



**MICROBIOLOGICAL, BIOCHEMICAL AND MOLECULAR  
IDENTIFICATION (PCR-RFLP-ITS) OF THE YEAST FROM  
SULTANA GRAPE CULTIVATED IN AIN MERANE  
(WILAYA OF CHLEF)**

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### Abstract:

Grapes defined as the fruit of the vine, the most cultivated in the world. It is considered a major habitat of many yeast species. This work aims to isolate, purify and characterize species of yeast from the sultana grape (*Thompson's seedless*) cultivated in the region of Ain Merane (from Chlef, Algeria). Several tests were performed (microbiological and molecular). Two strains isolated from grape juice obtained by simple grinding. *Pichia fermentans* and *Metschnikowia pulcherrima* were confirmed by a genetic approach (ITS-RFLP) using two restriction enzymes (Taq I, and HaeIII), of the region (ITS1, 5.8S rRNA, ITS2) amplified by PCR.

**Keywords:** Grape, Sultana (*Thompson's seedless*), Taq I, HaeIII, PCR, ITS rDNA.

### 1. Introduction :

Sultana (*Thompson Seedless*) plays a leading role as grape drying. It is characterized by the practical absence of seeds, rich in sucrose, ease of drying, and it is grown in regions marked in winter and dry summer (Levadoux *et al.*, 1971). Its considerable economic importance is at the level of fruit production. Its large sugar content promotes the growth of yeast, which is considered animal food. The producing yeast extracts was used as nutrients or flavoring agents in food. In addition, most recently yeast is used as probiotics. It is since ancient times, at the base of the bread and manufacture of alcoholic beverages. In addition, today it continues to be indispensable for these industrial activities (Larpent, 1991). This may be added the production of biofuels such as ethanol. It is in this context that fits the purpose of this study, in the laboratory of Algeria. At the end of installing a clean bank yeast species in our country, bringing a major interest in the use of the grapes.

### 2. Material and methods:

#### *Harvest grape fruit (Sultana)*

The samples were taken 14 March 2014, with a winemaker in the town of Ain Merane, located halfway between the capital of the province (Chlef) and Sidi Moussa (45 km). A quantity of 500 g of grape collected in sterile bags was broken and crushed, to get a mash. Then, it was left to ferment for a day at 25°C, which aims to increase the viability and quantity of yeast (Renouf *et al.*, 2006).

#### *Microbiological study*

##### *Study of characters crop:*

The step of isolating and yeast purification was carried out several times, by the method of the quadrants (Denis *et al.*, 2007) on the surface of the medium (YPG + Gentamicin). Then, the strains were maintained on YPG medium inclined at 4 °C for a period not exceeding 15 days (Guiraud, 2003). Identification of cropping and morphological characteristics was determined using Yeast Biotechnology book (Larpent, 1991).

##### *Sporulation test:*

After inoculation of grape juice on the culture medium (YPG + Gentamicin), colonies were picked and plated on a sporulation medium (Fowell + Gentamicin). Then, it was incubated for 3 days at 25 °C. The microscopic observation is used to examine the ascospores and the number and position of ascospores in asci (Didier, 1997).

*Molecular study:*

*DNA extraction:*

A quantity of colonies was inoculated from a biomass push on the middle surface (YPG + chloramphenicol). Then, a centrifugation was performed at a rate of separate cells. The bursting of the cells was realized by adding a volume of 500 µl of lysis buffer based on 30 TE / SDS resulting pellet, followed by incubation at 65 °C for 5 min. A volume of 340 µl of potassium acetate was added to the mixture and it was placed in the refrigerator for 30 min. Then, it comes another centrifugation to remove the lysis buffer. A volume of 750 µl of supernatant was added with an equal volume of isopropanol. A final centrifugation step was performed to precipitate DNA. Then, the pellet from the previous step was rinsed with 120 µl of 95% ethanol, then suspended in 300 µl of TE 1X, located 5 minutes at 65 °C and kept in the refrigerator until use (Renouf *et al.*, 2006).

*The identification technique by PCR-RFLP (ITS1-5.8S rRNA-ITS2):*

An amplification step was performed at the ITS1-5.8S-ITS2 sequence located on the ITS region of ribosomal DNA. The latter has a conserved region in the majority of yeast species and the variable regions. Described as universal primers (White *et al.*, 1990): ITS1 (5'-TCCGTAGGTGAACCTGCGG-3'), - ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The reaction mixture is summarized in Table 1. Then, the amplified sequence was digested with two restriction enzymes Hae III and Taq I to end strengthens identification.

Purification of a DNA fragment (PCR-RFLP-ITS) double strand was done by electrophoresis on Agarose gel. 18-20 µl of the digested mixture mixed with 5 µl of loading buffer, were deposited on wells (Renouf *et al.*, 2006). Markers were also submitted, they are used to correlate the migration distance of the DNA fragments to their size (in base pair). The migration was performed at 120 volts at a constant voltage for 30 min (Chantal, 1996).

### 3. Results and Discussion:

*Microbiological study:*

Two strains were determined from the maceration of the grape juice. The first was characterized macroscopically by the presence of small colonies of red-orange color, with a rough surface and with a clearance of an intense odor. However, the microscopic observation was able to round cells with a monopolar budding. The second strain showed colonies creamy, white, medium size, smooth and with a clearance of an intense odor. While microscopic observation has given ovoid cells with a unipolar budding.

This allowed us to predict that the first and second strain may belong to the kinds of *Metschnikowia* and *Pichia*, respectively. Our results are consistent with the work of Euzéby (2008) where he recorded the genus *Pichia* gives oval colonies. Note that the kind of *Metschnikowia* could give ovoid colonies, characterized by a red-brown color. Indeed the works of Kwan *et al.*, (2016) were reported the genus *Metschnikowia* from a scraping of the skin of a dermatitis patient was able to provide non-pigmented colonies. Renouf (2006) has

been reported that the identification of colonies from the juice shows at the beginning of alcoholic fermentation: *Hanseniaspora* and *Pichia* dominant with *Candida* species. While Coarer(2008) was mentioned that after settling grapes develop different species of apiculated and oxidative yeasts *Kloeckera*, *Candida* and *Metschnikowia*.

#### ***Test of sporulation:***

In our experience the two strains were submitted to the laboratory tests sporulation. The first strain has given two spores per ascus while the second give two to four spores. According to Ripert (2013), the genus *Pichia fermentans* was characterized by the appearance of one to two spores per ascus quickly released after training. Pitt *et al.*, (1968) have been reported in their works that the species *Metschnikowia pulcherrima* form two distinct spores per ascus. Comparing our results to previous work cited above. They indicate that the two strains may belong to the species *Metschnikowia pulcherrima* and *Pichia fermentans* respectively.

#### ***Molecular identification:***

The DNA of the two strains was subjected to the quality of verification tests and quantity. Actually, we found that the DNA of the first strain showed a size to about 1100pb et 1200 pb for the second strain. Note that the amplification of ITS I-5.8S-ITS II region of rDNA of the two strains could give fragments characterized by a size in the order of 380 bp for the first and 450 bp for the second (Figure 1).

The results are identical to the work of Renouf *et al.* (2006) made on yeast species where he recorded the species *Pichia fermentans* with 450pb size of the amplified ITS region. While *Metschnikowia pulcherrima* was characterized by a size equal to 380 bp. Two enzymes were chosen to digest the amplified ITS fragment of rDNA: TaqI, and Hae II. Digestion results were shown in the figures (2 and 3) illustrated below.

According to the work of Renouf *et al.* (2006), molecular characterization of the first strain showed that yeast belonging to the genus *Metschnikowia pulcherrima* while the second was *Pichia fermentans*. The use of restriction enzymes on a PCR fragment was used to achieve the main purpose (differentiate a species) from another or a group from another having RFLP polymorphisms (Gardes *et al.*, 1991; Henson *et al.*, 1993; Mugnier and Grosjean, 1995).

#### **4. Conclusion:**

This study based on the evaluation of a grape grown in Algeria (Sultana) showed that this variety is home to two major identified yeast *Pichia fermentans* and *Metschnikowia pulcherrima* by several tests. In view of this work, we plan to study several different varieties of Algerian regions. To better enrich our own DNA bank for the pharmaceutical and food purposes.

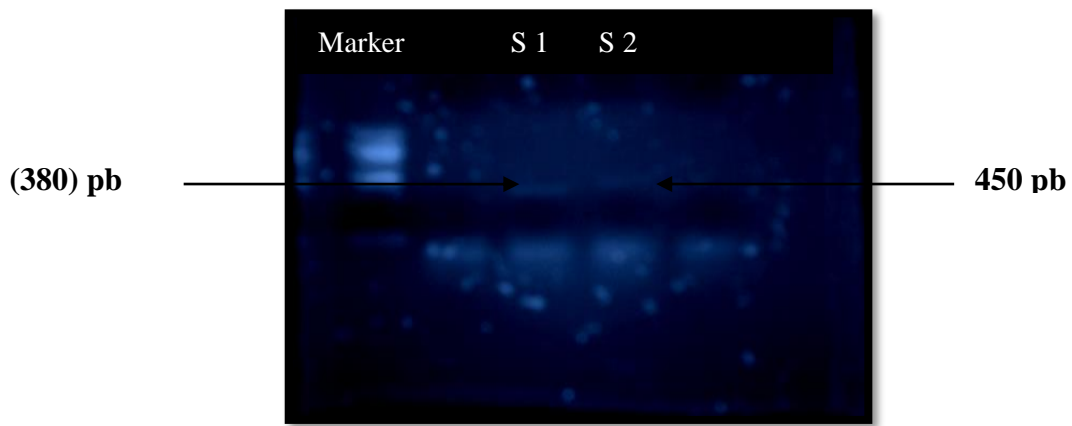
#### **References:**

1. Levadoux, L. *et al.*, 1971. Cépages de cuve et de table cultivés en Algérie. Ed. Société nationale. Algérie, 110 p.
2. Larpent, J. P. 1991. Biotechnologie des levures. Ed. Masson. Paris, p 426.
3. Renouf, V., Miot-Sertier, C., Strehaiano, P., Lonvaud-Funel, A. 2006. Le consortium microbien du vin : une réelle caractéristique du terroir.
4. Denis *et al.*, 2007. Bactériologie médicale (Techniques usuelles). Ed. Masson. Paris, 20p.
5. Guiraud, J.P. 2003. Microbiologie alimentaire. Ed. DUNOD. Paris. PP : 651-655.

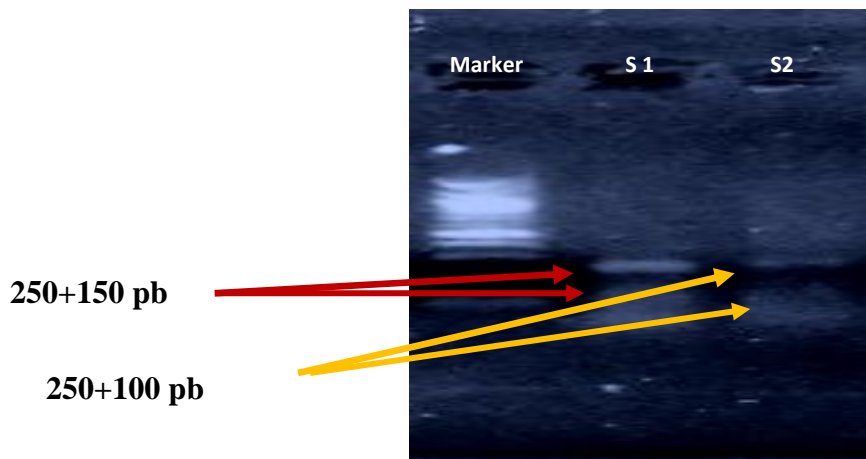
6. Didier, P. 1997. Le livre des travaux pratiques de biologie des levures. ED. Aubin. P 83-84, 147-149.
7. Chantal, G. 1996. Caractérisation génétique des régions ITS des *phytophthora* spp. qui causent le pourridié des racines du framboisier au Québec.
8. Euzéby, J. 2008. Grand dictionnaire illustré de parasitologie médicale et vétérinaire. Ed. Lavoisier. P 115.
9. Kuan, CS., Ismail, R., Kwan, Z., Yew, SM., Yeo, SK., Chan, CL., Toh, YF. 2016. Isolation and Characterization of an Atypical *Metschnikowia* sp. Strain from the Skin Scraping of a Dermatitis Patient. 10.1371/journal.
10. Coarer, M. 2008. Flores et fermentations spontanées. 10p
11. Ripert, C. 2013. Mycologie médicale. Ed. Lavoisier. P229.
12. Pitt, J.I., and Miller, M.W. 1968. Sporulation in *Candida pulcherrima*, *Candida Reukaufii* and *Chlamydozoma* Species: Their Relationships with *Metschnikowia*. Vol. 60. No.3. pp. 663-685
13. White, T. J., Bruns, T., Lee, S. et Taylor, J. 1990. Analysis of phylogenetic relationships by amplification and direct sequencing of ribosomal RNA genes tiré de PCR Protocols: A guide to Methods and Applications.
14. Gardes, M., White, T. J., Fortin, J. A., Bruns, T. D., Taylor, J. W. 1991. Identification of indigenous and introduced symbiotic fungi in ectomycorrhizae by amplification of nuclear and mitochondrial ribosomal DNA. Can. J. Bot. 69:180-190.
15. Henson, J. M. et French, R. 1993. The polymerase chain reaction and plant disease diagnosis. Ann. Rev. Phytopathoi. 31:81-109.
16. Mugnier, J., Grosjean, M. C. 1995. PCR catalogue: In plant pathology, *Pythium*. Lyon. France. 82 p.

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

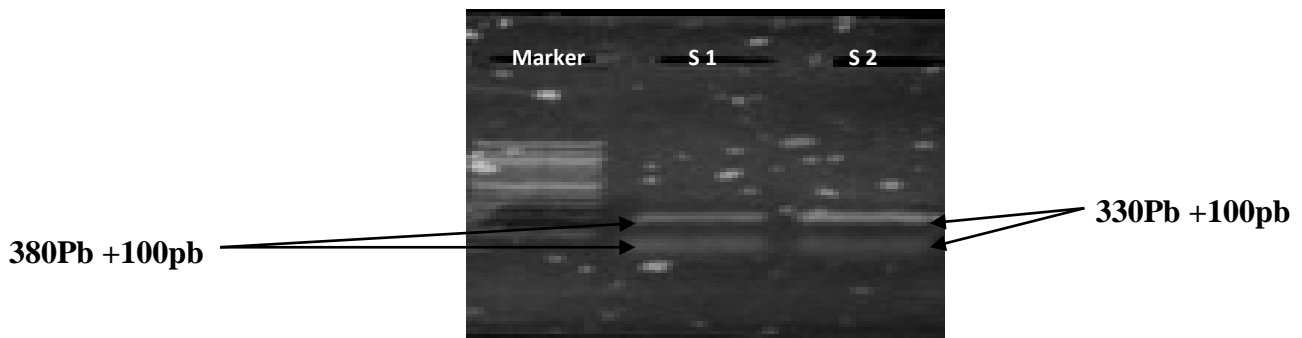
Product	Volume (µl)
H <sub>2</sub> O	915
10X buffer	120
dNTP	48
ITS1	9,6
ITS4	60
MgCl <sub>2</sub>	60
DMSO (2%)	28
DNA amplified	3
Taq DNA Pol	18



**Figure 1:** Viewing the amplified region (rRNA-ITS1-5.8S -ITS1)



**Figure 2:** Viewing the region (5.8S rRNA-ITS1-ITS1) digested by TaqI



**Figure 3:** Viewing the region (5.8S rRNA-ITS1-ITS1) digested by HaeII.