Advances in Bioresearch
Adv. Biores., Vol 8 (6) November 2017: 127-134
©2017 Society of Education, India
Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
DOI: 10.15515/abr.0976-4585.8.6.127134

Advances in Bioresearch

ORIGINAL ARTICLE

Microbiological and Molecular Identification (Pcr-Rflp-Its) Of The Yeast From Cinsault Grape Cultivated in Abdelmalek Ramdane (Wilaya of Mostaganem, Algeria)

[1]Nadia BERBER, [2]Rachid AISSAOUI, [1]Ahmed Mohamed Ali BEKADA, [3]Morvan COARER
[1]Technology of Food and Nutrition Laboratory (University of Abdelhamid Ibn Badis, Mostaganem), 27000, ALGERIA.
[2]Bioconversion Laboratory (University of Mustapha STAMBOULI, Mascara), 29000, ALGERIA.
[3]Microbiology and Biotechnology Laboratory (French Institute of Vine and Wine of Nantes), 44000, France.
E-mail of Corresponding author : nadia.berber27@gmail.com

ABSTRACT
The grape is an exemplary fruit of the microbial diversity, it is considered as the habitat of multiple wild microorganisms (yeasts). In this study, we explored the divergence of the indigenous yeast flora in the vineyards of the Abdelmalek Ramdane region (Wilaya de Mostaganem, Algeria) by collecting grape samples (Cinsault). Extraction of DNA as well as a PCR-ITS-RFLP has been developed for the ITS1-ADNR 5,8S-ITS2 region; it is sensitive enough to detect the biodiversity of the different species of isolated yeasts. Then 4 species of yeast out of the 11 studied belonging to 4 different genera were characterized according to their molecular profiles. Thus, the strains studied were characterized with two restriction enzymes (Hinf I and HaeIII). Meanwhile, classical microscopic yeast identification studies were able to enhance the results. Among the yeast species identified: Pichia fermentans, Torulaspora delbrueckii, Zygosaccharomyces microellipsoide and Saccharomyces cerevisiae. According to the results obtained, the Cinsault variety presents an excellent reservoir of yeasts Non saccharomyces. However, the tools of molecular biology have brought a notorious revolution in precise yeast identification tests. The PCR-RFLP-ITS is one of the most widely used methods of identification.
Key words: *Grape, Yeasts, Cinsault, PCR-ITS-RFLP, Hinf I and HaeIII.*

Received 28.05.2017 Revised 10.08.2017 Accepted 21.08.2017

How to cite this article:
N Berber, R Aissaoui, A Mohamed Ali Bekada, M Coarer. Microbiological and Molecular Identification (Pcr-Rflp-Its) Of The Yeast From Cinsault Grape Cultivated in Abdelmalek Ramdane (Wilaya of Mostaganem, Algeria). Adv. Biores., Vol 8 [6] November 2017.127-134.

INTRODUCTION

The grapes accumulate a great importance until a relatively recent time, when the very numerous works devoted to the ecology of the microbial flora of grape and especially the distribution of the species of the yeasts encountered on the film of the berries in maturation. Yeasts are part of the natural microbial communities associated with fresh grapes and are the most important micro organisms in wine production [9]. The yeast community on grapes is influenced by biotic and abiotic factors, including climatic conditions (mainly temperature and rainfall), geographical location and vineyard factors (age, size, grape variety and vintage year), vineyard treatments, physical grape damage, microbial vectors, microbial interactions and enzymic activities [1]. Spontaneous alcoholic fermentation of grape must is characterized by the presence of a high number of yeast genera and species. Cinsault is an old black grape with white juice, its clusters are large, composed of large berries with very juicy flesh. It is a late grape variety that needs sunlight and is resistant to drought, quite productive but fragile in the face of diseases. It prefers poor soils for quality production. However, the high sugar content of the Cinsault grape must promote the growth of yeast, these microorganisms are an essential part of the food industry. They participate in the development of many food products (bread, dairy, brewery) and in the production of metabolites but also in the revaluation of agricultural and industrial waste. Biotechnologies and

biomedical research largely exploit these microorganisms for the production of molecules of medical interest. The aim of this study is to isolate, characterize microscopically and identify the indigenous species of yeasts found in the grape variety (Cinsault) grown in the region of Abdelmalek Ramdane (Mostaganem, Algeria) to develop and express the diversity of the phylogenetic heritage based on the molecular technique (PCR-RFLP-ITS). Our work is an initiative for the creation of a "Souchier" typical yeast in our country Algeria.

MATERIAL AND METHODS

1-Yeast sampling source (Cinsault grape)

The grape harvest (Cinsault grape) was carried randomly in the vineyards of the region of Abdelmalek Ramdane (Mostaganem, Algeria) in the month of September 2014. The samples taken are transported directly to the Laboratory of Food Microbiology of the Department of Agronomy of the University of Mostaganem. Series of isolation and purification are carried by successive subcultures.

2-Isolation, purification and conservation of isolates

• Yeast sampling techniques:

We collected 500 g of healthy grapes (Cinsault grape) with sterile scisors and we collected them in sterile bags. A laboratory arrival, the grape berries are deposited independently on a bed of agar culture medium (YPG + Gentamicin) in a petri dish for a time of 10 to 12 hours, then the berries are removed and part of the yeasts supposed to be present on the grape has settled on our growing medium. The grapes are scraped and crushed in order to obtain a must and let ferment for one day at 25 ° C in order to increase the viability and quantity of the desired yeast [22].

• Isolation, purification and conservation of cultures:

We carried a series of seedings by the method of the streaks on plates of agar cultures (YPG + Gentamicin) in order to have pure cultures. The operation is renewed by taking at random an isolated colony. This leads to obtaining a culture whose purity is estimated by microscopic observation. Then the purified strains are placed in a glycerol solution (sterile) with approximately 25% and stored in the freezer at -18 ° C.

3-Microbiological identification

•Study of characters crop:

After incubating the cultures for 3 days at 25-28 ° C on agar medium (YPG + Gentamicin), macroscopic observation can describe the appearance of colonies (size, pigmentation, contour, viscosity ...) [13].

•The cellular characteristics:

Microscopic observation to define the shape, arrangement and mode of cell division. These characteristics are observed on microscopic slides to fresh (40 x objective) by collecting a colony from a solid medium by a loop and depositing it in a tube containing 10 m of distilled water and stirring vigorously up to the colony is dissociated, a drop of suspension is removed and deposited between a slide and a slide and then examined microscopically [18].

4-Molecular identification

•DNA extraction:

The technique developed by Ausubel et al., 1995 makes it possible to efficiently extract genomic DNA from yeasts in the case of liquid pure cultures but also cell suspensions collected on the surface of petri dishes. When isolated colonies of dishes are obtained, it is possible to extract the genomic DNA from the biomass present on the surface of the agar (YPG agar). When relatively fresh (maximum 15 days), a small amount of biomass is collected and treated by several extraction steps. The first step consists in mixing the collected biomass with 660 µl of 50% TE / SDS mixture (lysis buffer), then vortexing the mixture and incubating it at 65 ° C/5 minutes. To neutralize the medium, a volume of 340 µl of potassium acetate was added to the mixture, the latter is placed in the refrigerator for 30 minutes until the suspension became semi-solid. Then a second centrifugation was performed for 10 minutes and a volume of 750 µl of supernatant was mixed with 750 µl of isopropanol followed by a final centrifugation to precipitate DNA. Then, the collected DNA pellet was rinsed gently with approximately 120 µl of 95% ethanol, then suspended in a volume of 300 µl of TE1x (storage buffer) and incubated at 65 ° C for 15 minutes. Finally, the DNA was stored refrigerated 4°C until use (According to IFV Nantes, 2012).

•Amplifying the target region by the molecular techniques (PCR-RFLP-ITS):

1- Amplification of the ITS region

The sequence ITS1 5.8S rRNA ITS2 present a conserved region in the majority of yeast species and the variable regions. The primersITS1 (5'-TCCGTAGGTGAACCTGCGG-3 ') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') described as universal primers by [28]. The reaction mixture is

summarized in Table 1.The amplification is carried out in the thermocycler (Biométra) according to the program described by **[16]**.

2- Enzymatic digestion (According to Renouf, 2006)

In order to identify the different species of yeast. ITS PCR products are treated with restriction enzymes that recognize specific DNA sequences or motifs (the restriction site). Two enzymes were used in this study HinfI and HaeIII.

The reaction mixture is summarized in Table 2 and Table 3.

After restriction, the restriction profiles are obtained by electrophoresis (migration 120-130 for 30 minutes). The number and length of the fragments are compared with the size marker according to (IFV).

3- Electrophoresis on agarose gel

The purification of a double-stranded DNA fragment (ITS PCR products) is carried out by agarose gel electrophoresis, which consists of a mixture of 1.5% agarose and 1X TBE buffer , The gel is mixed with ethidium bromide (20µl BET / 200ml TBE) and hot-dipped in the electrophoresis tank. 20 µl of the mixture contained in each PCR microtube, mixed with 5 µl of loading buffer containing bromophenol blue, are deposited on the wells. Fragments of DNA of known molecular mass called markers are also deposited: they make it possible to correlate the migration distance of the DNA fragments to their size (in base pairs).

Then, applying a voltage of 120V / 30 minutes until the migration of the fragments **[3]**.The nucleotide different bands resulting from the electrophoresis are shown on the gel under UV light (254 nm) and photographed with a UV camera .

RESULTS AND DISCUSSION

Isolation and purification by successive subcultures made after collection of yeasts in September 2014 from the Cinsault grapes grown in the ABDELMALEK RAMDANE area (Wilaya of Mostaganem). This allowed us to get a collection of 11 yeast isolates.

Study cultivation of isolated yeasts:

Macroscopic examination of "yeast" cultures after incubation at 25 for 4 to 5 days shows generally creamy, well-isolated colonies that are whitish or yellowish in colour and sometimes pink in the form of powder with irregular and opaque contours. The Colonies are small to medium in size with smooth or rough surfaces and have an intense odor. Microscopic observation allows us to authenticate the cell form of isolated strains and their vegetative reproductive modes. The 11 isolated strains are ovoid or spherical, elongated or short and represent budding as a mode of vegetative reproduction. The uniformity of the cells confirms the perfect purification of the strains studied.

According to **[13]**, yeasts are in the form of independent free single cell or combined in pairs with characteristic morphology for example: spherical, ovoid, cylindrical, apiculé, bottled, pyramidal.

Molecular study of isolated yeasts:

DNA extraction:

DNA extraction has allowed us to observe a small white mass rushed to the microtube bottom, then it was inoculated in a buffer prior TE1x and must keep to the time of use.

PCR-RFLP-ITS:

A total of 11 isolates of yeasts isolated from the Cinsault grape juice were analyzed. To identify these yeasts, the region of the rRNA repeat unit comprises two non-coding regions referred to as internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene were amplified and digested by two enzymes of restriction (HinfI and HaeIII). The profiles obtained from each isolate were compared with reference strains in the determination of the IFV Guide (2012). The results of this study are summarized in Table 5. The size of the PCR products and restriction fragments of the major species identified in this study are shown in Table 6.

The species of yeasts isolated from the grape (Cinsault):

11 yeast isolates were identified as belonging to 4 genera and 4 different species: *Torulaspora* (05 strains), *Pichia* (04 strains), *Zygosaccharomyces* (01 strain), *Saccharomyces* (01 strain) (Table 5). These different kinds of yeast are well documented in the literature as present on grapes and the start of alcoholic fermentation [29, 1].

The review of the results indicates that 4 selected yeast strains (strain 1, 2, 3, 4) belong to the genus *Pichia* (Table 5), according to the determination of IFV guide (2012. The molecular profile of five yeast strains (strain 5,6,7,8,9) indicates that they look like the species *Torulaspora delbrueckii,* according to the determination of IFV guide (2012). However, the molecular characterization and micro-macroscopic characteristics show that the strains (10) belonging to the species *Zygosaccharomyces microellipsoide*.

Furthermore, the combination of the two results (morphological and molecular studies) indicated that the strain (11) belongs to the species *Saccharomyces cerevisiae.*

Grape berries are the primary source of yeast during the fermentation of the must [8] (Fig 1, 2, 3). In different wine regions in the world, insulation works and identification of yeasts showed that *Pichia,Candida, Metschnikowia, Kluyveromyces, Cryptococcus, Rhodotorula, Debaryomyces, Issatchenkia, Zygosaccharomyces, Saccharomycodes, Torulaspora Dekkera, Schizosaccharomyces* and *Sporidiobolus* are most frequently found [23, 8, 19, 20, 14]. Other species Non-Saccharomyces as *Candida zemplinina*, *Torulaspora delbrueckii and Hanseniaspora spp* are also an important part of the diversity of the community of the bay and are present during the fermentation, in particular during the pre fermentative stages [29].

In spontaneous fermentations, non-Saccharomyces such as *Torulaspora*, *Candida*, *Hanseniaspora* and *Pichia* usually develop at the beginning of alcoholic fermentation before being gradually replaced by *Saccharomyces*. Among the non-Saccharomyces, *Torulaspora delbrueckii* arouses a lively interest in the oenological world for its ability to ferment sugar, increase typicity and aromatic complexity of the wine, and also to reduce its volatile acidity. In our study, the latter represents the major species isolated from the mash of our "Cinsault" grape variety (5 strains) .This species is a non-Saccharomyces yeast naturally present in the must and the grape berries which has been described in the literature for its positive impact on the quality and complexity of wines [4, 5, 11] and for the purity its fermentation with especially low production of volatile acidity, acetaldehyde, diacetyl and acetoin [2, 17, 26]. This yeast is also described as cryophile and osmotolerant [12].

Among the Non-Saccharomyces yeasts isolated from our grape must, we have the species *Pichia fermentans* (4 strains), The genus Pichia represents a part of the native flora of grape skins. Used for beer making sometimes (lambic). The use of *Pichia fermentans* in pure cultures and sequential mixtures with *Saccharomyces cerevisiae* has been studied to improve the aromatic compounds and characteristics of a wine. *P. fermentans* has proved to be a good starter strains for must fermentation in the winemaking industry. It has shown the same level of sulphur tolerance and the same growth rate as *S. cerevisiae* [6]. According to [21, 22, 1], at the earliest stages, the Basidomycetes species are dominant, and the increasing number of Ascomycetes species, especially those that have fermentation capacity is observed at maturity stages (Pichia, *Metschnikowia, Hanseniaspora* and *Candida*).

However, only one species of *Zygosaccharomyces microellipsoide* (Strain 10) and *Saccharomyces cerevisiae* (Strain11) was isolated from the must. Fermentations are initiated by the growth of various species of *Pichia*, *Candida*, *Debaryomyces*, *Hanseniaspora*, *Metschnikowia*, *Schizosaccharomyces*, *Torulaspora*, and *Zygosaccharomyces*. Their growth is generally limited to the first two or three days of fermentation, after which they die off. Subsequently, the most strongly fermenting and more ethanol tolerant species of *Saccharomyces* take over the fermentation [8].

Species belonging to the genera *Pichia*, *Candida*, *Torulaspora* and *Zygosaccharomyces* isolated in our study were also frequently isolated in other studies of grape fermentation for wine production [7, 6, 10, 25, 24, 15, 27]. In our study, the grape must (Cinsault) is an excellent reservoir of Non-Saccharomyces yeasts as evidenced by the results obtained, major yeast species were represented by Non Saccharomyces yeast *T. delbrueckii, P.fermentans and Zygosaccharomyces.microellipsoide* while only one isolate was identified as Saccharomyces (Strain 11), so our work highlights the predominance of Non-Saccharomyces species or sound samples isolated from the grape must of Cinsault grape. During a spontaneous fermentation with native flora, Non-Saccharomyces yeasts predominate in the must during the pre-fermentation stage and at the beginning of the alcoholic fermentation before the yeast *S. cerevisiae* colonize the middle to complete the fermentation [29, 9, 22].

Table 1: The composition of the reaction mixture used for PCR-ITS according to (IFV)

Products	Volume (µl)	Volume (µl)
H2O	1220	1230
Tampon 10X	160	160
dNTP	64	64
ITS1	12,8 (40pmol/reaction)	12,8
ITS4	12,8 (40pmol/reaction)	12,8
Mgcl2	80	80
DMSO	31(2%)	31(2%)

Table 2: The restriction by the Hinf I.

Products	Volume en µl
Tampon 10X –Mix III	2,5
BSA	0,2
Enzyme Hinf I (10U/µl)	1

The reaction mixture is incubated at 37° C for 4 hours.

Table 3: The restriction by the Hae III.

Products	Volume en µl
Tampon 10X –Mix III	2,5
BSA	0,2
Enzyme Hae III (10U/µl)	1

The reaction mixture is incubated at 37° C for 4 hours.

Table 4: Example of macroscopic and microscopic observations in two isolated strains.

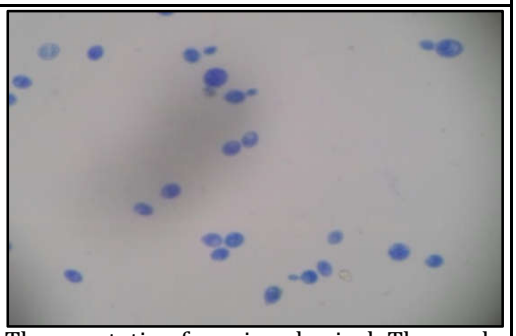
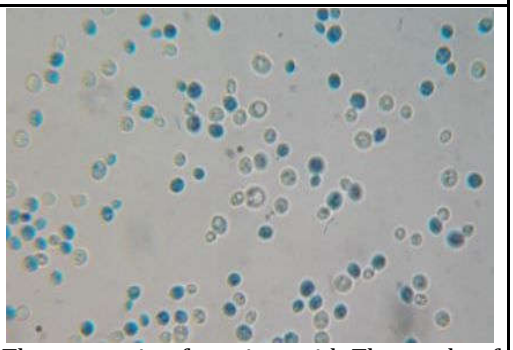
Souche	Macroscopic observation	Microscopic observation
(1)	 Colonies are pink, small in color, have a rough surface, have a rounded shape and have an intense odor.	 The vegetative form is spherical. The mode of reproduction is monopolar by budding.
(2)	 Creamy colonies of white, medium-sized, smooth surface and have a yeasty smell of beer.	 The vegetative form is ovoid. The mode of reproduction is monopolar by budding.

Table 5: Frequency of yeast species isolated from the grape "Cinsault"

Isolation source	Species	Frequency of isolation(Number of strains)
Cinsault grape variety	*Pichia fermentans* *Torulaspora delbrueckii* *Zygosaccharomyces microellipsoide* *Saccharomyces cerevisiae*	04 05 01 01

Table 6: Size in bp of the PCR products and the restriction fragments obtained with two different endonucleases (Hinf I and HaeIII) of the major species identified in this study.

Species	Amplified product (pb)	Restriction fragments (pb)	
		Hinf I	HaeIII
Pichia fermentans (Strains 1,2,3,4)	450	250/200	340/90
Torulaspora delbrueckii (Strains 5,6,7,8,9)	800	410/380	800
Zygosaccharomyces microellipsoide (Strain 10)	825	420/400	800
Saccharomyces cerevisiae (Strain 11)	850	370/360/110	320/230/170/120

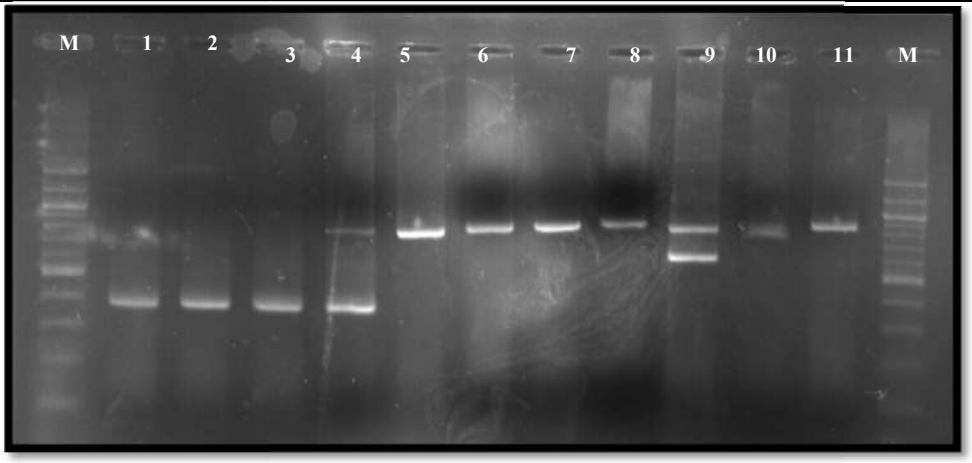


Fig. 1: Viewing the amplified region (rRNA 5,8S ITSI-ITSII) in 11 isolates. Lanes M correspond to molecular size standards (100-bp DNA ladder from IFV)

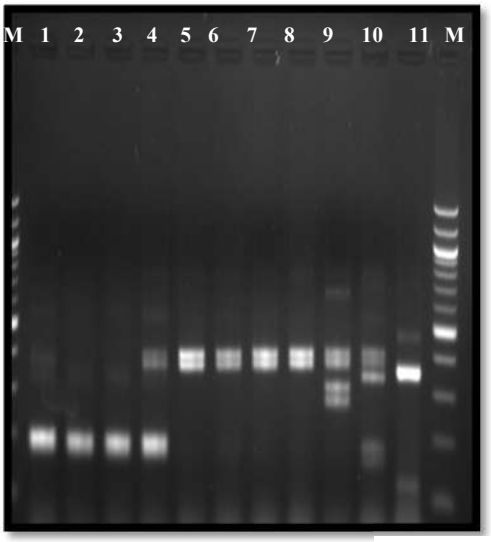


Fig. 2: Viewing the region (rRNA 5, 8S ITSI-ITSII) digested by HinfI in 11 isolates.

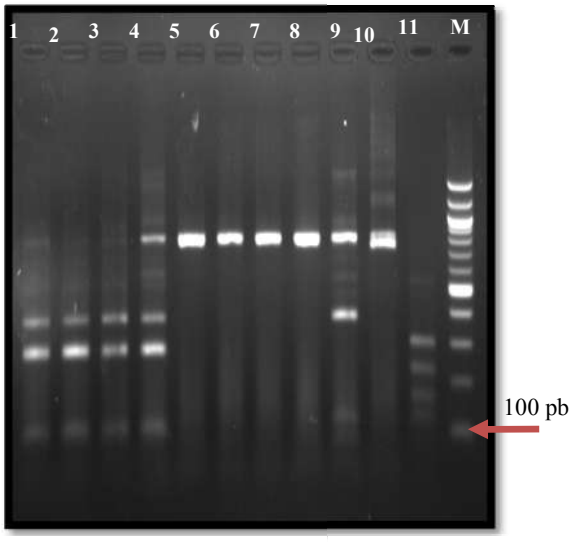


Fig. 3: Viewing the region (rRNA 5, 8S ITSI-ITSII) digested by HaeIII in 11 isolates.

This work is based on the evaluation of a grape variety grown in Algeria (Cinsault). A genetic approach has been developed and allowed the identification of 11 yeast isolates belonging to 4 different genera and species: *Pichia fermentans*, *Torulaspora delbrueckii*, *Zygosaccharomyces microellipsoide* and *Saccharomyces cerevisiae*. The results showed that the strains isolated on clusters are genetically distant from each other, but in parallel a cultural study (macro and microscopic) of the strains of isolated yeasts has allowed to reinforce the molecular study. However, the grape berries of this variety constitute an excellent reservoir of yeasts Non saccharomyces as evidenced by the results obtained. In terms of our study, we strongly encourage investigations to characterize biotechnologically these identified strains,

and to encourage all studies concerned with the identification and characterization of other grape varieties in all regions of Algeria. The final objective, drawn in the short and long term, is set in the development of the yeasts identified and then selected to serve mainly the agro-food domains (bread, dairy, brewery...) and new biotechnologies.

REFERENCES

1. Barata, A. *et al*., (2012). The microbial ecology of wine grape berries. International Journal of Food Microbiology, 153(3): 243-259.
2. Bely, M., P. Stoeckle, I. Masneuf-Pomarède and D. Dubourdieu, (2008). Impact of mixed *Torulaspora delbrueckii*–*Saccharomyces cerevisiae* culture on high-sugar fermentation. International Journal of Food Microbiology, 122: 312-320.
3. Chantal, G., (1996). Genetic characterization of the ITS regions of phytophthora spp that cause root rot of raspberry roots in quebec.
4. Ciani, Maurizio, et Maccarelli, Francesco, (1998). Oenological properties of non- *Saccharomyces* yeasts associated with wine-making. World Journal of Microbiology and Biotechnology, 14: 199-203.
5. Ciani, M., et G. Picciotti, (1995). The growth kinetics and fermentation behaviour of some non-*Saccharomyces* yeast associated with wine-making. Biotechnol Lett.,17: 1247-1250.
6. Clemente-Jimenez JM, Mingorance-Cazorla L, Martínez-Rodríguez S, Las Heras Vázquez FJ, Rodríguez-Vico F., (2004). Molecular characterization and oenological properties of wine yeasts isolated during spontaneous fermentation of six varieties of grape must. Food Microbiol. 2004;21:149–155.
7. Esteve-Zarzoso B, Gostíncar A, Bobet R, Uruburu F, Querol A.,(2008). Selection and molecular characterization of wine yeasts isolated from the 'El Penedès' area (Spain) Food Microbiol. 17:553–562.
8. Fleet, G.H., et G.M. Heard, (1993). Yeasts-growth during fermentation. In: Fleet, G.H. (Ed.), Wine Microbiology and Biotechnology. Harwood Academic Publishers, Chur, Switzerland.,pp: 27-57.
9. Fleet, G.H., (2008). Wine yeasts for the future. FEMS Yeast Research, 8: 979-995.
10. González SS, Barrio E, Querol A., (2007). Molecular identification and characterization of wine yeasts isolated from Tenerife (Canary Island, Spain) J Appl Microbiol.;102:1018–1025.
11. Herraiz, T., G. Reglero, M. Herraiz, P.J. Martin-Alvarez, M.D. Cabezudo, (1990). The influence of the yeast and type of culture on the volatile composition of wines fermented without sulfur dioxide. American Journal of Enology and Viticulture. 41: 313-318.
12. Hernández-Orte, P., M. Cersosimo, N.Loscos, J. Cacho, E. Garcia-Moruno and V. Ferreira, (2008).The development of varietal aroma from non-f oral grapes by yeasts of different genera. Food Chemistry, 107: 1064 - 1077.
13. Larpent, J.P., (1991). Biotechnology des levures. Ed. Masson. Paris, p: 426.
14. Nisiotou, A. andG.-J.E. Nychas, (2007). Yeast Populations Residing on Healthy or Botrytis-Infected Grapes from a Vineyard in Attica, Greece Applied and Environmental Microbiology, 73(8): 2765-2768.
15. Ortiz MJ, Barrajón N, Baffi MA, Villena MA, Briones A., (2013). Spontaneous must fermentation: Identification and biotechnological properties. LWT - Food Sci Technol. 2013;50:372–377.
16. Phalip Vincent, Hatsch Didier, Jeltsch Jean-Marc., (2004). Application of a yeast method for DNA extraction associated with database interrogations for the characterization of various filamentous fungi from diseased hop. Biotechnology Letters.March, Volume 26, Issue 5, pp 409-413.
17. Plata, C., C. Millan, J.C. Mauricio, J.M. Ortega, (2003). Formation of ethyl acetate andisomayl acetate by various species of wine yeasts. Food Microbiol.,20: 217-224.
18. Pol., D., (1996). Travaux pratiques de biologie des levures. Edition marketing, 158: 21-56.
19. Prakitchaiwattana, C.J., (2004). Application and evaluation of denaturing gradient gel electrophoresis to analyse the yeast ecology of wine grapes. FEMS Yeast Research, 4(8): 865-877.
20. Raspor, P., (2006). Yeasts isolated from three varieties of grapes cultivated in different locations of the Dolenjska vine-growing region, Slovenia. International Journal of Food Microbiology, 109(1-2): 97-102.
21. Rementeria, A., (2003). Yeast associated with spontaneous fermentations of white wines from the « Txakoli de Bizkaia » region (Basque Country, North Spain). International Journal of Food Microbiology, 86(1-2): 201-207.
22. Renouf, V., (2006). Description and characterization of microbial diversity during wine making: Interactions and balances - Relationship with the quality of wine. PhD thesis, Faculty of oenology, University Bordeaux 2.
23. Sabate, J., (2002). Isolation and identification of yeasts associated with vineyard and winery by RFLP analysis of ribosomal genes and mitochondrial DNA. Microbiological Research, 157(4): 267-274.
24. Settanni L, Sannino C, Francesca N, Guarcello R, Moschetti G., 2012. Yeast ecology of vineyards within Marsala wine area (western Sicily) in two consecutive vintages and selection of autochthonous *Saccharomyces cerevisiae* strains. J Biosci Bioeng. 2012;114:606–614.
25. Urso R, Rantsiou K, Dolci P, Rolle L, Comi G, Cocolin L., (2008). Yeast biodiversity and dynamics during sweet wine production as determined by molecular rmethods. FEMS Yeast Res. 2008;8:1053–1062.
26. Viana, F., J.V. Gil, S. Genovés, S. Vallés, P. Manzanares, 2008. Rational selection of non-Saccharomyces wine yeasts for mixed starters based on ester formation and enological traits.Food Microbiology, 25:778-785.
27. Wang C, Liu Y., (2013). Dynamic study of yeast species and *Saccharomyces cerevisiae* strains during the spontaneous fermentations of Muscat blanc in Jingayang, China. Food Microbiol. 2013;33:172–177.

28. White, T.J., T. Bruns, S. Lee and J. Taylor, (1990). Analysis of phylogenetic relationships by amplification and direct seq uencing of ribosomal RNA genes tiré de PCR Protocols: A guide to Methods and Applications. M. A. Innis, D. H.
29. Zott, K., C. Miot-Sertier, O. Claisse, A. Lonvaud-Funel and I. Masneuf-Pomarede, (2008). Dynamics and diversity of non-*Saccharomyces* yeasts during the early stages in winemaking.International Journal of Food Microbiology, 125: 197-203.