ENOLOGICAL SUITABILITY OF A YEAST STRAIN INDIGENOUS TO CASTILLA-LA MANCHA FOR RED WINE PRODUCTION

LEVURE DE LA RÉGION CASTILLA-LA MANCHA SÉLECTIONNÉE POUR SES APTITUDES ŒNOLOGIQUES À LA FERMENTATION DU VIN ROUGE

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Abstract: The suitability of an indigenous yeast strain, selected in Castilla-La Mancha by the Instituto de la Vid y el Vino de Castilla-La Mancha (IVICAM), both from a microbiological and an enological point of view wes studied in order to evaluate its potential use as starter of the alcoholic fermentation for red wine production.
Several grape musts of the varieties Cencibel and Garnacha were inoculated with the selected strain (L6) and it was proven that this strain was capable of dominating other yeasts, being thus responsible for the fermentative process. As for the aroma composition, a lower content of volatile phenols was identified in the wines produced by the selected strain, compared to the wine produced by the indigenous strain. Moreover, a panel of tasters positively scored the wines produced by the selected strain. Finally, it was proven that the L6 strain is genetically stable under different stress conditions.

Résumé: L'emploi de levures sélectionnées permet d'homogénéiser la flore initiale et de réussir des vins de qualité homogène et prévisible. La tendance actuelle est d'utiliser comme starter des souches de levures sélectionnées à partir de la flore autochtone avec la conviction qu'une meilleure adaptation des souches au milieu œnologique peut profiter à la qualité du vin et maintenir les particularités sensorielles typiques de chaque région.
Cinq vinifications ont été réalisées en cuves de 100 l, avec contrôle de la température entre 28 et 30 ºC. L'une d'elles a été effectuée à partir de moût de Cencibel fermenté de façon spontanée par les souches indigènes, les quatre autres cuves ont été ensemencées avec la souche sélectionnée L6, deux à partir de moût de Cencibel et les deux autres à partir de moût de Grenache.
Tous les moûts levurés ont présenté une bonne cinétique de fermentation, avec une bonne dégradation des sucres et une production d'acidité volatile modérée et inférieure à celle de la fermentation spontanée. Les différences les plus remarquables entre les vins Cencibel et ceux de Grenache, en termes physico-chimiques, sont dues à la réalisation de la fermentation malolactique dans les premiers.
Dans les vins ensemencés avec la souche L6, les profils PCR obtenus à partir de la biomase levurienne récoltée par centrifugation à la moitié de la fermentation alcoolique, ont été comparés au profil témoin de la souche L6. On a constaté que cette dernière s'est implantée dans tous les cas, ce qui fait donc d'elle la responsable du processus de fermentation.
La composition aromatique des vins a été déterminée par chromatographic en phase gazeuse. Des composés déterminés caractéristiques des moûts apparaissent en concentrations similaires dans tous les vins étudiés, quel que soit le cépage étudié. En revanche, certains composés variétaux apparaissent en proportion variable selon le cépage. Les teneurs en esters éthyliques sont semblables dans les vins fermentés par la souche L6 et par les souches indigènes, les premiers présentant cependant des teneurs en acétates moindres que celui fermenté de manière spontanée. Par ailleurs, on observe que les vins fermentés par la souche L6 présentent une teneur moindre en phénols volatils, 4-vinylguaïacol et 4-vinylphénol ; ces composés sont, en quantité élevée, indésirables du point de vue aromatique, car ils sont responsables d'arômes phénolés et fumés. Les vins élaborés avec cette souche L6 ont également été évalués positivement par un panel de dégustateurs. Ils présentent une meilleure intensité et une bonne qualité olfactive, ainsi que gustative. Il a été montré que la souche L6 est génétiquement stable grâce à l'évaluation de profils génétiques obtenus par électrophorèse sur gel en champ pulsé (PFGE), après avoir soumis cette souche à différentes conditions de stress : pH bas et élevé, températures basses et élevées, croissance en différents milieux nutritifs : carencés, salins, additionnés de SO_2, d'alcool, et déshydratation et croissance en conditions changeantes. Les résultats obtenus permettent de conclure que la souche de levure sélectionnée L6 est stable pour être produite industriellement comme LSA, et qu'elle présente des aptitudes œnologiques favorables à l'élaboration de vins rouges.

Key words: sensory analysis, PFGE, genetic stability, selected indigenous strains, PCR.

Mot-clés : analyse sensorielle, PFGE, stabilité génétique, souches indigènes sélectionnées, PCR.

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INTRODUCTION

The influence of yeast strains responsible for alcoholic fermentation on the chemical composition and quality of wine is well known (BOULTON *et al.*, 1996). The spontaneous fermentation of must is the result of the combined action of several indigenous yeast strains.

To obtain wine with a homogeneous and predictable quality, it is necessary to use cultures of selected yeast that allow a secure fermentation. The variability identified in the yeast microbiota in a region year after year is controlled by inoculating a large and physiologically active (DELTEIL, 1992) pure yeast culture, previously selected to ensure its predominance over the indigenous flora and to control the fermentation, thus avoiding any heterogeneity in the different vintages (VEZINHET and PINEAU, 1990). Yeast strains selected from the indigenous flora are often used as starters, as it is believed that a better adaptation to the enological environment can enhance the quality of wine and preserve the sensory features typical of each region.

The use of selected yeasts demands some microbiological controls. On the one hand, it is necessary to monitor the industrial production of all the batches to ensure that the yeast produced is the one originally selected rather than a contaminating one. Furthermore, it would be advisable to check that the inoculated culture is capable of imposing itself during the fermentation and will be the driving force of the process. These two objectives can be achieved by means of molecular biology techniques i.e., Polymerase Chain Reaction (PCR), Pulsed Field Gel Electrophoresis (PFGE).

This work was aimed at evaluating, from both a microbiological and an enological point of view, a yeast strain indigenous to the region of Castilla-La Mancha selected by the IVICAM, in order to evaluate its potential use as starter of the alcoholic fermentation in the local production of red wine.

MATERIALS AND METHODS

I - FERMENTATIONS

Fermentations were carried out in an experimental winery with grape musts of the varieties Cencibel and Garnacha. The musts were sulphited at 5 g/hl and the fermentations were carried out in self-evacuating tanks of 100 l under temperature control until the devatting, without exceeding the limit of 28-30 ºC.

The total number of preparations was five; one must of the variety Cencibel, spontaneously fermented with indigenous strains; two musts of the variety Cencibel and two other musts of the variety Garnacha, all five inoculated with the indigenous strain selected by the IVICAM, called L6 (Colección Española de Cultivos Tipo nº 11.814). The inoculation was carried out from liquid, fully active cultures with an initial population of $1x10^6$ cells/ml.

The wines of the variety Cencibel were subjected to spontaneous malolactic fermentation. The wine was decanted and the free sulphur dioxide was corrected at 25 mg/l. Later, it was clarified with gelatine and bentonite (6 g/hl and 30 g/hl, respectively), filtered by sheets and stabilized at –5 ºC. At the bottling stage, it was again sulphited at 25 mg/l and filtered by sheets with 0.2 micron dimensions.

II - CONTROL OF THE DOMINATION BY THE SELECTED STRAIN

The technique implemented was based on the genetic analysis of the yeast biomass, collected by centrifugation of the isolated samples of wine, inoculated with the selected strain. Two analyses were performed for each preparation, one roughly in the middle and the other at the end of the alcoholic fermentation.

The comparison of the genetic profiles allowed to conclude with high chances of success the domination or not of the selected strain. The analysis was performed by the PCR technique, which involves the amplification of genomic-specific selected sequences (NESS *et al*., 1993).

The result of the implantation analysis is positive if the profile of more than 80 % of the colonies developed from the yeast mass obtained in the fermentation coincides with the profile used as indicator. These analyses were performed in the Laboratory SIGMO (Lallemand - ITV, France).

III - GENETIC STABILITY TESTS

The L6 yeast was subjected to a genetic stability study, to evaluate the genetic profiles obtained by PFGE (CASEY *et al.*, 1989), after subjecting the yeast strain to different stress conditions: low and high pH, low and high temperatures, growth in nutritive medium very poor, growth in saline medium, growth in sulphur dioxide, growth in alcohol, dehydration conditions and growth in changeable conditions. These analyses were also performed in the Laboratory SIGMO (Lallemand, - ITV, France).

IV - PHYSICOCHEMICAL ANALYSIS

The most common physicochemical parameters of the musts were identified: density, pH, total acidity and malic acid. For the wines, the following parameters

were determined: density, pH, total acidity, total and free sulphur dioxide, malic acid, lactic acid, alcohol content, volatile acidity and reducing sugars (EUROPEAN UNION, 1990).

V - VOLATILE COMPOUNDS ANALYSIS

An extract was obtanined by XAD4 amberlite resin treatment and was analysed by gas chromatography with a FID detector following the method described by AZNAR *et al.* (2001).

VI - SENSORY ANALYSIS

The sensory analysis was performed by a panel composed of 15 trained tasters. The scorecard used was a penalizing one, covering all the individual gustative features, with on overall score for each wine sum of individual scores.

RESULTS AND DISCUSSION

The results of the analysis of the physicochemical parameters of the initial musts and corresponding wines are shown in table I. The main differences were due to the different level of maturity of the musts of both varieties. The must of the variety Cencibel had a lower Baumé and pH, and a higher total acidity and malic acid content than the other variety. All the wines produced by the selected strain showed good fermentation kinetics and their volatile acidity was lower than in the indigenous one. The main differences were due to the malolactic fermentation of the wines of the variety Cencibel, in contrast to the wines of the variety Garnacha, which were not subjected to this process. In the wines of the variety Cencibel, the malic acid almost disappeared and was converted into lactic acid. Moreover, as a consequence of this process, a higher volatile acidity, due to the degradation of citric acid by lactic bacteria was observed (DAVIS *et al.*, 1985).

The results of the domination analysis by the PCR technique show that the L6 strain dominated during the alcoholic fermentation, as the profiles obtained from the yeast mass in the middle and at the end of alcoholic fermentation in the four preparations performed, coincided with the profile used as L6 indicator. Figure 1 shows the PCR profile of the L6 strain and the profiles obtained from the yeast mass in the wines of the variety Cencibel, using 100 bp DNA Ladder as an indicator of the molecular weight.

Among the organoleptic features typical of wine, the bouquet is one that plays a major role as far as quality is concerned. The aromatic composition of the wines obtained with the studied yeast are shown in table II. Some compounds typical of the musts had a similar concentration, irrespective of the variety, for instance, *cis*-3-hexenol and the acids on the whole. On the other hand, some varietal compounds, such as 1-hexanol, β-phenyletanol, eugenol and phenylacetic acid, had a higher concentration in the wines of the variety Cencibel. The wines of the variety Garnacha had a higher concentration of benzyl alcohol, β-damascenone, acetovanillone, vanillin and methyl vanillate, ethyl vanillate and α-terpineol. These results confirmed other studies of the composition of the must and

Table I - Analysis of the physicochemical features of must and wine

Analyse des paramètres physico-chimiques du moût et du vin

	LIC	L61C	L62C	L61G	L62G
Must					
Baumé	11.74	11.74	11.74	12.35	12.47
Density	1088	1088	1088	1093	1094
pH	3.21	3.21	3.21	3.41	3.39
Total acidity[1]	7.89	7.89	7.89	4.93	4.83
Malic acid[2]	3 035	3 035	3 035	2 115	2 230
Wine					
pH	3.67	3.56	3.56	3.50	3.48
Total acidity[1]	5.23	5.30	5.59	4.68	4.64
Malic acid[2]	0.211	0.185	0.196	1 688	1 703
Lactic acid[2]	1 590	1 737	1 599	0.020	0.010
Alcoholic content[3]	12.0º	12.5º	12.2º	12.4º	12.4º
Volatile acidity[4]	0.62	0.43	0.50	0.27	0.30
Reducing sugars[2]	5	4.7	4.2	2.0	2.1
Days of fermentation	5	5	5	6	6

L6: selected strain; LI: indigenous strain; C: variety Cencibel; G: variety Garnacha
1 g/L tartaric acid; 2 g/L; 3 % vol.; 4 g/L acetic acid

Table II - Volatile compounds

Composés volatils

Wine	LIC	L61C	L62C	L61G	L62G
Alcohols					
Isobutanol	58.3	52.1	51.6	45.5	48.2
1-butanol	2.55	2.5	2.04	1.46	1.44
Isoamilic alcohol	427.8	413.5	402.9	247.9	275.7
1-hexanol	3.77	3.26	3.45	2.19	2.00
cis-3-hexenol	0.23	0.18	0.24	0.16	0.22
Methionol	2.72	4.1	4.53	1.11	1.11
Benzilic alcohol	0,65	0,56	0,46	1.37	0.99
β-phenylethanol	45.9	66.2	58.5	14.1	15.7
Guaiacol*	1.44	3.55	5.94	4.89	2.51
Furfuryl alcohol*	13.2	12.8	12.9	15.1	12.8
4-vinylguaiacol*	372.5	34.2	12.6	29.9	42.8
4-ethylguaiacol*	2.65	2.58	0	11.2	2.63
m-cresol*	0.43	0.38	0.38	3.58	0.82
Eugenol*	2.41	2.35	1.77	0.58	0.19
4-ethylphenol*	0.17	0.1	0.09	1.03	0.18
2.6-dimethoxyphenol*	0	19.7	12.3	0	6.73
Isoeugenol II*	0	4.01	3.8	0	3.73
4-vinylphenol*	75.4	0	0	5.31	2.92
Total	542.38	542.47	523.76	313.96	345.43
Acids					
Propanoic acid	3.11	1.84	1.95	1.44	1.71
Isobutyric acid	2.19	1.85	1.38	1.36	1.18
Butyric acid	1.64	2.06	1.70	1.57	1.44
Hexanoic acid	3.33	3.20	3.20	3.58	3.87
Octanoic acid	3.89	3.54	3.49	5.57	6.04
Decanoic acid	0.7	0.7	0.69	0.73	0.77
Isovalerianic acid	1.95	1.82	1.39	1.24	1.04
3-methyl butyric acid*	206.6	37.2	162	198.1	286.7
2-methyl butyric acid*	115.2	83.1	108.4	89.3	104.0
Phenyl acetic acid*	14.8	22	10.9	1.74	7.27
Benzoic acid*	29.2	10.2	7.71	8.80	21.2
Total	17.17	15.16	14.08	15.78	16.46
Lactones					
γ-butyrolactone	13.9	13.0	10.6	5.61	4.34
γ-nonalactone*	0	5.33	6.15	15.5	17.0
β-damascenone*	2.31	1.96	2.11	4.91	5.76
β-damascone*	98.5	96.7	104	87.9	108.0
β-ionone*	0.16	0.14	0.15	0	0.10
δ-decalactone*	0	5.17	4.47	0.99	6.97
δ-octalactone*	1.19	2.90	3.35	0	4.35
Total	14.00	13.11	10.72	5.64	4.48

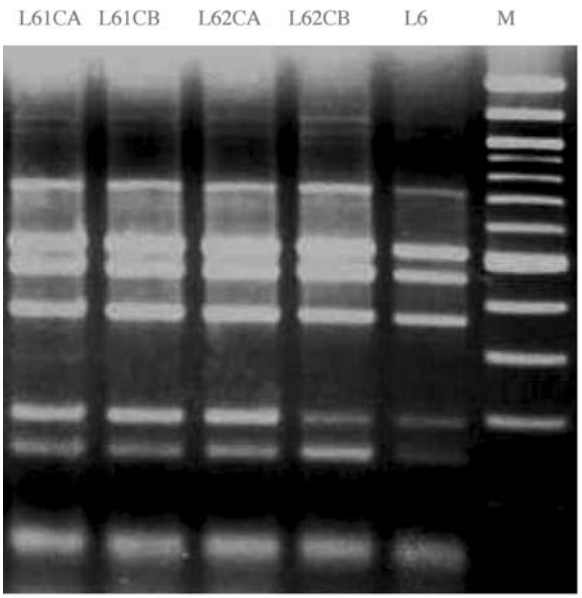
L6: selected strain; LI: indigenous strain; C: variety Cencibel; G: variety Garnacha; Results in mg/L; *µg/L

Table II - Volatile compounds (suite)

Composés volatils

Wine	LIC	L61C	L62C	L61G	L62G
Carbonilic compounds					
Acetaldehyde	53.4	55.2	51.8	63.0	56.0
Diacetyl	8.36	14.2	10	1.91	2.13
3-octanone*	215.7	249.1	186.4	165.1	181.2
Acetoine	16.4	7.8	14.0	2.56	2.75
Total	61.99	69.72	62.00	65.07	58.31
Acetates	2.72	4.1	4.53		
Phenyl ethyl acetate	7	0.07	0.17	0.06	0.10
Isoamyl acetate	2.42	1.56	1.76	3.07	2.35
Hexyl acetate*	27.3	23.1	21.4	33.6	35.9
Butyl acetate*	3.77	4.93	4.49	5.35	4.67
Isobutyl acetate*	44.3	88.3	53.3	53.9	63.5
Total	9 495	1 746	2.00	3.22	2.55
Ethyl and methyl esteres	0.43	0.38	0.38		
Ethyl butyrate	0.27	0.30	0.18	0.31	0.25
Ethyl hexanonate	0.78	0.64	0.53	0.50	0.58
Ethyl lactate	132.0	117.0	103.0	25.0	20.4
Ethyl octanate	0.40	0.39	0.28	0.34	0.48
3-OH-ethyl butyrate	0.60	0.66	0.64	0.57	0.41
Ethyl decanoate	0.46	0.39	0.36	0.31	0.33
Diethyl succinate	11.1	16.8	16.0	0.92	0.72
Ethyl isobutyrate*	39.9	45.2	24.7	16.0	22.9
Ethyl vanillate*	24.7	21.0	24.1	49.1	49.3
Methyl vanillate*	1.84	1.90	3.19	13.8	11.3
Methyl benzoate	0	0.07	0.06	0.14	0.08
Ethyl isovalerate*	3.99	4.89	3.31	2.71	3.42
Ethyl furoate*	1.65	1.77	1.81	0.56	0.73
Ethyl cinamate*	0.46	0.41	0.18	0.13	0.46
Total	145.67	136.25	121.04	28.03	23.25
Terpenes	115.2	83.1	108.4		
α-terpineol*	1.26	1.02	0.96	1.68	1.84
Linalool*	5.38	2.8	2.36	4.17	4.25
β-citronellol*	0	4.05	3.9	4.17	6.30
Total*	6.64	7.87	7.22	10.02	12.39
Other compounds	13.9	13.0	10.6		
Furfural*	1.76	3.02	1.81	2.22	3.54
Acetovanillone*	36.2	22.8	30.4	51.3	50.0
Vanilline*	0	1.03	1.25	2.61	2.19
4-allyl-2,6-dimethoxyphenol*	7.25	3.58	2.84	0	1.14
Total*	45.21	30.43	36.30	56.23	56.87

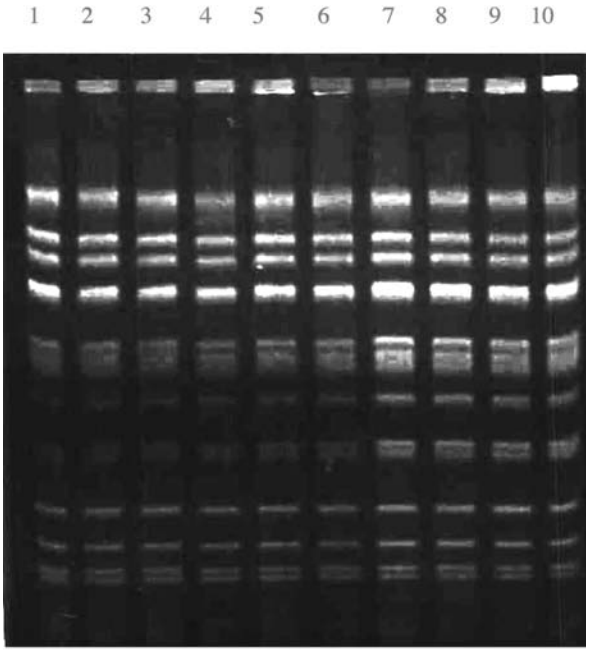
L6: selected strain; LI: indigenous strain; C: variety Cencibe ; G: variety Garnacha; Results in mg/L; *ug/L



L6: selected strain; C: Cencibel variety; 1 and 2: replications; A y B middle and end (of the alcoholic fermentation.; M: 100 bp DNA Ladder indicator.

Fig. 1 - PCR profile of the strain L6 and profiles obtained from the yeast mass in the middle and at the end of the alcoholic fermentation of wines of the variety Cencibel.

Profil PCR de la souche L6 et profils obtenus à partir de la masse levurienne au milieu et en fin de fermentation alcoolique des vins de cépage Cencibel.



1, 2 etc.; stress conditions applied to the L6 strain.

Fig. 2 - Results of the profiles obtained by Pulsed Field Gel Electrophoresis (PFGE) to check the genetic stability of the L6 strain under different stress conditions.

Résultats des profils obtenus par électrophorèse sur gel à champ pulsé (PFGE) pour vérifier la stabilité génétique de la souche L6 sous différentes conditions de stress.

skin of grapes of the varieties Cencibel and Garnacha (CHACÓN *et al.*, 2002).

If the wines are compared, considering the malolactic fermentation of the wines of the variety Cencibel, it is observed that they had a higher content of ethyl lactate, diacetyl (acetoin semi-reduction) and γ-butyrolactone, and a lower content of octanoic acid. These results were in good agreement with the results obtained by other authors in studies of the influence of the malolactic fermentation on the aromatic composition of wine (MAICAS *et al.*, 1999). Diacetyl is associated with the sensory character of butter. This compound, an intermediate product in the degradation of sugars and citric acid by lactic bacteria, can make a positive contribution to the bouquet when present in small amounts and can contribute to the general organoleptic quality (BRIGITTE *et al.*, 1995).

If the wines of the variety Cencibel produced by the indigenous strains were compared to the wine produced with the L6 strain, it was observed that the former had a higher content of isoamyl alcohol and isobutanol. These results coincided with the experiments carried out by ALEIXANDRE *et al.* (2000) to compare productions of selected commercial strains and indigenous strains. At high concentrations, these compounds have a negative impact, as they can impart off-odours.

The ethyl ester content was similar in the wines fermented by the selected strain and the indigenous strains. Besides, these wines had a lower content of acetate esters than the wine fermented by indigenous strains. According to (FERREIRA *et al.*, 1995), wines with a higher content of ethyl esters had a higher aromatic intensity than the wines with a higher proportion of acetate esters.

It was also observed that the wines produced by the selected strain had a lower content of volatile phenol, i.e. 4-vinylguaiacol and 4-vinylphenol than the indigenous strains. In general, the amounts produced were larger in white wine than in red wine (BAUMES *et al.*, 1986). These compounds are formed by the *Saccharomyces cerevisiae* yeasts by enzymatic decarboxylation of the ferulic and coumaric acids (DUBOURDIEU *et al.*, 1990). A high concentration of these compounds is undesirable from an aromatic point of view as they are responsible for the phenolic, pharmaceutical and smoked odours. A high concentration can also be due to contamination by

Table III - Results of the sensory analysis

Résultats de l'analyse sensorielle

Wine	LIC	L61C	L62C	L61G	L62G
Visual stage	1	3	1	4	4
Nose intensity stage	6	8	6	2	6
Nose quality stage	8	12	8	2	6
Taste intensity stage	6	8	2	2	8
Taste quality stage	12	18	3	9	9
Harmony	9	12	3	9	12
Total score	42	61	23	28	45

L6: selected strain; LI: indigenous strain; C: variety Cencibel; G: variety Garnacha

Brettanomyces/Dekkera (CHATONNET *et al.*, 1995) yeast strains.

The results of the sensory analysis are shown in table III. With the exception of the wine L61C, which obtained a higher score than the wine produced with indigenous strains, two of the other wines produced by the selected strain obtained lower scores than the wine produced by indigenous strains, and another one obtained a score similar to that of the wine produced by indigenous strains. The wines produced by the selected strain had a higher aroma and taste intensity and quality.

The wine that obtained the highest score was the wine L6C2, which was indicaled by the tasters as a wine with a very intense, fruity bouquet of very ripe fruits, sloe-gin and banana, and very mature on the palate. As for the wines produced by the L6 strain and the variety Garnacha (L61G and L62G), they were described as floral and fresh wines with a taste of cherry and milk, and light on the polate. The wine produced by indigenous strains had a varnish bouquet, with a taste of small fruits, and dry and rough tannins.

As the results obtained seem to justify that the L6 yeast selected by the IVICAM should be available to the wineries in Castilla-La Mancha, Lallemand has collaborated with the IVICAM to study the genetic stability and the feasibility of its industrial production as an active dry yeast. Figure 2 shows the profiles obtained by PFGE from the L6 yeast after subjecting it to stress conditions, (bands 1 to 10, respectively). This strain has proven to be genetically stable as the 10 profiles obtained by PFGE are identical after being subjected to the stress processes.

CONCLUSIONS

The results contribute to the characterization of an indigenous yeast strain selected by the IVICAM and its potential application as starter of the alcoholic fermentation in the production of red wine from the varieties Cencibel and Garnacha.

The productions where the selected yeast (L6) was used were characterized by good fermentation kinetics and the exhaustion of all the sugars, which resulted in a moderate volatile acidity.

The indigenous yeast selected was capable of domination in all the preparations where it was used as starter, and thus it can be concluded that it was responsible for the alcoholic fermentation.

Gas chromatography proved that it did not form volatile compounds, which were undesirable at high concentrations, and the wine produced was positively scored by a panel of trained tasters.

When the strain was subjected to different stress conditions, it was proven that all the profiles obtained were identical. This supports its genetic stability and the feasibility of its industrial production as an active dry yeast to produce red wine in commercial wineries in the region of Castilla-La Mancha.

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