MOLECULAR IDENTIFICATION OF *SACCHAROMYCES CEREVISIAE* STRAINS IN WINE

IDENTIFICAREA MOLECULARĂ A SUŞELOR DE *SACCHAROMYCES CEREVISIAE* DIN VINURI

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Abstract: *The present study analyses the use of PCR method in determining yeast strains which lead to wine alcoholic fermentation. Determination of the strains is important in the vinification process by improving the alcoholic degree, emphasising varietal aromas, colour fixation, acidity maintaining. Strains selection was made by extracting samples from 4 fermentation tanks (Brestnik and Caedinie regions in southern Bulgaria) at three definite times (beginning, middle and end of fermentation). Molecular identification was realised according to the working protocol used by the researchers within the Vine and Wine Institute „Jules Guyot" Dijon- France. Extraction of the DNA from the yeast cells, its fragmentation and multiplication was made by PCR method while the emphasis of the specific DNA fragments in UV was obtained by using the UV transilluminator. The obtained results show a number of common yeast strains, strains which are responsible of the wines' tipicity from the two viticultural areas.*

Rezumat. *Studiul de fata evidentiaza aplicativitatea metodei PCR-ului (polymerase chain reaction) in evidentierea suselor de levuri ce determina si sustin procesul de fermentatie alcoolica a mustului. Cunoasterea acestor suse intereseaza in procesul de vinificatie prin contributiile pe care le pot aduce la imbunatatirea randamentului alcoolic, evidentierea aromelor varietale, fixarea culorii, mentinerea aciditatii, etc. Selectia suselor s-a facut prin prelevarea probelor din 4 vase de fermentatie (din 2 centre viticole Brestnik si Caedinie din zona viticola din Sudul Bulgariei) in trei momente ale fermentatiei alcoolice (inceputul fermentatiei, fermentatia propriu-zisa si finalul fermentatiei). Identificarea moleculara s-a realizat conform protocolului recomandat de cercetatorii din cadrul Institutului Viei si Vinului „Jules Guyot" Dijon- Franta. Ea consta in extragerea ADN-ului din celulele levuriene, fragmentarea si multiplicarea acestuia (prin PCR) si evidentierea fragmentelor specifice de ADN in mediu UV (transiluminatorul UV). Rezultatele obtinute evidentiaza un numar de suse de Saccharomyces cerevisiae comune celor doua centre viticole, suse ce dau specificitate vinurilor din cele doua centre viticole.*

The modern methods of yeast identification show a complex approach that includes modern techniques of microbial genetic, molecular biology and immunology. The taxonomy of different *Saccharomyces* yeast species develops at the same time with the development of the new techniques of microbial physiology or those of genetics. A complex research about the evolution of yeast taxonomy principles have been made by Ribereau- Gayon et al. (1998) and Kurtzman

(1998). Using this classification and the results of his own research Grossman M.K (1999) presents a study concerning the yeasts identification methods in general and of those from *Saccharomyces* species in particular.

The nucleic acid research, the hereditary holders of living beings, can determine the genetic relationship of yeasts species by the determination of the basic DNA segments (method known as DNA recombination). The obtaining of the DNA fragments by pulse field electrophoreses made possible the construction of the genetic map for eukaryotes. Thus appeared the electrophoretic karyotype using the pulse field electrophoreses, we can have direct dates on the *Saccharomyces cerevisiae* and *Candida albicans* karyotypes.

The purpose of this study is to identify the common strains of *Saccharomyces cerevisiae* yeasts, from two different viticulture centres from Bulgaria (Saedinie şi Bresnik), which are specific to Bulgarian Merlot wine, yeasts that could be selected for an industrial and commercial purpose.

MATERIAL AND METHOD

The molecular identification was made by prelevation of the must sample from 4 fermentation tanks (two for each enterprise) in three different moment of A. F. (alcoholic fermentation; A, B, C). We were especially interested in the strains that maintain and finish the A.F. that's why we did not use the strains from the beginning of the A.F. The drawing method of the yeast from the must sample uses the sterile filtration of the untreated must followed by the micro-organisms' incubation on a solid specific culture medium.

The identification process of the strains was made after the extraction of the DNA from the levurian cells. The steps of the molecular identification process are:

1. The DNA extraction and purification.

The cell membrane' breaking is done by a succession of physicochemical treatments and the DNA is liberated. The SDS detergent, the ice and the vortex helps in membrane breaking. The phenol/clorophorm/izoamilic alcohol solution eliminates the proteins and the unsolvable nucleic acids. Centrifugation permits the separation of the aqueous phase, a solution of nucleic acids, from the organic phase from the Ependorf vessel's bottom (phenol + lipids) and the interface between those is represented by the precipitate proteins.

The purified DNA is precipitated with alcohol 100%.

2. The DNA amplification by PCR

The PCR by Kary Mullis in 1985, is a technique of genetic amplification which permits the obtaining of important quantities of DNA's specific fragments, using a complex but quantitatively reduced sample. The PCR principle is repetitively use of a DNA polymerase properties of synthesize a complementary DNA chain from a primer. It's about a subsection of replication reaction of a double DNA matrix chain. Each reaction sets into action 2 primer oligonucleotidics whose 3' extremities reaching towards the other. The primers define the fragment for multiplication, bordering it. The multiplication grade will be of million copies in few hours, which is in general sufficient for the next step.

The key of the procces is using the products of each steps as a matrix for the next one, and reseparating them for reusing the original matrixs. The obtained amplification is exponential. A PCR reaction coresponds to 30 succesives cicles, with their 3 steps:

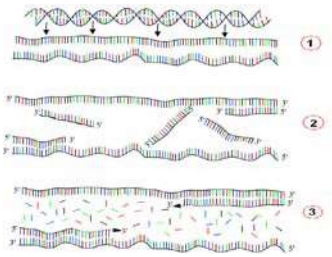

Fig. 1. The steps of the DNA chain multiplication procces through PCR method
1. Denaturation; 2. Hybridation; 3. Elongation

All the necessary elements for the reaction are introduced in an Ependorf tube that will be submitted to different temperatures, corresponding to each step; these temperatures cycles are automatically controlled by the thermocycler.

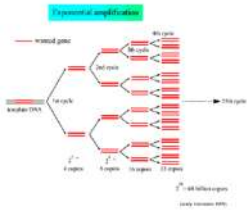

Fig. 2. Exponential amplification of a single chain of DNA

3. The electrophoresis of PCR products in an agarose gel electrophoresis (1, 5 concentration)

The electrophoresis is a technique of DNA separation fragments, based on the migration of the DNA fragments in an electric field. In a basic medium the DNA is negatively charged, but when submitted to an electric field it will move away to the positive pole; having the same electrical charge is its molecular weight that differentiates the speed of migration through the electrophoresis gel.

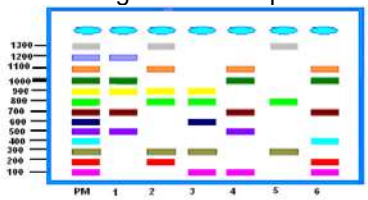

Fig. 3. The DNA fragments migration from the negative pole to the positive one

4. The profiles reading is made by the UV transilluminator

The last step is reading and comparing the strains profiles, and identification of common profiles

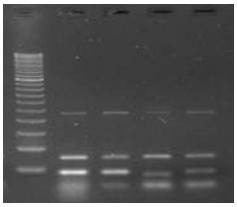

Fig. 4. The evidentiation of common strains of *Saccharomyces cerevisiae* profile

After the comparing of the profiles obtained, 8 common profiles were observed: A2, B2, C2, A3, C3, D4, E5, and A6. The others profiles are found only once.

RESULTS AND DISCUSSIONS

The results obtained by using the molecular method of yeasts strains identification, for *Saccharomyces cerevisiae* yeasts from the two viticulture centres Bresnik and Saedinie (Bulgaria), are presented in the tables 1 and 2.

Table. no. 1

No.	Saedinie enterprise / The A.F. tank / Year / Selected strain number	The A.F. stage	Profile
1	С I 2041	B	A3
2	С I 2043	B	B3
3	С I 2052	B	A2
4	С I 2055	B	A6
5	С I 2101	C	D3
6	С I 2130	C	B4
7	С I 2109	C	B2
8	С I 2120	C	G3
9	С I 2112	C	B3
10	С I 2123	C	F5
11	С I 2124	C	F5
12	С I 2164	C	L3
13	С II 2060	B	A6
14	С II 2062	B	B5
15	С II 2069	B	A8
16	С II 2070	B	B8
17	С II 2130	C	E3
18	С II 2133	C	D4
19	С II 2139	C	E4
20	С II 2140	C	D2
21	С II 2144	C	D4
22	С II 2145	C	H3
23	С II 2148	C	G5
24	С II 2044	C	G4
25	С II 2126	C	H4

Table no. 2

No.	Bresnik enterprise / The A.F. tank / Year / Selected strain number	The A.F. stage	Profile
26	Б I 2075	B	C5
27	Б I 2080	B	B2
28	Б I 2081	B	A4
29	Б I 2082	B	C3
30	Б I 2155	C	F3
31	Б I 2158	C	C2
32	Б I 2160	C	E5
33	Б I 2161	C	H5
34	Б I 2166	C	A2
35	Б I 2170	C	I3
36	Б I 2172	C	E2
37	Б I 2173	C	C2
38	Б II 2093	B	A3
39	Б II 2090	B	D5
40	Б II 2097	B	D3
41	Б II 2100	B	E5
42	Б II 2177	C	J3
43	Б II 2178	C	B2
44	Б II 2180	C	B3
45	Б II 2185	C	F4
46	Б II 2188	C	B3
47	Б II 2190	C	K3
48	Б II 2194	C	B3
49	Б II 2200	C	B2

From the initial 100 yeast strains which have been rigorously selected, only 49 remained by the end of the research; 21 strains have common profiles (8) after the

molecular identification. From these ones only 4 have the attributes required to be selected for preservation and lyophilization, as we can see in the table number 3.

The B2 and B3 profiles fulfil, but not completely, the conditions for selection for an eventual next step, because they have been found in the B moment of the A.F. but also at the end of the A.F. which proved that they have an important role in maintaining and ending of the spontaneous A.F. of the Bulgarian Merlot wine.

Another important characteristic is that B2 and B3 profiles were found in both enterprises' fermentation tanks (even if not in all four tanks). So we can conclude that somehow they are specific strains for that viticulture area, contributing at the preservation of the specific character of the Bulgarian wines; the B2 profile was found in both tanks from Bresnik enterprise and in both A.F. moments, but also in the first tank of Saedinie enterprise. The B3 profile was found only in the first tank from Saedinie winery, but in both moments of A. F. (B and C) and in the Bresnik firm only in the second tank in the final moment of the A. F.;

- the E5 profile performs only half of the required conditions; it was found in the two A. F moments but only in Bresnik enterprises tanks.

The other profiles have different characteristics as follows:

- the A3 profile is specific only for the B moment of the A. F. and only for one tank from each enterprise (C I and Б II);

- the C2 profile was found only in the first fermentation tank from Bresnik winery and only at the end of the A. F. which means that this strain can end the A. F. but it is not specific for this area, that's why it won't be selected for the step;

- the D4 profile is found only in the II tank of Saedinie enterprise and only in the C moment of A. F. (C II C);

- the A6 profile was found in both A. F. tanks from Saedinie firm but only in the B moment of the A. F. ;

- the A2 profile is a special case because it was found in both moments of the A. F. fermentation and in both enterprises but in a special distribution: for Saedinie enterprise only at the B moments of A. F of the first tank (C I B) and for Bresnik enterprise only at the C moment of A. F. of the first tank (Б I C).

The next table presents the 8 common strains distributed in the 4 tanks of the two Bulgarian enterprises:

Table 3

PROFILE	Strain yeasts number	Enterprise and A. F.'s tank	A. F.'s moment
A2	30	C I	B
	62	Б I	C
B2	37	Б I	B
	49	C I	C
	67	Б II	C
	74	Б II	C

PROFILE	Strain yeasts number	Enterprise and A. F.'s tank	A. F.'s moment
C2	47	Б I	C
	65	Б I	C
A3	28	C I	B
	40	Б II	B
B3	29	C I	B
	51	C I	C

CONCLUSIONS

The result of this *Saccharomyces cerevisiae* yeast strains study from the four A. F. tanks of the two Bulgarian enterprises (Saedinie and Bresnik) shows a considerable instable distribution of the strains during the A. F. process, none of these strains being found in all the 4 tanks from the two geographically close Bulgarian viticulture enterprises.

From the initial 100 strains which have been rigorously selected, only 49 remain by the end of the research; 21 strains have common profiles (8) after the molecular identification (with the profiles A2, B2, C2, A3, C3, D4, E5, A6). From these ones, only 4 have the characteristics to be selected for preservation, industrialisation / lyophilization and commercialisation.

The PCR method used according to the protocol recommended by the scientists from the Vineyard and Wine Universitary Institute of „Jules Guyot", Dijon-France, proved to be sufficiently sever in the differentiation of the yeasts strains profiles, this fact recommending it for all kinds of analyses in this domain.

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