation injectable (PPI) water with 1 µql of RNase is used to rehydrate DNA overnight at 4 °C. After rehydratation, DNAs are stored at -20°C.

References:

1. Baleiras-Couto M.M., Eiras-Dias J.E. (2006). Detection and identification of grape varieties in must and wine using nuclear and chloroplast microsatellite markers. Analytica Chimica Acta 563: 283–291.
2. Jara C., Mateo E., Guillamón J.M., Torija M.J., Mas A. (2008) Analysis of several methods for the extraction of high quality DNA from acetic acid bacteria in wine and vinegar for characterization by PCR-based methods. Int. J. Food Microb., 128: 336–341.
3. Marques A.P., Zé-Zé L., San-Romão M.V., Tenreiro,R. (2010) A novel method for identification of Oenococcus oeni and its specific detection in wine. Int. J. Food Microb., 142: 251-255.
4. Pinzani P., Bonciani L., Pazzagli M., Orlando C., Guerrini S. and Granchi L.(2004) Rapid detection of Oenococcus oeni in wine by real-time quantitative PCR. Lett. Appl. Microb., 38: 118–124.
5. Savazzini F., Martinelli L. (2006) DNA analysis in wines: Development of methods for enhanced extraction and real-time polymerase chain reaction quantification Analytica Chimica Acta, 563: 274–282.

2. Identification of the species

2.1. Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) and sequencing

Scope

Identification of wine lactic acid bacteria at species level in a mixed population

Principle

The PCR-DGGE is performed after DNA extraction from the sample. It consists in the

amplification of hypervariable regions of genes encoding the 16S rRNA, or other genes such as rpoB gene (encoding the □ subunit of the RNA polymerase), that are surrounded by consensus regions. The DNA amplification products, all identical in size but different in their sequence, if they come from different species, are separated by electrophoresis in a polyacrylamide gel containing a gradient of denaturing components (normally urea and formamide). The double-stranded DNA fragments migrate through the gel until they are denatured by the chemical conditions so that their migration is slowed and eventually immobilised. However, the fragments do not completely denature because of a "GC clamp" formed thanks to the use of a PCR primer showing a GC-rich 5 end. The position in the gel where the double-stranded DNA fragment is denatured and becomes single-stranded DNA is dependent on the nucleotide sequence and % G+C content of the fragment. Different sequences have different melting domains and as a consequence also in different positions in the gel where the DNA fragment halts. DNA amplification products of different species of bacteria are separated by the distance of migration in the gel. The final identification of each species is obtained either by identifying the migration distance in relation to a reference DNA strand of the species, or by the purification and sequencing of each band and by comparison with the available data bank (Gen Bank, www.ncbi.nlm.nih.gov/gov/blast/)

This technique has been successfully employed in the identification of several wine lactic acid bacteria species in complex mixture. At first 16SrDNA regions were used as DNA target (Lopez et al., 2003) but more recently it has been demonstrated that the housekeeping rpoB gene, coding the RNA polymerase beta subunit, is a better target for species differentiation by direct PCR-DGGE analyses than the 16S rRNA gene (Rantsiou et al. 2004; Renouf et al. 2006; Renouf et al. 2007, Spano et al. 2007).

References:

1. Lopez I., Ruiz-Larrea F., Cocolin L., Orr E., Phister T., Marshall M., VanderGheynst J., and Mills D.A. (2003) Design and Evaluation of PCR Primers for Analysis of Bacterial Populations in Wine by Denaturing Gradient Gel Electrophoresis. Appl. Environ. Microbiol. 6801–6807
2. Rantsiou, K., Comi, G., Cocolin, L., 2004. The rpoB gene as a target for PCR-DGGE analysis to follow lactic acid bacteria population dynamics during food fermentations. Food Microbiol. 21, 481–487
3. Renouf V., Claisse O., Miot-Sertier C.,.Lonvaud-Funel A (2006) Lactic acid bacteria evolution during winemaking:Use of rpoB gene as a target for PCR-DGGE analysis.

Certified in conformity Izmir, 22nd June 2012
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

Food Microbiol., 23: 136–145

4. Renouf V., Strehaiano P. and Lonvaud-Funel A. (2007) Yeast and bacteria analysis of grape, wine and cellar equipments by PCR-DGGE. Int. Vine Wine Sci., 41: 51-61.
5. Spano G., Lonvaud-Funel A., Claisse O., Massa S. (2007) In Vivo PCR-DGGE Analysis of Lactobacillus plantarum and Oenococcus oeni Populations in Red Wine. Cur. Microbiol., 54: 9–13.

2.2. Polymerase Chain Reaction (PCR) with species-specific primers

Scope

Identification of lactic bacteria species mixed into a sample of wine or must.

Principle

The general principle is that primers specific to the species anneal on the DNA template of the DNA mix extracted from the wine or must, if they find complementary sequences. Their hybridization causes the amplification reaction. Only the DNA of the species targeted by the choice of primers will be amplified, if such a species is present.

Procedure

The identification of the presence of Oenococcus oeni is possible because the primers specific to that species have been developed. After DNA extraction from grape juice or wine samples PCR reactions are performed according to conditions described in specific protocols. Zapparoli et al., (1998) and Bartowsky and Henschke, 1999 developed the PCR conditions to identify O. oeni using primers specific for malolactic enzyme gene or the 16 S rRNA gene.

References:

1. Zapparoli G, Torriani S, Pesente P, Dellaglio F., 1998.Design and evaluation of malolactic enzyme gene targeted primers for rapid identification and detection of Oenococcus oeni in wine. Lett Appl Microbiol. 27:243-246.
2. Bartowsky E.J., Henscke P.A. 1999 Use of a polymerase chain reactionfor specific detection of the malolactic fermentation bacterium Oenococcus oeni (formerly Leuconostoc oenos) in grape juice and wine samples. Austr. J. Grape Wine Res. 5: 39-44.

2.3. Detection of specific strains of lactic bacteria marked by the presence of a particular function-coding gene: detection of alteration bacteria

The principle of the protocol is the same as for the detection of a species. However, the primers are sequences deduced from the genes that determine the alteration functions: production of biogenic amines (see OENO-MICRO 10-449), bitterness caused by hydroxyl-propionaldehyde/acrolein (Claisse and Lonvaud-Funel, 2001) and ropiness (Gindreau et al 2001).

1. Claisse, 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.
2. Gindreau, E., E. Walling, and A. Lonvaud-Funel. 2001. Direct polymerase chain reaction detection of ropy Pediococcus damnosus strains in wine. J Appl Microbiol 90:535-42.

3. Identification of a bacterial species in a mixed population without any DNA extraction

3.1. In situ hybridization: Fluorescence Hybridization in situ technique (FISH)

It is based on the design of specific species probes marked with a fluorescence label. The ribosomal RNA is often the target for these probes. In the first step, bacterial cells are permeabilised, which allows the probe to penetrate through the bacteria wall and reach the DNA. The probe anneals if it finds it's complementary sequence in the 16s RNA of the ribosome. Therefore, during this step the fluorochrome will be fixed to the ribosome of cells where the species corresponds to the probe. Observation is performed using a epifluorescence microscope. It is possible to simultaneously detect, count and identify various types of microorganisms in a sample if several probes with different-coloured fluorochromes are used. One of the main advantages of this method is that it is very fast, because it does not require the cultivation of the sample. The main inconvenience is that its low sensitivity limits the microscopic observation.

This method can be adapted to the detection of specific strains by choosing a DNA probe that hybridizes with a gene or any other region of the genome.

References:

Certified in conformity Izmir, 22nd June 2012
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

1. Sohier, D. Lonvaud- Funel, A. (1998). Rapid and sensitive in situ hybridization method for detecting and identifying lactic acid bacteria in wine. Food Microbiol. 15, 391-397.

2. Blasco L., Ferrer S.; Pardo I. (2003). Development of specific fluorescent oligonucleotide probes for in situ identification of lactic acid bacteria. FEMS Microbiol. Lett. 225, 115-123.

Certified in conformity Izmir, 22nd June 2012
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI