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Isolation and Molecular Identification (PCR-Delta and PCR-RFLP-ITS) of the yeast from Black muscat grape cultived in El malah (Wilaya of Ain Temouchent, Algeria)

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ABSTRACT

The grape is an exemplary product of microbial diversity, it is considered as home to many wild microorganisms (yeast). In this context, we explored in this study, the divergence of the indigenous yeast flora in the vineyards of the region El Malah (Wilaya of Ain Temouchent, Algeria) by collecting varietal grape samples (Black Muscat). A high molecular diversity of the flora has been demonstrated, using two molecular techniques, where one is interested in the study of variability of rDNA, more precisely the region 5,8S rRNA ITS1-ITS2 by PCR-RFLP-ITS and the region Delta by PCR-Delta is an additional study which helped sort the yeast Saccharomyces and non Saccharomyces.So 6 yeast species belonging to the 11 studied at 4 different types were characterized according to their molecular profiles. Thus the strains studied were characterized by two restriction enzymes (HinfI and HaeIII). Meanwhile, microscopic and macroscopic studies of classical identification of yeast were able to strengthen these results. Among the yeast species identified: *Torulaspora delbrueckii*, *Torulaspora pretoriensis*, *Candida solani*, *Candida pseudointermedia* and *Saccharomyces cerevisiae*. This variety is an excellent tank of non Saccharomyces yeasts as evidenced by the results.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.

KEYWORDS: Grape, Yeast, Black Muscat, PCR-Delta, PC-RFLP-ITS, HinfI, HaeIII.

INTRODUCTION

Yeasts are naturally present on soils, plant surfaces, especially grape berries, or in wine-growing areas [3,23]. Their dissemination is assured by wind, insects and humans through its various interventions on the environment [1,13].The yeast species present on the surface of the grape berries are significantly limited in number. The density of the yeasts evolves during the maturation of the grapes. The evolution of yeast populations may be related to the increase in the area of the bay and the availability of nutrients: during the maturation of the bunch, the berries grow, the nutrient content Surface area increases, sugar concentration increases and acidity decreases [4,9].The representation of the different yeasts varies during the development of the grape berry according to several parameters: grape variety, climatic conditions, soil, wine practices, age of vines, health status of the cluster and the degree of maturity of the grape [8,10,33].

Black Muscat is a black table grape varietal. It is characterized by beautiful black cohosh, juicy flesh, staining grain is sometimes clear. This variety is fairly resistant to drought and likes in sunny areas. The grape

whether table or tank is the habitat of many wild microorganisms since it is considered the kingdom of yeasts. However, the high sugar content of the Muscat grape must (300 g / l) promotes the growth of yeast, these microorganisms play an essential role in the food industry. They participate in the development of many food products (bread, dairy, brewery) and the production of metabolites (lipids, proteins, enzymes), but also the upgrading of agricultural and industrial waste. However, many yeast species produce a very important primary metabolite namely ethanol, used as fuel or for other industrial purposes [17]. Two strains of bacteria were isolated from the gut of termite in different media. It was observed that these isolates have produced ethanol from rice straw (7.52 ± 0.5 to 9.33 ± 0.4 g/L) followed by corn stove (6.35 ±0.6 to 6.95 ±0.5 g/L) having theoretical yields of ethanol 43.31 % (rice straw) and 39.62 % (corn stove) [16]. The aim of this study is to isolate, characterize microscopically and identify the indigenous species of yeasts found in the grape variety (Black Muscat) grown in the region of El Maleh (Ain Temouchent, Algeria) to develop and express the diversity of the phylogenetic heritage based on the two molecular techniques (PCR-Delta and PCR-RFLP-ITS). Our work is an initiative for the creation of a "Souchier" typical yeast our country Algeria.

MATERIAL AND METHODS

Grape picking Source (Black Muscat):

The samples are taken randomly in the month of September 2014 in the vineyards of the city El Malah, a town located 11 Km from Ain Temouchent. We collected with sterile scissors, about 500 g healthy grapes, and it was collected in sterile bags. A laboratory arrival, they are scuffed and crushed to obtain a mash and ferment for one day at 25 ° C in order to increase the viability and quantityof the desired yeast [30].

Isolation, purification and conservation of cultures:

We conducted a series of seeding by the method streaks on plates of agar cultures (YPG + Gentamicin) which aims to have pure cultures. The operation is repeated in each time taking random an isolated colony. Next, the purified strains are placed in a glycerol solution (sterile) with approximately 25% and stored in the freezer at -18 ° C.

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 ...) [17].

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) [25].

Molecular identification:
DNA extraction:

The isolation of DNA from biological samples is a crucial step in the process of DNA-based molecular biological assays [27]. Before extracting genomic DNA from strains levuriennes, these have been cultured in the medium (liquid YPG + Gentamicin), then a small amount of culture was taken and centrifuged to separate the cells. After the supernatant was removed and the lysis of the resulting pellet of cells was performed by addition of a volume of 660 µl of SDS-50TE mix (lysis buffer).Then the mixture was incubated at 65°C for 10 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 100 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 two molecular techniques (PCR-Delta and PCR-RFLP-ITS):
PCR-Delta (Delta 12 / Delta 21):

Typing of strains of *Saccharomyces cerevisiae* is performed by PCR using the method developed by [18] with the primers: δ 12 (5'-TCAACAATGGAATCCCAAC-3 ') δ 21 (5'-CATCATTAACACCGTATATGA-3 ').The reaction mixture is summarized in Table 1.

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

Products	Volume (µl)	Volumes (µl)
H2O	1220	1230
Tampon 10X	160	160
dNTP	64	64
Delta 1 or 12	8(40pmol/reaction)	8
Delta 2 or 21	8(40pmol/reaction)	8
Mgcl2	80	80

PCR-RFLP-ITS:

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 [35]. The reaction mixture is summarized in Table 2. Then, in order to identify the different species of yeast, the products of PCR-ITS are treated with two restriction enzymes HinfI and HaeIII [3].

Table 2: 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%)

Purification of a DNA fragment (PCR-Delta +PCR-RFLP-ITS) double-strand is performed by electrophoresis on Agarose gel. The gel is mixed with ethidium bromide and cast hot in the electrophoresis tank. 20 µl contained in each tube PCR, mixed with 5 µl of loading buffer containing bromphenol blue, are deposited on the wells. Fragments of known molecular weight DNA called markers are also deposited: they are used to correlate the migration distance of the DNA fragments to their size (in base pairs). In this study, the number and length of the fragments were compared to the size marker (100 bp). according to (IFV).Then, applying a voltage of 120V / 30 minutes until the migration of the fragments [5].The nucleotide different bands resulting from the electrophoresis are shown on the gel under UV light (254 nm) and photographed with a camera UV.

RESULTS AND DISCUSSION

Isolation and purification by successive subcultures made after collection of yeasts in September 2014 from the Black Muscat grapes grown in the El Maleh area (Wilaya of Ain Temouchent). This allowed us to get a collection of 11 yeast isolates.

Study cultivation of isolated yeasts:

Macroscopic examination of cultures "levuriennes" after incubation at 25 ° C for 4-5 days shows generally well isolated colonies are whitish, cream or sometimes yellowish opaque and irregular outline, they have an intense smell. Microscopic observation has allowed us to identify cell shape of isolated isolates and vegetative reproduction modes. Of the 11 individual isolates, 10 are spherical or cylindrical, elongated or short form and are budding as vegetative reproduction mode. The remaining isolate is characterized by an oval shape and has a monopolar budding, it has a smell of yeast. The uniformity of cells confirms the purification of isolates studied. According to [17], 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-Delta:

This technique allows to distinguish between the species Saccharomyces and Non Saccharomyces our results, the graph (Fig.1) shows that the first 10 isolates of the species Non Saccharomyces since no amplification profile is observed on their columns, so they belong to other species or they will be identified by

PCR-RFLP-ITS. Regarding the last isolate (isolate 11), we see the emergence of three bands on the different molecular size column (300 + 350 + 800 bps), then we can predict that this isolate belongs to the species Saccharomyces. The study of the ecology of Non Saccharomyces yeast known significant growth since the last few years, we focus not only on the distribution of species found on grapes but also their succession during alcoholic fermentation [1,6,20,30,32]. Many of the Non Saccharomyces yeast can colonize the surface of grape berries. Indeed, school supports the idea that although minority, Saccharomyces yeasts from the surface of the grapes, and that their presence or absence exchange of a cluster to another, within the same plot [9,19,28].[21]do consider that grape berry on 1000 carries *Saccharomyces cerevisiae.* However, these same authors found that damaged grapes (naturally in the vineyard) are significant populations with Bay 1 of 4 carriers of *Saccharomyces cerevisiae.* However, according to other authors, *Saccharomyces cerevisiae* is undetectable on grapes [9,28].

In our study, major yeast species were represented by Non Saccharomyces yeast (10 isolates) while only one isolate was identified as Saccharomyces(isolate 11), so our work highlights the predominance of Non-Saccharomyces species or sound samples isolated from the grape must of Muscat black 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 [36,12,30]. However, Non-Saccharomyces yeasts are very present, even the majority, in the grape must (Black Muscat) compared to Saccharomyces yeasts.

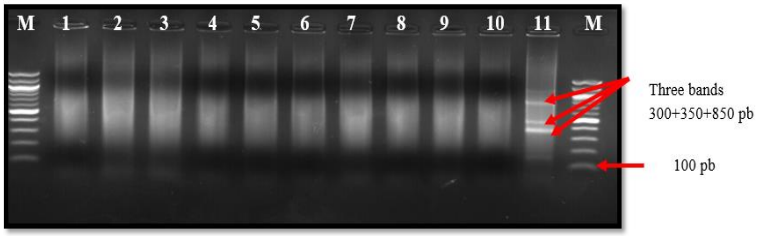

Fig. 1: Profils PCR-Delta of 11 isolates. Lanes M correspond to molecular size standards (100-bp DNA ladder from IFV)

PCR-RFLP-ITS:

A total of 11 isolates of yeasts isolated from the Black Muscat 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 3. The size of the PCR products and restriction fragments of the major species identified in this study are shown in Table 4.

The species of yeasts isolated from the grape (black Muscat):

11 yeast isolates were identified as belonging to 4 genera and 6 different species: *Torulaspora* (05 strains), *Hanseniaspora* (03 strains), *Candida* (02 strains), *Saccharomyces* (01 strain) (Table 3). These different kinds of yeast are well documented in the literature as present on grapes and the start of alcoholic fermentation [36,1].

Table 3: Frequency of yeast species isolated from the grape "Black Muscat"

Isolation source	Species	Frequency of isolation(Number of strains)
Black Muscat grape variety	*Torulaspora delbrueckii*	04
	Hanseniaspora uvarum	03
	Candida solani	01
	Candida pseudointermedia	01
	Torulaspora pretoriensis	01
	Saccharomyces cerevisiae	01

Table 4: 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
Torulaspora delbrueckii (Strains 1, 2, 3,4)	800	410+380	800
Hanseniaspora uvarum (Strains 5, 6,7)	770	350+190+160	750
Candida solani (Souche 8)	600	280+300	400
Candida pseudointermedia (Strain 9)	400	200+110	400
Torulaspora pretoriensis (Strain 10)	800	380+200+190	800

The review of the results indicates that 5 selected yeast strains (strain 1, 2, 3, 4.10) belong to the genus *Torulaspora* (Table 4), according to the determination of IFV guide (2012), while 4 strains were identified as belonging to the species *Torulaspora delbrueckii* and the remaining strain (strain 10) belongs to the species *Torulaspora pretoriensis.* The molecular profile of three yeast strains (strain 5, 6, 7) indicates that they look like the species *Hanseniaspora uvarum*, according to the determination of IFV guide (2012). However, the molecular characterization and micro-macroscopic characteristics show that the strains (8) and (9) belonging to the species *Candida solani* and *Candida pseudointermedia* respectively. 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 [11](Fig 2, 3, 4). 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 [31,12,26,30,22]. 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 stages pre fermentative [36].

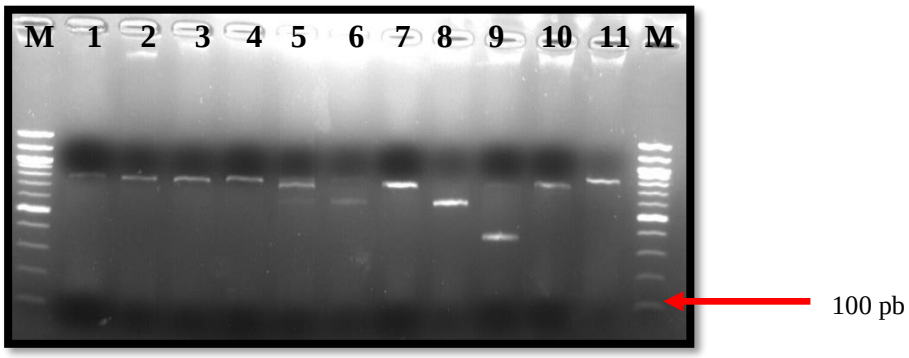

Fig. 2: Viewing the amplified region (rRNA 5,8S ITSI-ITSII) in 11 isolates

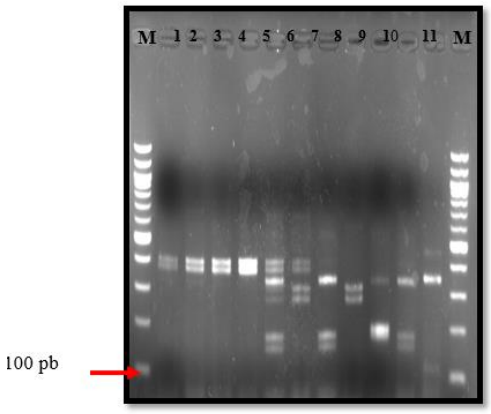

Fig. 3: Viewing the region (rRNA 5, 8S ITSI-ITSII) digested by HinfI in 11 isolates

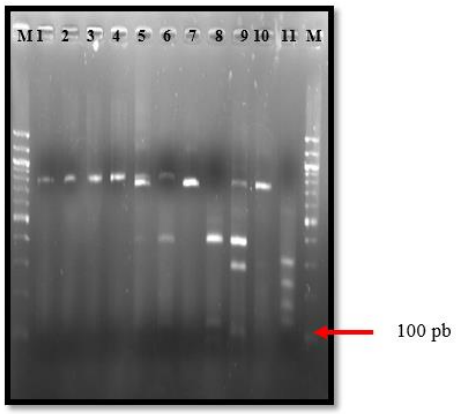

Fig. 4: Viewing the region (rRNA 5, 8S ITSI-ITSII) digested by HaeIII in 11 isolates

In our study, the grape must (Black Muscat) is an excellent reservoir of Non-Saccharomycesyeasts as evidenced by the results obtained, we met different fermentative species such as *T. delbrueckii* 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[6,7,15] and for the purity its fermentation with especially low production of volatile acidity, acetaldehyde, diacetyl and acetoin[2,24,34]. This yeast is also described as cryophile and osmotolerant [14].Among the Non-Saccharomyces yeasts isolated from our grape must, we have the species *Hanseniaspora uvarum, Candida solani* and *Candia pseudointermedia*. Indeed yeast fermentation activity low, mainly as the species of the genus *Hanseniaspora* and to a lesser degree the genera *Candida* predominate on grapes and in the early stages of fermentation. These results are in agreement with the literature. According to[2,30,1], 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 (*Metschnikowia, Hanseniaspora, Candida , Pichia*). This study highlights the variability of species in each genus of yeast especially in *Torulaspora* genres (*T.delbrueckii, T. pretoriensis*) and *Candida* (*C.Solani, C.psendointermedia*). However, only one species of *Saccharomyces cerevisiae* was isolated from the must. This is the main agent of fermentation. Fermentations are initiated by the growth of various species of Candida, Hanseniaspora, Metschnikowia, Pichia, 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 [11].

Conclusion:

This study is based on the evaluation of a grape variety grown in Algeria (Black Muscat) .A genetic approach has been developed in this work and has achieved an identification of 11 isolates belonging to 4 genera and 6 different species: *Torulaspora*, *Hanseniaspora*,*Candida* and *Saccharomyces*. This variety is an excellent tank of Non Saccharomyces yeasts as evidenced by the results. 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.

However, yeasts open up avenues of research for the future. Constantly improved, production systems make it possible to envisage the production of very different proteins, for the human or veterinary pharmacy. 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] Bokulich, N.A. *et al.*, 2013. Monitoring Seasonal Changes in Winery-Resident Microbiota. PLoS ONE, 8 (6): e66437.
[4] Cadez, N. *et al.*, 2010. The effect of fungicides on yeast communities associated with grape berries. FEMS Yeast Research, 10(5): 619-630.
[5] Chantal, G., 1996. Caractérisation génétique des régions ITS des *phytophthora* spp qui causent le pourridié des racines du framboisier au québec.
[6] 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.
[7] 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.
[8] Clavijo, A. *et al.*, 2010. Diversity of Saccharomyces and non-Saccharomyces yeasts in three red grape varieties cultured in the Serranía de Ronda (Spain) vine-growing region. International Journal of Food Microbiology, 143(3): 241-245.
[9] Combina, M., 2005. Yeasts associated to Malbec grape berries from Mendoza, Argentina. Journal of Applied Microbiology, 98(5): 1055-1061.
[10] Cordero-Bueso, G. *et al.*, 2011. Influence of the farming system and vine variety on yeast communities associated with grape berries. International Journal of Food Microbiology, 145(1): 132-139.
[11] 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.
[12] Fleet, G.H., 2008. Wine yeasts for the future. FEMS Yeast Research, 8: 979-995.

[13] Ganter, P.F., 2006. Yeast and Invertebrate Associations. In D. G. Péter & P. C. Rosa (Éd.), Biodiversity and Ecophysiology of Yeasts, 303-370. Springer Berlin Heidelberg.
[14] 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.
[15] 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 fermentedwithout sulfur dioxide. American Journal of Enology and Viticulture. 41: 313-318.
[16] Iram batool *et al*, 2016. Ethanol production from agricultural residues by simultaneous saccharification and fermentation process (SSF) by using termites and *Saccharomyces cerevisiae.* Adv. Environ. Biol., 10(7) July 2016, Pages: 107-115.
[17] Larpent, J.P., 1991. Biotechnologie des levures. Ed. Masson. Paris, p: 426.
[18] Legras, J-L.,F. Karst, 2003. Optimisation of interdelta analysis for *Saccharomyces cerevisiae* strain characterisation. FEMS Microbiology Letters, 221: 249-255.
[19] Martini, A., 1993. Origin and domestication of the wine yeast *Saccharomyces cerevisiae*. Journal of Wine Research, 4(3): 165-176.
[20] Mora, J. andA. Mulet, 1991. Effects of Some Treatments of Grape Juice on the Population and Growth of Yeast Species During Fermentation. American Journal of Enology and Viticulture, 42(2): 133-136.
[21] Mortimer, R. andM. Polsinelli, 1999. On the origins of wine yeast. Research in Microbiology, 150(3): 199-204.
[22] 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.
[23] Ocón, E. *et al.*, 2010. Quantitative and qualitative analysis of non-Saccharomyces yeasts in spontaneous alcoholic fermentations. European Food Research and Technology, 230(6): 885-891.
[24] 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.
[25] Pol., D., 1996. Travaux pratiques de biologie des levures. Edition marketing, 158: 21-56.
[26] 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.
[27] Raghad J. Fayyad and Ahmed S. Dwaish., 2016. New modified protocol of DNA extraction Comparison with other extraction method for polymerase chain reaction analysis of gnomic DNA from Cyanophyceae isolates. Adv. Environ. Biol., 10(9) September 2016, Pages: 77-82.
[28] 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.
[29] 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.
[30] Renouf, V., 2006. Description et caractérisation de la diversité microbienne durant l'élaboration du vin : Interactions et équilibres – Relation avec la qualité du Vin. *Thèse* de doctorat, Faculté d'oenologie, Université Bordeaux 2.
[31] 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.
[32] Setati, M.E., 2012.The vineyard yeast microbiome, a mixed model microbial map. PloS One, 7(12): e52609.
[33] Valero, E. *et al.*, 2007. Biodiversity of Saccharomyces yeast strains from grape berries of wine-producing areas using starter commercial yeasts. FEMS Yeast Research, 7(2): 317-329.
[34] 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.
[35] 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.
[36] 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.