Effect of Macerating Enzymes on Red Wine Aroma at Laboratory Scale: Exogenous Addition or Expression by Transgenic Wine Yeasts

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The effects of a *Trichoderma longibrachiatum* endoglucanase and an *Aspergillus nidulans* endoxylanase on the concentration of free and bound volatiles, color, and phenolics during maceration in red wine vinification has been studied. Two different approaches have been considered for the utilization of these enzymes: (i) direct addition of the enzymes to must and (ii) inoculation of must with recombinant wine yeasts overexpressing these activities. An experimental design based on a Taguchi orthogonal array was carried out in order to evaluate the effects of the enzymatic treatments. The data show that these fungal activities are able to increase the concentrations of free and glycosidically bound flavor compounds, color, and phenolics to similar or greater extents as compared to a commercial pectolytic enzyme preparation. The effects of the two different ways of addition of the enzymes were not always equivalent. These enzymes could be considered to be of potential application in the red wine maceration process.

Keywords: *Endoglucanase; endoxylanase; maceration; transgenic yeast; aroma precursors; wine*

INTRODUCTION

In wine production, maceration refers to the breakdown of grape solids following grape crushing and is always included in the initial phase of red wine production. The rupture of grape cells and consequent release of enzymes facilitates the liberation and solubilization of those compounds bound to the cells of the skin, flesh, and seeds such as phenolic derivatives (*1, 2*) and the glycosidic precursors of wine aroma (*3*−*5*). Properly conducted maceration can thus enhance wine quality since phenolic compounds (anthocyanins and tannins) are major components of red wine color (*6, 7*) and the release of glycosidic precursors increases wine aroma upon their subsequent hydrolysis by glycosidase activities (*8*−*10*).

The use of macerating enzymes is common practice in the winemaking process. The enzyme preparations used, normally termed "pectinases", contain pectolytic activities (polygalacturonase and pectin-lyase) in addition to hemicellulases (e.g., xylanases), cellulases, and occasionally glycosidase activities (*11, 12*). Traditionally, these preparations are used to increase juice yields by degrading structural polysaccharides that interfere with juice extraction, clarification, and filtration (*13*). In addition to the pectolytic enzymes, the cellulases and xylanases also improve the extraction of phenolic compounds (*14*) and contribute to wine aroma by increasing the amount of flavor precursors in the must.

In our laboratory, we have purified fungal enzymes such as endoglucanases and xylanases (*15*), and analyses of grape macerations conducted in the presence of fungal culture fluids enriched in endoglucanase activities indicate the increased release of glycosidically bound compounds, particularly in the case of the Muscat grape variety (*16*). The genes encoding these enzymes have been cloned, sequenced, and expressed in wine yeast strains (*9, 17*−*19*). The use of recombinant wine yeasts expressing β-(1,4)-endoglucanase (EGL1) and β-(1,4)-endoxylanase (X_{22}) in microvinification experiments resulted in increased levels of some volatile compounds (*9, 10*).

The Bobal grape variety is widespread in the Utiel-Requena region (Spain) and possesses low levels of terpenyl compounds in both free and glycosidically bound forms. In common with similar grape varieties, it also contains other glycosidically bound aroma compounds that produce a characteristic varietal aroma in the finished wine (*20, 21*). Cell wall degrading enzymes may therefore play a useful role in grape varieties of this type.

Two strategies can be adopted to achieve increases in phenolics, color, or volatile precursor content: (i) addition of enzymes directly to the must or (ii) inoculation of must with wine yeasts capable of producing the enzymatic activities during the alcoholic fermentation of the grape juice. This paper reports the results of both strategies during the vinification of the Bobal grape must.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions. The selection and molecular characterization of the *Saccharomyces cerevisiae* industrial yeast strain T_{73} (CECT 1894) have been published previously (*22, 23*). Strain T_{73}/pTLEGY3 (T_{73}-EGL1) (*9*) expresses the *T. longibrachiatum* endoglucanase activity (EGL1), and strain T_{73}/YepCA1-A (T_{73}-X_{22}) (*10*) expresses the *A. nidulans* 22-kDa xylanase activity (X_{22}).

To obtain starter cultures, yeast cells were grown for 36 h at 30 °C in YPD-rich medium (1% w/v yeast extract, 1.5% w/v peptone, 2% w/v glucose; all components from Sigma, St. Louis, MO) containing 1 μg/mL cycloheximide (Sigma, St. Louis, MO).

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Table 1. Factorial Composition of the Microvinification Experiments

	factors				
trial	EGL1	X_{22}	T_{73}-EGL1	T_{73}-X_{22}	Lzym
1	no	no	no	no	no
2	no	no	no	yes	yes
3	no	no	yes	no	yes
4	no	no	yes	yes	no
5	no	yes	no	no	yes
6	no	yes	no	yes	no
7	no	yes	yes	no	no
8	no	yes	yes	yes	yes
9	yes	no	no	no	yes
10	yes	no	no	yes	no
11	yes	no	yes	no	no
12	yes	no	yes	yes	yes
13	yes	yes	no	no	no
14	yes	yes	no	yes	yes
15	yes	yes	yes	no	yes
16	yes	yes	yes	yes	no

Table 2. L16 Taguchi Orthogonal Array

	colum no.														
trial	1[a]	2[a]	3	4[a]	5	6	7	8[a]	9	10	11	12	13	14	15[a]
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
2	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2
3	1	1	1	2	2	2	2	1	1	1	1	2	2	2	2
4	1	1	1	2	2	2	2	2	2	2	2	1	1	1	1
5	1	2	2	1	1	2	2	1	1	2	2	1	1	2	2
6	1	2	2	1	1	2	2	2	2	1	1	2	2	1	1
7	1	2	2	2	2	1	1	1	1	2	2	2	2	1	1
8	1	2	2	2	2	1	1	2	2	1	1	1	1	2	2
9	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
10	2	1	2	1	2	1	2	2	1	2	1	2	1	2	1
11	2	1	2	2	1	2	1	1	2	1	2	2	1	2	1
12	2	1	2	2	1	2	1	2	1	2	1	1	2	1	2
13	2	2	1	1	2	2	1	1	2	2	1	1	2	2	1
14	2	2	1	1	2	2	1	2	1	1	2	2	1	1	2
15	2	2	1	2	1	1	2	1	2	2	1	2	1	1	2
16	2	2	1	2	1	1	2	2	1	1	2	1	2	2	1

[a] Columns used to assign factors.

Enzyme Production. Yeast strains T_{73}/pTLEGY3 and T_{73}/YepCA1-A were used for the production of endoglucanase EGL1 and xylanase X_{22}, respectively. Yeasts were grown for 48 h in 4.75 L of YPD-rich medium containing 1 μg/mL cycloheximide during 48 h using a Bioflo III fermentor (New Brunswick Scientific, Edison, NJ). Cultures were stored overnight at 4 °C to permit yeast sedimentation, and the supernatant concentrated to 500 mL in a Pellicon Ultrafiltration System (Millipore Corp., Bedford, MA) using a 10 000-Da molecular mass cutoff polysulfone filter. Proteins were precipitated at 0 °C with ammonium sulfate (80% saturation) and separated by sedimentation at 4 °C overnight.

Enzyme Assays. Xylanase and endoglucanase activities were determined according to refs *15* and *24*, respectively. One unit of each activity (IU) is the amount of enzyme that released 1 μmol of xylose (xylanase) or glucose (endoglucanase) equivalents per minute.

Preparation of Wine. The factorial composition of microvinification experiments is shown in Table 1. Bobal variety grape mash was collected from a commercial winery in Requena (Valencia, Spain). The crushed grape mass was separated into a flesh + skins + seeds fraction and a grape juice fraction using a filter of pore diameter 2 mm. Sterile bottles (1 L) were filled with 500 g of the filter-retained fraction and 500 mL of grape juice fraction. The cap was not immersed during vinification. The initial reducing sugar content of the must was 233 g/L.

Table 3. Concentration (mg/L) of Free Volatile Compounds Present in the Wines Produced

	trial no.															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Alcohols																
1-propanol	0.803	3.465	0.881	3.747	0.515	2.180	2.215	2.310	0.333	0.213	0.880	2.524	2.554	2.592	1.507	0.363
isobutyl alcohol	12.71	70.76	17.97	45.35	33.67	35.46	67.44	45.02	6.42	16.60	38.11	35.91	29.83	31.62	46.54	7.81
1-butanol	0.460	1.468	0.647	1.753	0.629	2.074	1.276	1.488	0.363	0.500	1.111	1.979	1.266	0.919	0.502	0.337
isoamyl alcohol	164.8	288.1	116.3	234.3	299.6	307.1	248.4	340.1	102.5	130.6	149.2	237.5	252.7	231.2	274.0	57.5
1-pentanol	0.059	0.125	0.064	0.103	0.084	0.041	0.060	0.056	0.010	0.049	0.077	0.108	0.087	0.103	0.150	0.033
3-methyl-1-pentanol	0.075	0.082	0.031	0.077	0.122	0.106	0.033	0.092	0.062	0.069	0.028	0.081	0.089	0.072	0.049	0.022
1-hexanol	1.317	1.643	1.384	1.696	1.541	1.671	1.586	1.562	0.810	1.081	1.344	1.434	1.433	1.379	2.128	0.663
trans−3-hexen-1-ol	0.025	0.033	0.031	0.042	0.300	0.036	0.030	0.033	0.029	0.020	0.033	0.024	0.026	0.034	0.063	0.012
3-ethoxy-1-propanol	0.012	0.059	0.041	0.043	0.025	0.018	0.034	0.033	0.018	0.036	0.069	0.049	0.031	0.051	0.279	0.041
cis−3-hexen-1-ol	0.060	0.070	0.049	0.071	0.070	0.073	0.054	0.060	0.034	0.037	0.042	0.061	0.058	0.053	0.093	0.022
7-octen-4-ol	0.135	0.126	0.109	0.133	0.094	0.017	0.021	0.018	0.089	0.021	0.028	0.016	0.045	0.013	0.051	0.206
1-heptanol	0.988	0.116	0.034	0.027	0.134	0.095	0.097	0.103	0.111	0.094	0.100	0.059	0.097	0.087	0.094	0.083
1-octanol	0.086	0.047	0.038	0.042	0.030	0.024	0.049	0.038	0.078	0.047	0.048	0.030	0.037	0.047	0.098	0.064
3-methylthio-1-propanol	0.053	0.045	0.065	0.035	0.034	0.219	0.054	0.240	0.172	0.143	0.036	0.118	0.074	0.065	0.043	0.019
2-phenylethanol	17.73	19.08	27.24	24.63	44.18	70.75	36.63	65.81	99.55	37.62	25.68	27.89	24.57	21.36	21.99	19.72
total alcohols (%)	100.0	200.2	110.4	182.8	201.1	186.7	158.5	192.9	117.6	106.0	138.0	173.1	144.5	148.9	288.2	76.5
Esters																
ethyl hexanoate	0.059	0.103	0.072	0.157	0.074	0.086	0.058	0.067	0.061	0.021	0.077	0.084	0.084	0.092	0.182	0.053
ethyl lactate	7.46	23.39	4.70	13.40	4.51	3.97	4.79	5.47	3.90	6.21	6.33	11.26	12.16	16.04	11.76	3.60
ethyl octanoate	0.248	0.049	0.022	0.017	0.038	0.019	0.017	0.022	0.053	0.026	0.026	0.009	0.022	0.033	0.027	0.035
ethyl 3-hydroxybutanoate	0.320	0.306	0.281	0.267	0.109	0.445	0.274	0.411	0.104	0.545	0.361	0.841	0.532	0.588	0.555	0.398
diethyl succinate	0.262	0.104	0.025	0.125	0.192	0.303	0.122	0.266	0.213	0.106	0.138	0.187	0.173	0.184	0.347	0.274
ethyl 4-hydroxybutanoate	3.173	0.271	0.289	0.146	0.499	4.760	6.049	9.372	2.360	11.518	5.000	10.774	9.939	10.526	7.944	9.786
monoethyl succinic acid	0.237	0.057	0.039	0.043	0.147	0.068	0.062	0.087	0.073	0.006	0.009	0.122	0.043	0.026	0.034	0.153
ethyl vanillate	0.120	0.041	0.042	0.025	0.094	0.193	0.192	0.204	0.062	0.013	0.027	0.081	0.037	0.086	0.032	0.063
total esters (%)	100.0	88.6	43.8	78.3	58.1	99.9	84.8	115.9	56.0	89.5	71.7	136.0	113.5	131.4	134.0	100.7
Terpenes																
linalool	0.006	0.003	0.002	0.001	0.004	0.013	0.018	0.017	0.017	0.006	0.008	0.025	0.006	0.007	0.008	0.009
Acids																
isobutyric acid	0.370	0.321	0.500	0.341	0.073	0.444	0.317	0.425	0.153	0.233	0.142	0.431	0.421	0.151	1.260	0.711
butyric acid	1.714	0.519	0.351	0.210	0.124	1.519	0.611	1.253	7.388	1.696	0.871	1.450	1.337	1.375	0.911	1.934
isovaleric acid	0.329	0.290	0.216	0.336	0.479	0.565	0.194	0.401	0.940	0.385	0.213	0.325	0.348	0.266	0.376	0.361
hexanoic acid	0.712	0.114	0.087	0.050	1.146	0.353	0.121	2.260	4.006	2.209	0.979	0.076	0.090	0.623	2.433	5.184
octanoic acid	0.029	0.013	0.015	0.011	0.017	0.088	0.096	0.058	0.112	0.077	0.180	0.079	0.065	0.072	0.212	0.394
decanoic acid	0.263	0.071	0.096	0.069	0.080	0.091	0.037	0.086	0.131	0.031	0.013	0.012	0.048	0.043	0.029	0.033
lauric acid	0.066	0.038	0.056	0.061	0.087	0.038	0.035	0.045	0.041	0.010	0.009	0.018	0.030	0.029	0.015	0.047
total acids (%)	100.0	50.4	57.9	53.0	79.4	118.6	85.9	133.0	260.4	126.6	134.1	88.6	86.1	85.6	232.1	372.4
total free volatiles (%)	100.0	105.9	80.9	108.1	97.8	144.5	126.3	161.2	154.7	104.3	103.6	154.5	102.5	111.8	189.0	164.8

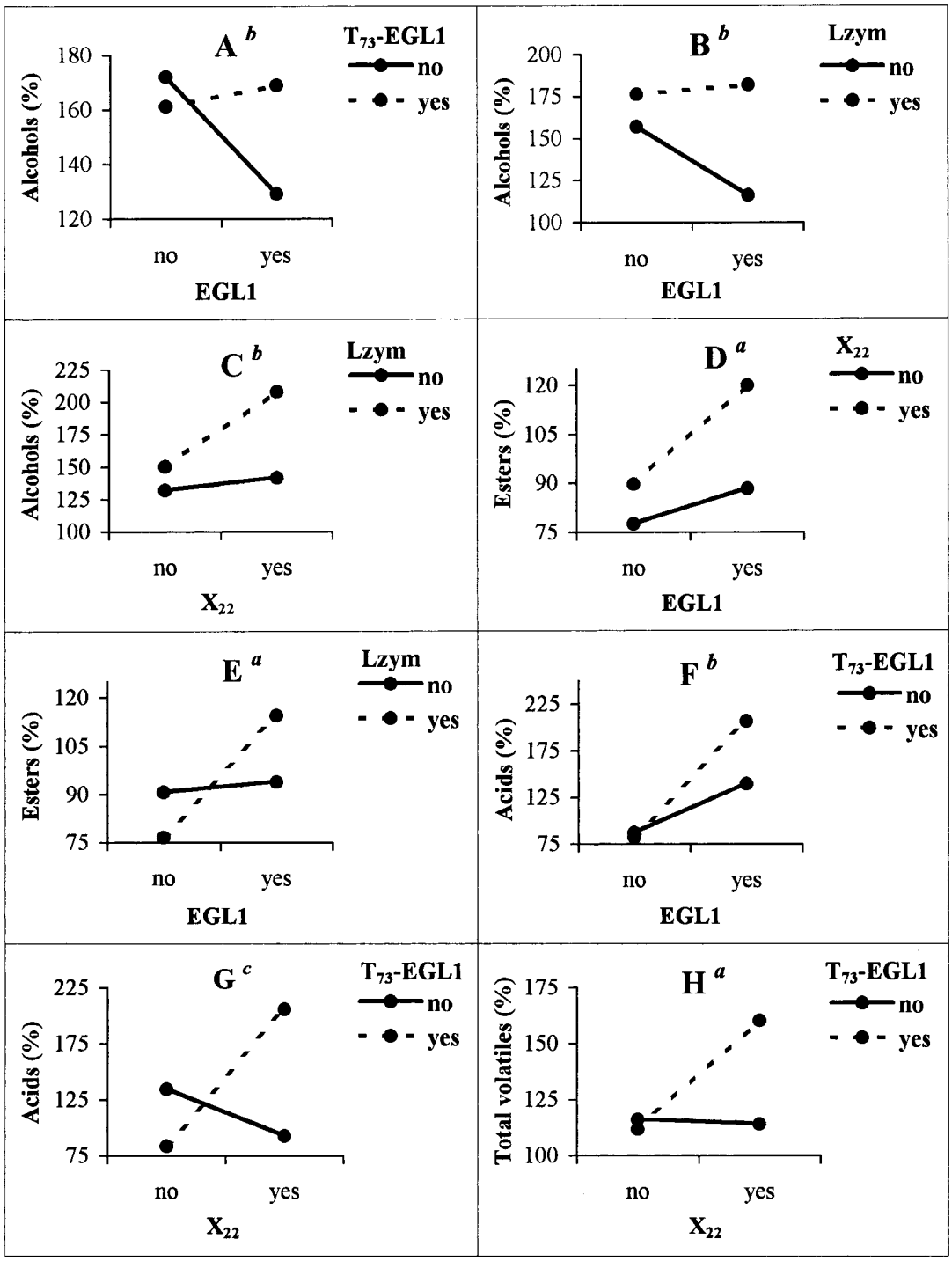

Figure 1. Effects of the interactions between two enzymatic treatments on the percentage of alcohols (panels A−C), esters (panels D and E), acids (panels F and G), and total free volatiles (panel H) detected in the free fraction of wines. a−c indicate significance at the $p < 0.05$, $p < 0.01$, and $p < 0.001$ levels, respectively.

Prior to inoculation, the must yeast population was reduced by the addition of 6.25 mg/L of dimethyl dicarbonate (Fluka) overnight. All must samples were subsequently inoculated to a final concentration of 10^6 cells/mL, and microvinifications were carried out at 25 °C. The development of the alcoholic fermentation was checked by measuring reducing sugar content using the Nelson-Somogyi method (*25*).

In the cases of microvinifications with direct addition of enzymes, the doses added were 1.3 and 7.7 U/mL for the EGL1 and X_{22} activities, respectively. An equal amount of 80% saturated ammonium sulfate solution was added to samples to which no exogenous enzymes were added in order to maintain the same amount of inorganic nitrogen in all samples. Lallzyme HC (Lzym) (Lalvin, Lallemand Inc., Montreal, Canada) was used as the commercial pectolytic enzyme preparation at 20 mg/L (twice the recommended dose).

Upon completion of fermentation, the mixtures of wine and grape skins were pressed, and the wine obtained was filtered through 0.45-μm pore size membranes (Millipore Corp., Bedford, MA). Finished wines were treated with 50 mg/L of SO_2, bottled, and stored at 4 °C until analysis.

Spectrophotometric Determinations. Spectrophotometric measurements of wine were made using 2-mm path length cells at 520 and 420 nm from which tint (A420/A520) and color density (CD) (A520 + A420) were calculated for a 10-mm cell and expressed in absorbance units (*26*). The index of total polyphenols was determined by absorbance at 280 nm of

Table 4. Effects of the Enzymatic Treatments on Free Volatile Compounds[a]

	enzymatic treatments (factors)					mean
	EGL1	X_{22}	T_{73}-EGL1	T_{73}-X_{22}	Lzym	(mg/L)
Alcohols						
1-propanol	−38.04[a]			56.92[a]		1.692
isobutyl alcohol	−42.69[a]	19.80[a]	24.80[a]	13.24[a]	12.78[a]	33.827
1-butanol	−33.60[a]		16.86[a]	50.85[b]		1.048
isoamyl alcohol	−32.83[a]	34.21[a]				214.62
3-methyl-1-pentanol	−26.79[a]	14.68[a]	−48.44[b]	20.55[a]	16.88[a]	0.068
1-octanol	23.66[b]			31.13[b]		0.050
3-methylthio-1-propanol				49.89[a]		0.088
total alcohols (%)	−11.06[a]	21.32[b]	9.15[a]		26.88[b]	157.84
Esters						
ethyl hexanoate			25.56[a]			0.083
ethyl lactate			−23.51[a]	39.88[b]	33.27[a]	8.685
ethyl 3-hydroxy-butanoate	47.69[a]		13.86[a]	39.92[a]		0.396
diethyl succinate		46.41[a]				0.189
ethyl 4-hydroxy-butanoate	93.69[b]	54.85[b]	13.67[a]	47.40[b]		5.775
monoethyl succinic acid	−45.44[a]					0.075
ethyl vanillate	−77.74[a]	74.70[a]				0.082
total esters (%)	21.77[b]	23.23[b]		23.75[b]		93.89
Terpenes						
linalool	29.33[a]	18.67[a]	34.67[a]		21.33[a]	0.009
Acids						
isobutyric acid			62.32[a]			0.393
isovaleric acid			−39.18[a]			0.377
hexanoic acid	105.24[c]	38.91[b]	18.95[a]	12.67[a]		1.278
octanoic acid	113.83[b]	−64.03[a]	75.36[a]			0.095
decanoic acid	−79.96[a]	42.19[a]	−67.61[a]	−46.07[a]		0.071
lauric acid	−72.64[c]		−16.96[a]			0.039
total acids (%)	68.57[c]	31.22[b]	24.19[b]		−8.64[a]	129.0
total free volatiles (%)	25.92[a]	22.77[a]	18.91[a]			132.34

[a] The letters a−c indicate significance at the $p < 0.05$, $p < 0.01$, and $p < 0.001$ levels, respectively.

diluted wine (1/50, v/v). Color and phenol determinations were made after 1 yr of aging.

Extraction of Free and Bound Aroma Compounds. Wine samples were fractionated on an Amberlite XAD-2 column (100 × 10 mm) (*27*) after the addition of an internal standard (10 μL, 2-octanol, 0.1% w/v) to each 30-mL sample. The fraction containing free aromatic compounds was eluted with pentane/dichloromethane (2:1, v/v), dried with anhydrous sodium sulfate, and concentrated at 40 °C using a Vigreux column. The remaining bound fraction was then eluted with ethyl acetate/methanol (9:1, v/v) (*28*) and concentrated to dryness using a vacuum concentrator (Büchi, Switzerland).

Enzymatic Hydrolysis of Grape Glycosides. Bound-fraction aroma compounds were dissolved in 500 μL of 50 mM citrate-phosphate buffer, pH 5.0, and extracted five times with 400 μL of pentane/dichloromethane (2:1, v/v) to remove any traces of free volatiles. A total of 5 μL of Pectinase AR2000 (Gist-Brocades, Seclin, France) solution (50 mg/mL) was added, and the mixture was incubated at 40 °C for 24 h. The mixture was subsequently extracted five times with 400 μL of pentane/dichloromethane (2:1, v/v), and 10 μL of 2-octanol (0.1%, w/v) was added as an internal standard. The extract was concentrated under a nitrogen stream prior to analysis.

GC and GC−MS Analysis. A HP5890 series II gas chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with a fused-silica capillary column coated with TR-WAX (60 m × 0.20 mm i.d. × 0.20 μm film thickness, Tracer Analítica S.A., Sant Cugàt del Vallès, Spain) and a flame ionization detector was used. The operating conditions were as follows: detector temperature 250 °C; injector temperature 200 °C; oven temperature programmed to rise from 50 (5 min) to 150 °C at 2.5 °C/min and from 150 to 215 °C at 5 °C/min and held at 215 °C for 45 min. Injections were made in splitless mode, and the sample size was 1 μL. Identification of compounds was determined by comparing retention times with those of standard compounds (Sigma, St. Louis, MO) and the use of a Fisons Trio 1000 mass spectrometric detector (Fisons Instruments, Rodano, Milan, Italy) under the same chromatographic conditions.

Statistical Analysis. Taguchi's orthogonal array L16 (Table 2) was used as the experimental design in order to elucidate the main effect of the factors studied as well as the effect of double interactions among them. The statistical significance of the effects of the factors was determined by analysis of the variance (ANOVA), and analysis of means was carried out by comparison of LSD intervals at the 95% confidence level.

RESULTS AND DISCUSSION

Microvinifications. Sixteen microvinifications were carried out as detailed in the factorial composition shown in Table 1. All alcoholic fermentations finished within 7 days, yielding final reducing sugar contents less than 3 g/L. No differences in fermentative progress were detected (data not shown). Seven days after inoculation of the must, cycloheximide resistant colonies ranged between 30 and 60% of total viable yeasts in those wines that had been inoculated with transgenic yeasts. No cycloheximide-resistant colonies were found in samples inoculated with the untransformed yeast strain T_{73}.

Free Aroma. The study of free aroma was based on 31 compounds comprising alcohols, esters, terpenes, and acids. Table 3 shows the individual concentrations and percentages (with reference to untreated sample no. 1) of the chemically grouped compounds. The percentage value gives equal weight to each compound independent of its absolute value. Significant increases and reductions in the levels of various compounds were detected depending on the enzymatic treatment employed. Table 4 shows those compounds whose concentrations are significantly affected by the enzymatic treatments as-

Table 5. Concentration (μg/L) of Aglycons Liberated from Wine Glycosides

	trial no.															
aglycon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
isobutyl alcohol	30.61	22.65	17.09	15.48	24.62	17.25	80.47	18.04	10.97	30.09	13.68	18.27	43.24	43.83	113.06	100.41
1-butanol	15.59	10.45	37.97	32.54	22.68	23.23	40.98	24.37	12.18	32.89	7.26	21.87	51.81	49.07	39.98	40.44
isoamyl alcohol	295.64	109.43	332.66	287.07	138.00	118.99	3667.86	190.00	166.47	2327.80	93.75	172.67	411.42	338.55	599.27	318.42
1-hexanol	46.84	39.13	161.80	156.95	314.36	53.55	132.94	76.61	73.31	61.62	162.61	48.93	221.20	210.30	24.43	22.82
trans−3-hexen-1-ol	4.64	15.12	18.08	19.36	19.55	7.90	32.74	11.13	9.52	15.48	11.27	10.87	37.40	34.27	34.00	43.98
cis−3-hexen-1-ol	29.91	23.81	45.96	48.15	26.85	16.60	33.97	31.04	39.82	28.28	18.36	21.23	46.57	51.16	18.86	51.86
benzyl alcohol	559.8	626.7	1079.8	1140.1	5095.1	1933.1	1149.2	1439.1	1379.7	958.3	2389.0	1009.8	1670.2	1780.0	915.8	1061.5
2-phenylethanol	357.7	352.7	890.6	929.2	2479.7	1249.8	1507.7	1043.5	1022.0	1289.6	5941.6	964.2	1430.0	1510.5	886.2	721.4
total aglycons (%)	100.0	109.7	217.8	216.7	382.3	160.1	437.1	167.0	149.7	273.5	357.8	139.8	343.2	334.0	261.0	282.1
total phenols	21.15	21.55	25.25	24.8	29.35	28.05	31.4	30.00	26.00	26.75	25.55	28.80	25.85	24.00	26.05	23.50
color desnsity	3.57	4.26	3.59	3.94	4.00	3.42	3.74	4.92	3.86	3.89	3.53	4.35	3.56	4.01	4.46	4.21
tint	0.74	0.67	0.80	0.77	0.86	1.03	0.88	0.78	0.74	0.74	0.87	0.68	0.69	0.72	0.71	0.67

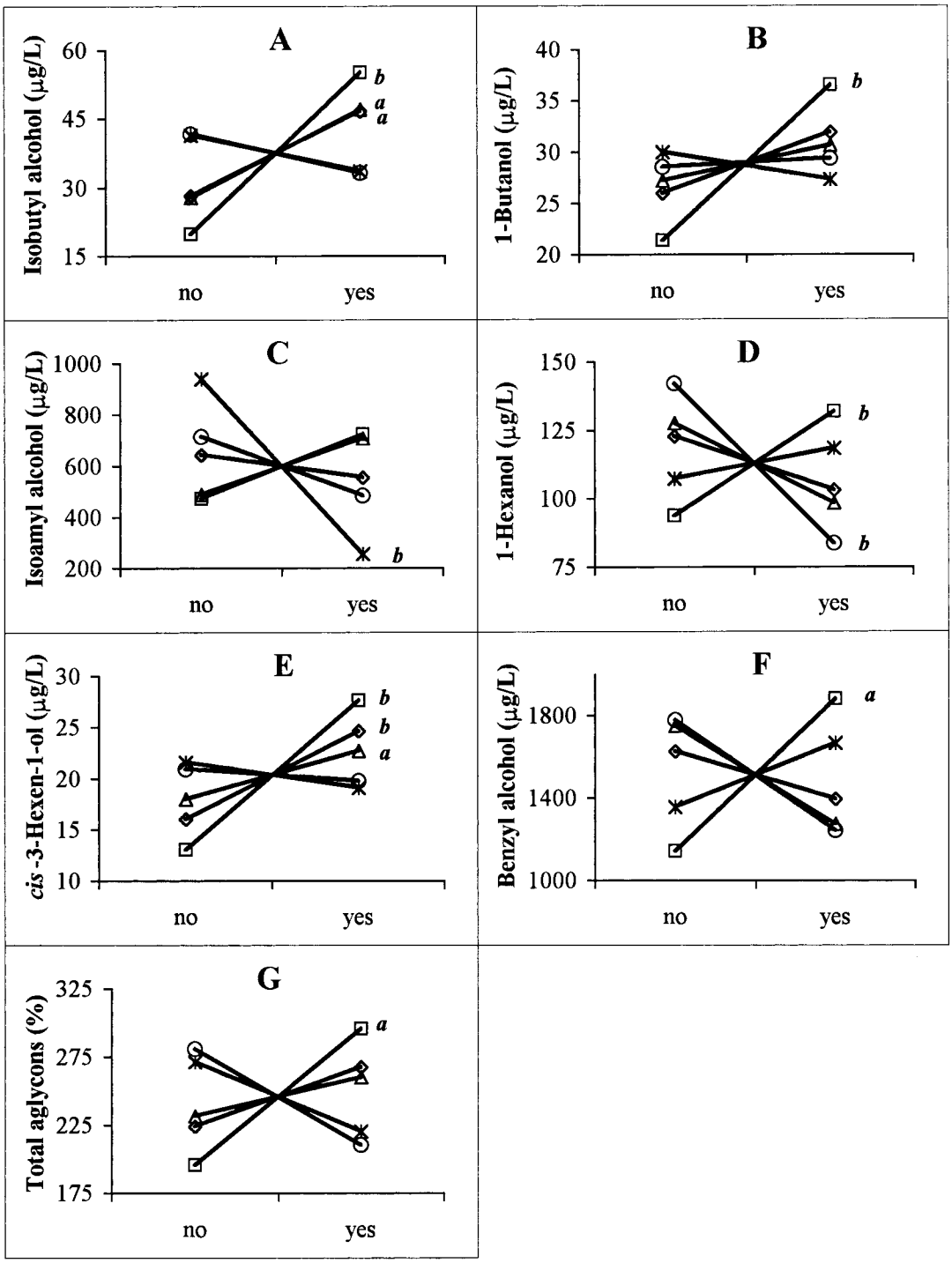

Figure 2. Effects of the enzymatic treatments on the glycosidically bound flavor compounds of wines: (◇) EGL1, (□) X_{22}, (△) T_{73}-EGL1, (○) T_{73}-X_{22}, and (*) Lzym. a and b indicate significance at the $p < 0.05$ and $p < 0.01$ levels, respectively.

sayed and the percentage increase or decrease with respect to the means of the 16 samples.

Direct addition of EGL1 caused significant reductions in the yields of nine compounds, mainly alcohols, and increases in six of them: 1-octanol, ethyl-3-hydroxybutanoate, ethyl-4-hydroxybutanoate, linalool, hexanoic acid, and octanoic acid. The effects of inoculation with the T_{73}-EGL1 yeast strain were similar for 3-methyl-1-pentanol, ethyl-3-hydroxybutanoate, ethyl-4-hydroxybutanoate, linalool, hexanoic acid, and octanoic acid but not so for isobutyl alcohol and 1-butanol. Direct addition of xylanase X_{22} resulted in increases in the concentrations of nine compounds and the diminution of decanoic acid. Similarly, inoculation with the T_{73}-X_{22} yeast strain yielded increases in 10 compounds (some of which are different from those compounds increased by direct addition of the enzyme) and the diminution of decanoic acid. Use of the commercial preparation Lzym resulted in the increased yields of isobutyl alcohol, 3-methyl-1-pentanol, ethyl lactate, and linalool. It is noteworthy that the levels of the only terpene detected (linalool) were increased by all the treatments employed (use of strain T_{73}-X_{22} resulted in an increase of 16% with respect to the mean although not statistically significant at 95% confidence).

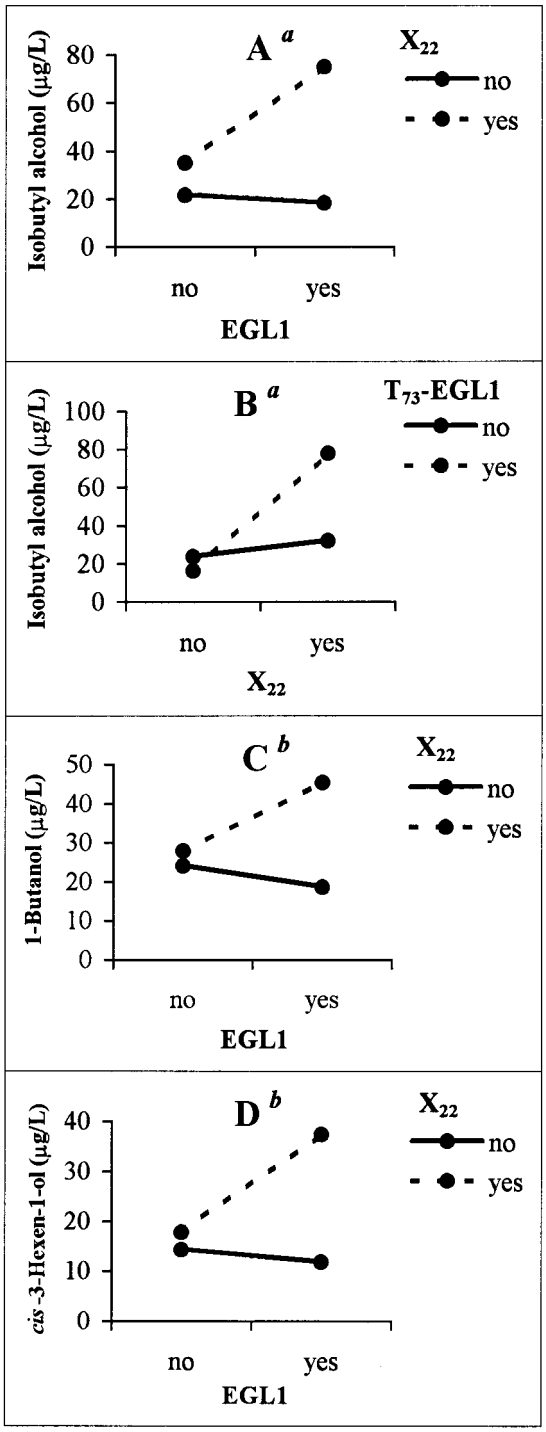

Figure 3. Effects of the interaction between two enzymatic treatments on the glycosidically bound flavor compounds of wines. a−c indicate significance at the $p < 0.05$, $p < 0.01$, and $p < 0.001$ levels, respectively.

A number of significant effects due to interactions between two factors were also detected. Figure 1 shows the most notable effects on the relative amounts of free volatile components grouped by chemical family. The decrease in alcohol levels upon addition of EGL1 was not observed in inoculations with the T_{73}-EGL1 strain (panel A) or addition of Lzym C (panel B). An additive effect increasing the relative amounts of alcohols can be observed between X_{22} and Lzym (panel C). The increase in esters due to the addition of EGL1 was greater in the presence of X_{22} (panel D) or Lzym (panel E). The yield of acids was greater when EGL1 or X_{22} were added and the must was inoculated with T_{73}-EGL1 (panels F and G). Total free aroma was increased due to the interactive effect between the addition of X_{22} and inoculation with T_{73}-EGL1.

Only the commercial preparation contains activities able to hydrolyze glycosidic precursors and thus liberate the corresponding aglycons. Our data showed that addition of this preparation produced increases mainly in compounds typically founded as glycosidic precursors, such as alcohols and terpenes (primary aromas). The effect of EGL1 or X_{22} should be the release of glycosides from grape cell walls but not their subsequent hydrolysis. Hydrolysis and consequent release of the aglycons could be produced by the action of endogenous glycosidases or via chemical hydrolysis. In this sense, an increase in the levels of glycosidically bound aromatic precursors due to the action of EGL1 or X_{22} would be expected to produce an increase in the corresponding aglycon in the resultant wine. However, these enzymes could also significantly change the composition of the must during maceration due to their action on grape skin polysaccharides. Such changes could influence yeast metabolism and result in increased or decreased levels of compounds responsible for secondary aroma, as the results of the present work suggest. Similar findings regarding the use of the EGL1 or X_{22} transgenic wine yeast strains and their effects on certain compounds of the free volatile fraction such as alcohols, esters, and terpenes as well as the increased fruitiness of the wines have been previously described (*9, 10*). Increased levels of glycosides after the maceration of musts in the presence of EGL1-enriched culture fluid from transgenic *A. nidulans* have also been reported (*16*). In certain cases (see Table 4), the effect of the direct addition of enzyme to the must was different from the effect observed upon its production by a transgenic yeast strain, suggesting the importance of factors often forgotten, such as enzyme dose and the most suitable point of application.

Glycosidically Bound Aroma. Glycosidically bound wine compounds were extracted, enzymatically hydrolyzed, and analyzed by GC and GC−MS. The concentrations of the resulting aglycons in the various wines are shown in Table 5.

The addition of EGL1 or X_{22} resulted in increases in the amounts of certain glycosides. Addition of EGL1 produced significant increases in isobutyl alcohol and *cis*-3-hexen-1-ol glycosides (Figure 2, panels A and E, respectively), and the addition of X_{22} yielded increases in the concentrations of isobutyl alcohol, 1-butanol, 1-hexanol, *cis*-3-hexen-1-ol, and benzyl alcohol glycosides as well as an elevation of the percentage of total aglycons (Figure 2, panels A, B, and D−G). The addition of Lzym produced a statistically significant reduction in the content of isoamyl alcohol glycoside (Figure 2, panel C) but no significant decrease in other glycosides.

Inoculation of must with the transgenic yeast strain T_{73}-EGL1 produced significant increases in the concentrations of isobutyl alcohol and *cis*-3-hexen-1-ol glycosides (Figure 2, panels A and E), similar to the effect observed upon direct addition of EGL1 enzyme to must. A significant decrease in 1-hexanol glycoside was the only effect observed as a consequence of inoculation with the T_{73}-X_{22} strain (Figure 2, panel D). Contrary to the effect of the direct addition of X_{22} (Figure 2, panel G),

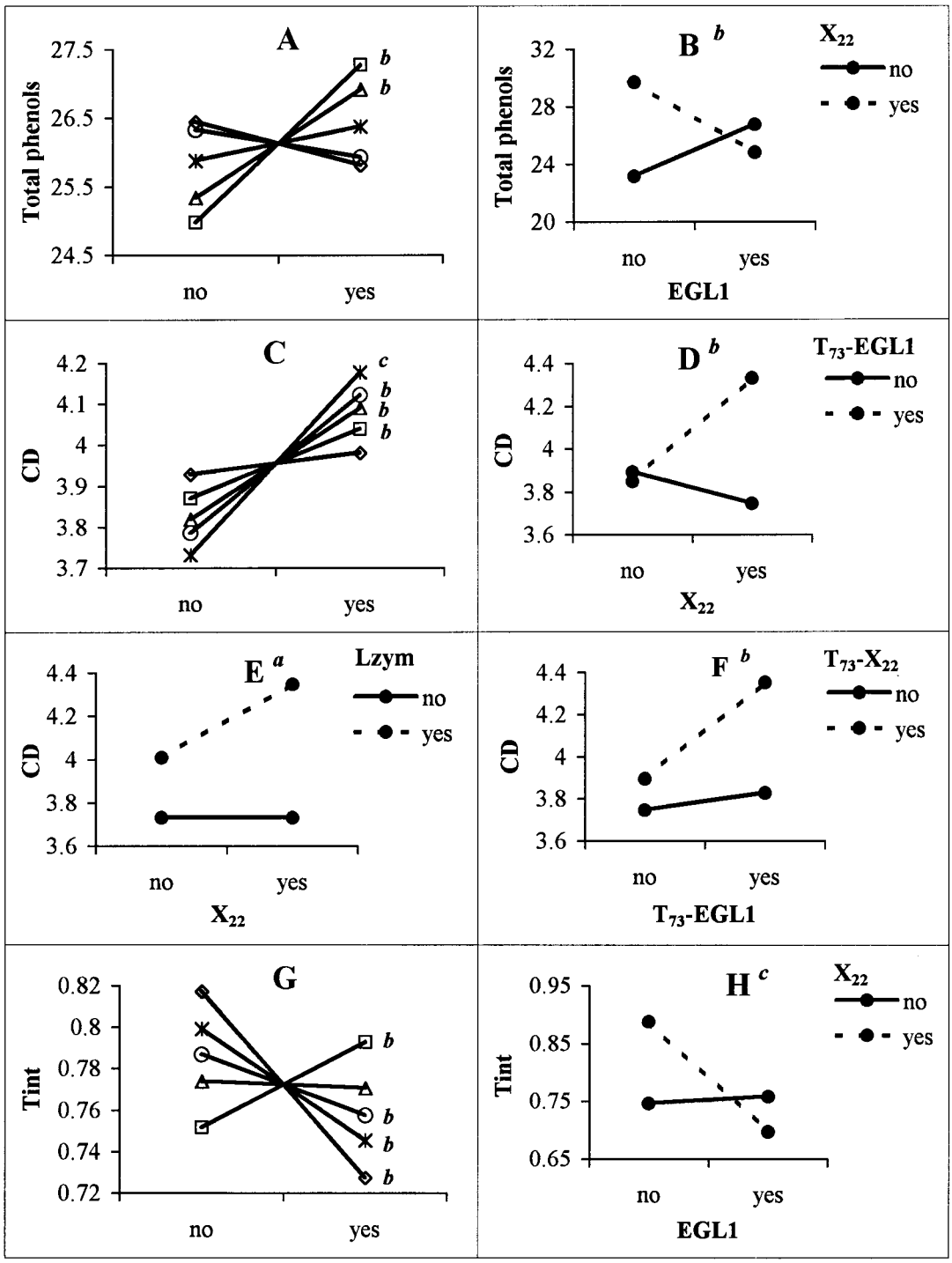

Figure 4. Effects of the enzymatic treatments and their interactions on total phenols, color density, and tint value of wines: (◇) EGL1, (□) X_{22}, (△) T_{73}-EGL1, (○) T_{73}-X_{22}, (*) Lzym. a−c indicate significance at the $p < 0.05$, $p < 0.01$, and $p < 0.001$ levels, respectively.

inoculation of must with the T_{73}-X_{22} strain tended to cause a decrease, though mainly not statistically significant, in the content of glycosidically bound precursors.

Interactions between the treatments applied also produced significant effects on the glycosidically bound wine fraction. As shown in Figure 3, an additive effect between EGL1 and X_{22} or between X_{22} and the inoculation with T_{73}-EGL1 was observed with regard to isobutyl alcohol content (panels A and B). The simultaneous action of EGL1 and X_{22} also produced higher concentrations of 1-butanol and *cis*-3-hexen-1-ol (Figure 3, panels C and D).

The data show that the enzymes employed in the microvinification experiments increased the amounts of glycosidically bound precursors, most notably in the case of direct addition of X_{22} to must. While the effects of the direct addition of EGL1 and inoculation with the corresponding transgenic yeast strain were equivalent, this was not the case for X_{22}. This difference is particularly evident in the case of the 1-hexanol glycoside (Figure 2, panel D). A study of the importance of the time of addition of the enzyme and the dose used would be necessary in order to clarify this observation.

The action of the glycosidases present in the commercial enzyme preparation Lzym could be responsible

for the decrease in the glycoside content of the resulting wines and related to the higher concentration of certain volatiles in the free fraction.

Wine Color and Phenolics. Wine color and the total content of phenolics were measured in all samples in order to assess the enzymatic treatments for the extraction of other cell wall associated components. Table 5 shows values for CD, tint, and total phenol content in the resulting wines. The data show that addition of X_{22} and inoculation with T_{73}-EGL1 each produced significant increases in total phenols (Figure 4, panel A). However, the simultaneous addition of X_{22} and EGL1 did not produce an increase in phenols (Figure 4, panel B).

CD was increased by all the enzymatic treatments and was statistically significant in the cases of direct addition of X_{22} and Lzym and inoculation with the T_{73}-EGL1 and T_{73}-X_{22} strains (Figure 4, panel C). The interaction between some enzymatic treatments such us X_{22}/T_{73}-EGL1, X_{22}/Lzym or simultaneous inoculation with both transgenic yeast strains T_{73}-EGL1/T_{73}-X_{22} produced an additive effect increasing the CD value (Figure 4, panels D−F).

A decrease in the tint value indicates a relative increase in redness as compared to brownness. Addition of EGL1 or Lzym or inoculation with the T_{73}-X_{22} strain produced significant decreases in tint values, whereas addition of X_{22} produced a significant increase (Figure 4, panel G). The decrease in tint due to EGL1 was specially marked in the presence of X_{22} (Figure 4, panel H).

Previous studies have reported that commercial enzyme preparations enhance color extraction during the production of red wines (*6*, *7*) and also produced an increase in the quantity of polyphenols (*29*) without any apparent sensory disadvantages. In Bobal wine, our data show that the commercial enzyme preparation (Lzym) increases CD and decreases the tint value. Treatments with the EGL1 or X_{22} fungal activities also produced this effect, with the advantage that only one or two activities were sufficient to obtain the improvement.

CONCLUSION

We have found a number of effects of enzyme treatments on aroma, color, and levels of phenolics in Bobal wine. In many cases, these effects are greater than those arising from the use of the commercial enzyme preparation, which possesses a complex mixture of pectolytic and glycolytic activities (and other collateral activities). Currently, enzyme preparations are widely employed in the wine industry, and the cellulase EGL1 and the hemicellulase X_{22} could play important roles or even substitute the use of complex enzyme mixtures for the extraction of components bound to cell walls. Further studies focusing on dosage and the most appropriate moment of addition are necessary in order to obtain a better optimization of the enological use of these activities.

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