Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Effect of physicochemical factors on glycerol production by simultaneous cultures of wine micro-organisms using the response surface method

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Keywords
glycerol production, mixed cultures, physicochemical factors, response surface method, winemaking.

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2014/1199: received 10 June 2014, revised 22 July 2014 and accepted 7 August 2014

doi:10.1111/jam.12621

Abstract

Aim: To evaluate the effect of temperature, pH and SO_2 on growth and glycerol production improvement by *Saccharomyces cerevisiae* mc_2, *Kloeckera apiculata* mF and *Oenococcus oeni* X_2L using the response surface method (RSM).

Methods and Results: Multifactorial design of cultures with physicochemical factors variations was performed. The micro-organisms grew in all cultures conditions. Overall, after 6 days yeasts prevailed, especially *S. cerevisiae* (10^9 CFU ml^{-1}), while *O. oeni* reached 10^7 CFU ml^{-1}. At initial fixed pH 5·5, metabolic behaviour of cultures showed a temperature-dependent response. Total malate consumption occurred at 26°C, 50 mg l^{-1} SO_2. Glucose and pentoses utilization was highly modified when varying SO_2. Ethanol showed negative interaction with temperature–SO_2 relationship. At low SO_2, glycerol and acetate production increased when temperature enhanced. Predictive results of RSM indicate that 26°C, 60·24 mg l^{-1} SO_2 and pH 5·5 were the optimal conditions for glycerol and organic acids synthesis compatible with wine quality.

Conclusions: We propose a predictive condition to improve the performance of mixed cultures for must fermentations.

Significance and Impact of the Study: To optimize the culture conditions to design mixed starters containing autochthonous yeasts and *O. oeni* strains for winemaking and to obtain products with high glycerol content, low acidity and maintenance of regional characteristics.

Introduction

The conversion of grape juice to wine is the result of complex interactions between yeasts (*Saccharomyces* and non-*Saccharomyces*), lactic acid bacteria (LAB), musts and physicochemical conditions prevailing during winemaking. The end products obtained also depend on the variety, origin and health status of the grapes as well as on the process used on its manufacture. During winemaking, yeasts and LAB are responsible for alcoholic fermentation (AF) and malolactic fermentation (MLF), respectively (Longo *et al.* 1991; Pretorius 2000). The growth and persistence of microbial populations related to winemaking depend on specific strain characteristics and culture conditions (Hansem *et al.* 2001; Mendoza *et al.* 2011; Elmaci *et al.* 2014). Thus, for successful winemaking, AF should reach a suitable ethanol level according to the wine variety, while MLF should be carried out completely to diminish acidity, which also allows the microbial stabilization of wines (Colagrande *et al.* 1994; Jay 1996; Alexandre *et al.* 2004).

At the end of the winemaking process, the wine has low pH and sugar contents, and high ethanol and organic acids concentrations, so that only a few species can proliferate

such as *Saccharomyces cerevisiae*, *Kloeckera apiculata* and *Oenococcus oeni* (Fleet and Heard 1993; Henschke 1997; Hansem *et al.* 2001). Moreover, by-products such as glycerol must be present at an adequate level to ensure the smoothness and roundness of wines on the palate and enhance their flavour (Ribereau-Gayon *et al.* 1975; Grazia *et al.* 1995). Low levels of organic acids and high levels of alcohols and esters (polyphenols and aldehydes to a lesser extent) are required to improve the organoleptic characteristics of wine (Rapp and Versini 1991), so non-*Saccharomyces* yeasts are relevant as other authors demonstrated their ability to produce some flavour-related compounds (Rojas *et al.* 2003; Romano *et al.* 2003).

Due to the selective pressure that musts exert on wine micro-organisms and the need to obtain reproducible conditions and/or products with particular characteristics, selected starter cultures are used for both AF and MLF (Henschke 1993; Nielsen *et al.* 1996; Henick-Kling *et al.* 1998; Ribéreau-Gayon *et al.* 2000; Hansem *et al.* 2001; Hong and Park 2013).

Many studies have examined the role of physicochemical factors on the growth and metabolic activity of wine LAB under winemaking conditions (Wibowo *et al.* 1985; Henick-Kling 1993) as well as the interaction of certain parameters (temperature, pH, inoculum size) in multifactorial experiments (Thomas *et al.* 1985; Vaillant *et al.* 1995; Nielsen *et al.* 1996; Kumar *et al.* 2009). Statistical inference techniques can be used to evaluate the significance of individual factors, of their combination and the sensitivity of the response to the modifications of different microbial systems (Mason *et al.* 1989). Thus, this kind of statistical experimental design can be used for bioprocess optimization. The response surface method (RSM) is a suitable tool to identify the effect of individual variables and determine the optimal conditions to analyse a multivariable system. This method has been successfully applied to optimize AF and other fermentation processes (Ambati and Ayyanna 2001; Ratnam *et al.* 2003; Kumar *et al.* 2009).

In a previous work, we evaluated the metabolic behaviour of a mixed system of *S. cerevisiae* mc_2, *K. apiculata* mF and *O. oeni* X_2L in sequential and simultaneous cultures in a basal medium and optimal inoculation and incubation conditions to carry out both AF and MLF with high glycerol yields were proposed (Ale *et al.* 2014). However, there are not reports concerning changes in this metabolic profile when physicochemical factors are modified. Therefore, the aim of this work was to use RSM to evaluate the effect of pH, SO_2 and temperature on the success of both AF and MLF, enhancing glycerol production and diminishing acetate synthesis during the simultaneous culture of *S. cerevisiae* mc_2, *K. apiculata* mF and *O. oeni* X_2L.

Materials and methods

Micro-organisms

Kloeckera apiculata mF (an apiculate yeast) and *S. cerevisiae* mc_2 (an elliptic yeast) isolated from Malbec grape must (north-western Argentina) and *O. oeni* X_2L isolated from an Argentinian wine were previously selected on the basis of their ability to produce glycerol in a culture medium formulated with natural grape juice (NGJ) (Ale *et al.* 2014).

All the micro-organisms were deposited in the wine yeasts and LAB culture collection at the Instituto de Microbiología 'Dr. Luis Verna', Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Argentina.

Culture media and growth conditions

Yeast strains were grown in YEPG medium (in g l^{-1}: yeast extract, 10; peptone, 20; glucose, 20), pH 5·5, for 24 h at 28°C, while *O. oeni* X_2L was grown in MRS medium (de Man *et al.* 1969) supplemented with 150 ml l^{-1} natural tomato juice (MRStj), pH 4·8, for 24 h at 30°C. To carry out the simultaneous cultures, micro-organisms were grown in NGJ medium (Ale *et al.* 2014) and cell enumeration (CFU ml^{-1}) was performed.

Physicochemical factors and growth conditions of the microbial strains

NGJ medium was formulated considering different initial pH values (3·5, 4·5 and 5·5) with 1N HCl, and sodium metabisulphite (Sigma-Aldrich) was supplemented to achieve 50, 100 and 150 mg l^{-1} molecular SO_2. The combination of these factors and the incubation temperature are shown in Table 1.

Yeasts and *O. oeni* X_2L were co-inoculated into 50 ml NGJ of each combination medium to reach 10^6 CFU ml^{-1} of each strain and incubated for 6 days in microaerophilia (unshaken capped tubes or flasks

Table 1 Factors in winemaking and levels used in the response surface method

	Factor level		
Independent variables	−1	0	1
Temperature (°C)	26	28	30
pH	3·5	4·5	5·5
SO_2 (mg ml^{-1})	50	100	150

two-thirds full). Samples were taken for both growth (every day) and analytical determinations (after 6 days).

Determination of microbial growth and differential cell enumeration

Growth was determined by counting the number of viable cells (CFU ml^{-1}) using the decimal successive dilution method using sterile distilled water. To differentiate *Saccharomyces* and non-*Saccharomyces* yeasts from mixed cultures, samples were plated on YEPG medium supplemented with ethanol (120 ml l^{-1}), sodium metabisulphite (0·15 g l^{-1}) and chloramphenicol (1 g l^{-1}) for the elliptic yeast, while YEPG medium supplemented with cycloheximide (0·01% w/v) was used for the apiculate strain.

The samples were also plated on MRStj supplemented with cycloheximide (0·1% w/v) to assess *O. oeni* X_2L growth.

Analytical determinations

Cell-free supernatants were obtained from each experiment and stored at −20°C until analytical determinations. Glucose, ethanol, glycerol and organic acids (total lactic, acetic and malic) were quantified with kits supplied by Boehringer-Mannheim, Inc. (Germany). Fructose concentration was determined using the Roe method, and total reducing sugars were evaluated with the technique of Somogyi-Nelson (Ale *et al.* 2014). Taking into account that musts mainly contain fructose, glucose and C5-sugars, pentoses concentration was calculated as follows:

Pentoses (mmol l^{-1}) = total reducing sugars (mmol l^{-1}) – [glucose+fructose] (mmol l^{-1})

Statistical analysis

All the combinations of physicochemical factors shown in Table 1 were performed using two repetitions of a complete factorial design 2^3 with four replications at the central points ($n_0 = 4$) leading to a total number of 20 separately randomized runs.

The combinations were designed using Design Expert® Software (7 ver., Stat-Ease, Inc., Minneapolis, MN), and the final design is shown in Table 2. For the predictive solution, one-way analysis of variance (ANOVA) was applied to the experimental data to analyse confidence intervals ($\alpha = 0{\cdot}05\%$) using INFOSTAT software (2012 student ver., Universidad Nacional de Córdoba, Córdoba, Argentina).

The three-dimensional (3D) plots were generated by keeping one variable at a constant value at the central point and changing the others within the experimental range.

Table 2 Combinations of the level of factors using the response surface method

Run	A:Temp	B:SO_2	C:pH
11	30	50	5·5
2	26	50	3·5
5	26	150	3·5
13	26	150	5·5
7	30	150	3·5
9	26	50	5·5
15	30	150	5·5
10	26	50	5·5
18*	**28**	**100**	**4·5**
16	30	150	5·5
1	26	50	3·5
4	30	50	3·5
12	30	50	5·5
14	26	150	5·5
8	30	150	3·5
20	**28**	**100**	**4·5**
3	30	50	3·5
17	**28**	**100**	**4·5**
6	26	150	3·5
19	**28**	**100**	**4·5**

*Bold numbers indicate four repetitions of the central point.

Results

Effect of physicochemical factors on microbial growth in natural grape juice

All the micro-organisms grew in different culture conditions and reached their maximum growth rate after 2–3 days (Table 3). Highest populations were detected at 6 days, especially for the yeast strains, which increased viable cell counts by approx. 3 log units. *Saccharomyces cerevisiae* showed the highest populations (maximum value = $4{\cdot}2 \times 10^9$ CFU ml^{-1}, 50 mg l^{-1} SO_2) especially at 30°C and pH 5·5, reaching a relative growth of 46·27%, while *K. apiculata* (maximum value = 4×10^9 CFU ml^{-1}, 150 mg l^{-1} SO_2) showed a relative growth of 16·75%.

Overall, the *O. oeni* strain grew until about 10^7 CFU ml^{-1} (maximum value = $2{\cdot}7 \times 10^7$ CFU ml^{-1}, 50 mg l^{-1} SO_2, 30°C). In the above conditions, maximum growth rates of the elliptic and apiculate strains were 0·27 and 0·13 h^{-1}, respectively, and 0·04 h^{-1} for *O. oeni* (Table 3).

At 26°C, pH 3·5 and 150 mg l^{-1} SO_2, only *K. apiculata* mF showed a decrease in growth at the end of the assay (relative growth = −33·19%) (Table 3), with a final population of 1×10^4 CFU ml^{-1} (data not shown).

Although maximum growth values were found at high temperature and low SO_2 concentrations, only for *S. cerevisiae* mc_2, this condition matched the maximum

Table 3 Growth parameters of *Saccharomyces cerevisiae* mc_2, *Kloeckera apiculata* mF and *Oenococcus oeni* X_2L in simultaneous cultures when using different combinations of the level of factors

Culture condition							
SO_2 (mg l^{-1})	pH	T (°C)	Strain	Initial cell count (CFU ml^{-1})*	Relative growth (%)†	K (h^{-1})‡	Maximum growth (CFU ml^{-1})*
50	5·5	26	mc_2	$1·26 \times 10^6$	26·70	0·22	$3·60 \times 10^9$
			mF	$1·02 \times 10^6$	16·84	0·20	$2·30 \times 10^9$
			X_2L	$9·80 \times 10^5$	25·38	0·01	$1·00 \times 10^7$
150	5·5	26	mc_2	$1·04 \times 10^6$	27·57	0·20	$2·80 \times 10^9$
			mF	$1·05 \times 10^6$	23·80	0·18	$2·10 \times 10^9$
			X_2L	$7·90 \times 10^5$	41·54	0·05	$2·00 \times 10^7$
50	3·5	26	mc_2	$1·36 \times 10^6$	24·43	0·21	$3·20 \times 10^9$
			mF	$1·54 \times 10^6$	17·38	0·20	$2·40 \times 10^9$
			X_2L	$9·20 \times 10^5$	42·04	0·01	$1·00 \times 10^7$
150	3·5	26	mc_2	$1·35 \times 10^6$	23·56	0·15	$1·80 \times 10^9$
			mF	$1·42 \times 10^6$	−33·19	0·15	$1·20 \times 10^9$
			X_2L	$9·70 \times 10^5$	45·09	0·01	$1·00 \times 10^7$
50	5·5	28	mc_2	$1·03 \times 10^6$	28·50	0·20	$3·80 \times 10^9$
			mF	$9·80 \times 10^5$	18·06	0·19	$3·70 \times 10^9$
			X_2L	$8·50 \times 10^5$	44·40	0·07	$1·00 \times 10^6$
150	5·5	28	mc_2	$1·13 \times 10^6$	42·62	0·30	$3·60 \times 10^9$
			mF	$1·24 \times 10^6$	17·95	0·29	$3·80 \times 10^9$
			X_2L	$8·60 \times 10^5$	41·24	0·07	$4·00 \times 10^6$
50	3·5	28	mc_2	$1·36 \times 10^6$	20·85	0·14	$3·63 \times 10^9$
			mF	$1·87 \times 10^6$	17·56	0·14	$2·75 \times 10^9$
			X_2L	$9·00 \times 10^5$	42·31	0·02	$2·30 \times 10^7$
150	3·5	28	mc_2	$1·26 \times 10^6$	24·91	0·23	$2·34 \times 10^9$
			mF	$1·17 \times 10^6$	16·67	0·22	$1·23 \times 10^9$
			X_2L	$1·00 \times 10^6$	45·30	0·02	$1·20 \times 10^7$
50	5·5	30	mc_2	$1·10 \times 10^6$	46·27	0·32	$4·20 \times 10^9$
			mF	$9·80 \times 10^5$	16·34	0·33	$3·50 \times 10^9$
			X_2L	$1·04 \times 10^6$	49·13	0·04	$2·00 \times 10^6$
150	5·5	30	mc_2	$8·70 \times 10^5$	29·69	0·30	$3·50 \times 10^9$
			mF	$1·02 \times 10^6$	16·75	0·31	$4·00 \times 10^9$
			X_2L	$9·90 \times 10^5$	41·60	0·03	$3·00 \times 10^6$
50	3·5	30	mc_2	$1·43 \times 10^6$	27·03	0·27	$2·88 \times 10^9$
			mF	$1·04 \times 10^6$	16·50	0·13	$3·80 \times 10^9$
			X_2L	$1·02 \times 10^6$	42·90	0·04	$2·70 \times 10^7$
150	3·5	30	mc_2	$1·34 \times 10^6$	24·93	0·25	$3·56 \times 10^9$
			mF	$1·24 \times 10^6$	17·01	0·24	$2·54 \times 10^9$
			X_2L	$9·60 \times 10^5$	15·15	0·06	$1·60 \times 10^7$

N_0, initial viable cell number; N_t, viable cell number in time considered.
*Represents the mean value of two randomized runs.
†Relative growth (%) = $(N_t - N_0/N_0) \times 100$.
‡K = $\{3·3 \times [\text{Log } (N_t/N_0)]\}/t$.

relative growth, as for *K. apiculata* mF, it was 17·01% at 30°C, pH 3·5 and 150 mg l^{-1} SO_2, and for *O. oeni* X_2L, it was 45·30% at 28°C, pH 3·5 and 150 mg l^{-1} SO_2 (Table 3).

Substrates consumption

To propose culture parameters for winemaking, microbial consumption of the main substrates (glucose, fructose, pentoses and malic acid) was determined.

At initial pH 5·5 as a fixed factor, differential substrate consumption was observed (Fig. 1). While fructose and malic acid were the only carbon sources that exhibited high consumption values at 26°C (2·9 and 38·2 mmol l^{-1}, respectively), glucose, fructose and pentoses showed the highest consumption pattern when temperature increased up to 30°C. The decrease in SO_2 concentration favoured the above behaviour with the exception of pentoses, which exhibited high consumption when the culture medium was supplemented with

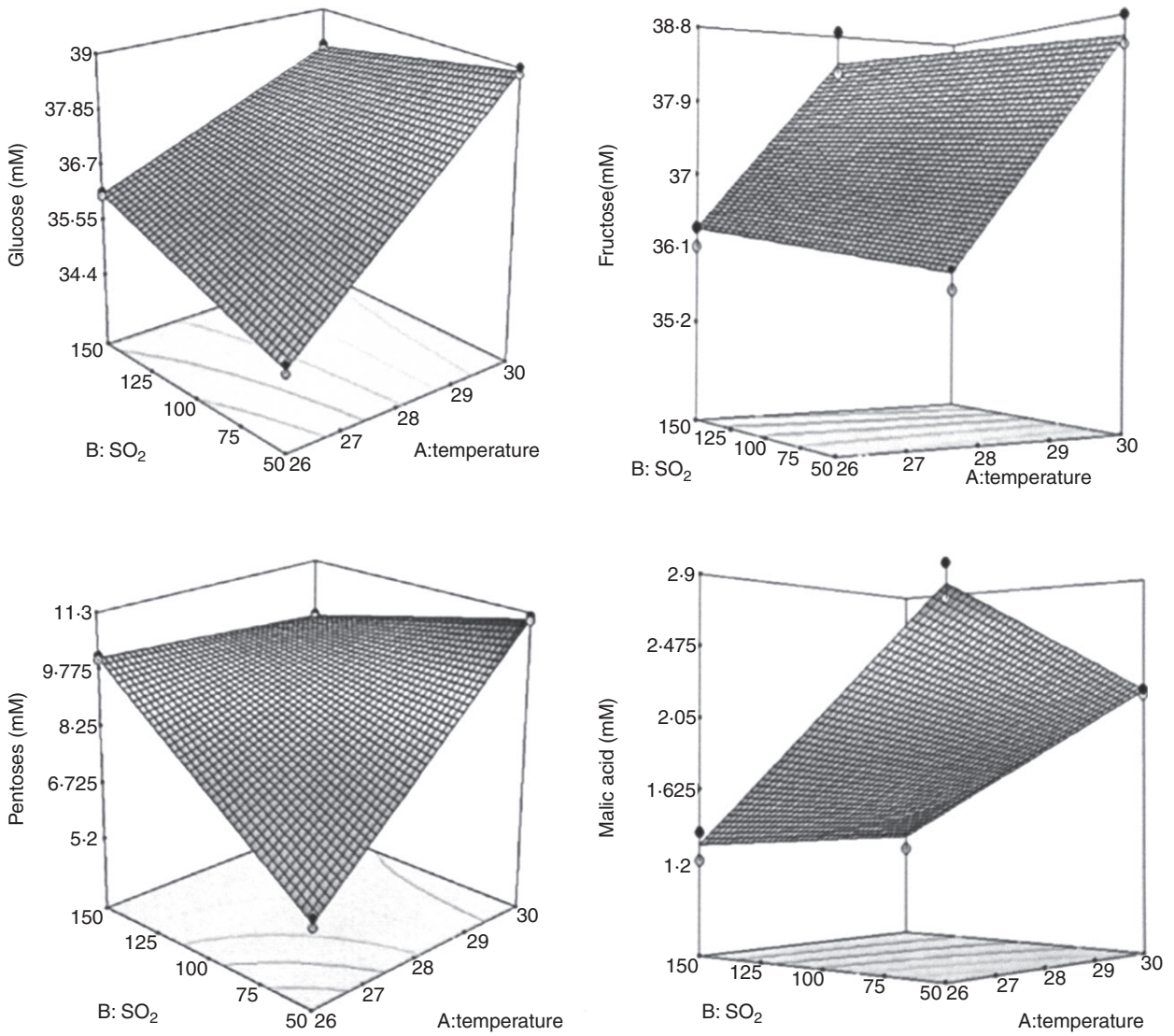

Figure 1 Response surface method of substrates consumption by simultaneous cultures of wine micro-organisms. SO_2 (mg l^{-1}), temperature (°C), initial pH = 5·5 (fixed factor).

150 mg l^{-1} SO_2 at high and low temperature values (approx. 11·3 mmol l^{-1}), and glucose, with high consumption (up to 38·8 mmol l^{-1}) only at 30°C. Lowest glucose and pentoses consumption was detected at 26°C and 50 mg l^{-1} SO_2 (Fig. 1).

Glucose consumption showed an increase when temperature increased at both high and low SO_2 concentrations (Fig. 2). For pentoses, however, this behaviour occurred only with the second condition (50 mg l^{-1} SO_2). In the above conditions, fructose consumption was not significant ($P \leq 0{\cdot}05$). Malic acid utilization increased when temperature fell at low SO_2 levels, while at high concentrations, no significant ($P \leq 0{\cdot}05$) modifications were observed (Fig. 2).

Products formation

The effect of physicochemical factors on products (D- and L-lactic and acetic acids, ethanol and glycerol) formation showed different responses. At 26°C, high ethanol (approx. 138 mmol l^{-1}) and L-lactic acid (approx. 2·8 mmol l^{-1}) production were detected when culture media were supplemented with 50 mg l^{-1} SO_2 (Fig. 3). However, highest glycerol (approx. 2·1 mmol l^{-1}) and D-lactic acid (approx. 13·2 mmol l^{-1}) concentrations were detected when SO_2 concentration increased (150 mg l^{-1}). Moreover, high glycerol synthesis (approx. 2 mmol l^{-1}) was observed when cultures grew between 50 and 150 mg l^{-1} SO_2 at 30°C. A similar behaviour was

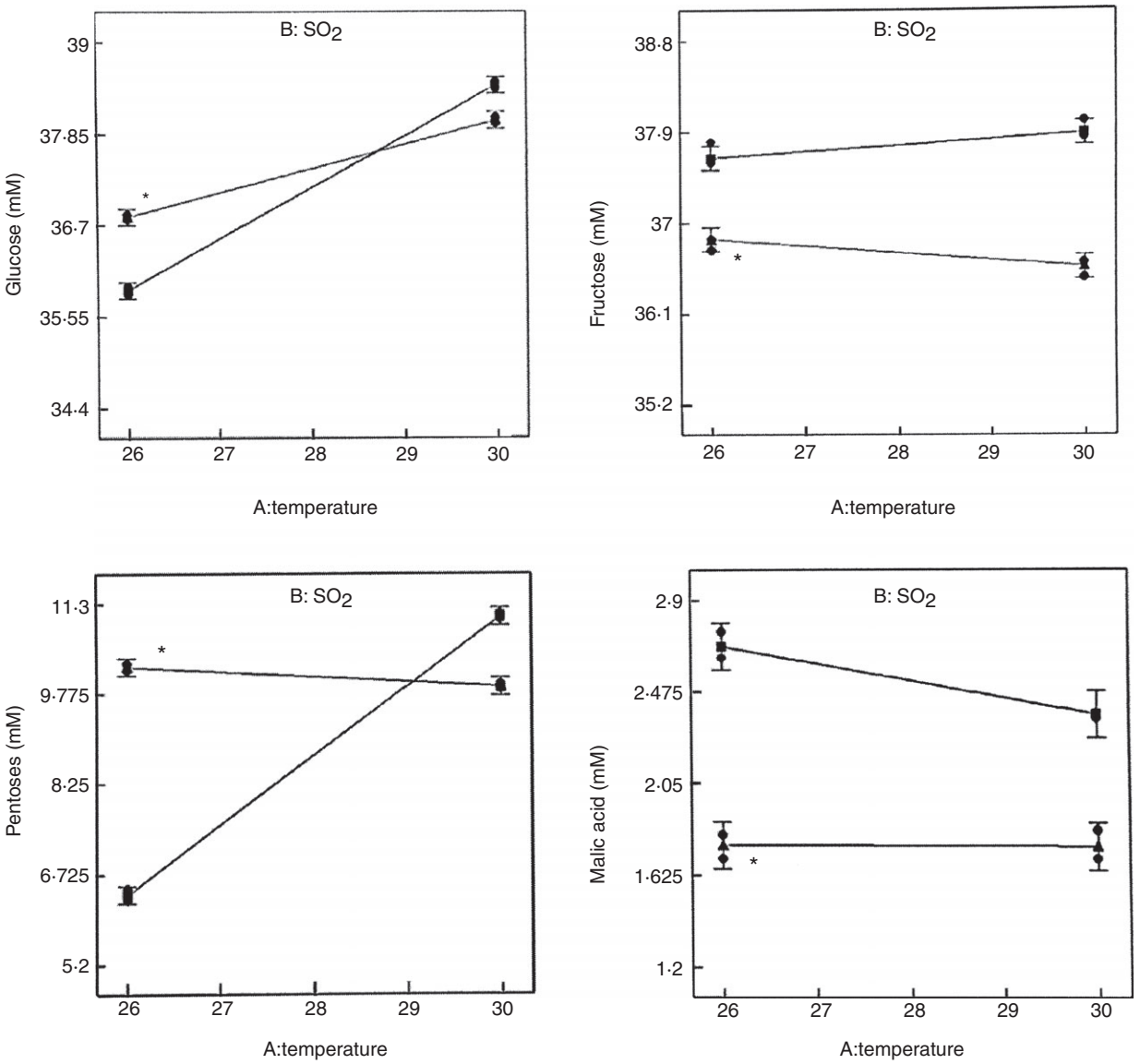

Figure 2 SO_2–temperature interaction on substrates consumption during simultaneous cultures of wine micro-organisms. [—] 50 mg l^{-1} SO_2, [*—] 150 mg l^{-1} SO_2, initial pH = 5·5 (fixed factor).

determined for acetic acid production, reaching a maximum of 11·7 mmol l^{-1} when culture conditions were 150 mg l^{-1} SO_2 and 30°C. In these conditions, ethanol synthesis also increased (Fig. 3).

Glycerol and D-lactic acid production showed an increase in negative interaction when temperature increased and SO_2 levels were low, the opposite effect being observed at high SO_2 concentrations (Fig. 4). A similar behaviour was observed for acetic acid production, but at high SO_2 concentrations, no differences were detected. However, ethanol synthesis exhibited an opposite negative interaction, increasing when the temperature rises at high SO_2 levels and diminishing at low SO_2 concentrations. With respect to L-lactic acid production, positive interaction was determined which increased when temperature diminished for both SO_2 concentrations (Fig. 4).

Optimal predictive estimations

Considering the requirements of the system to be designed, which involves increase in malic acid consumption and glycerol production, decrease in glucose and fructose consumption and maintenance of volatile acidity

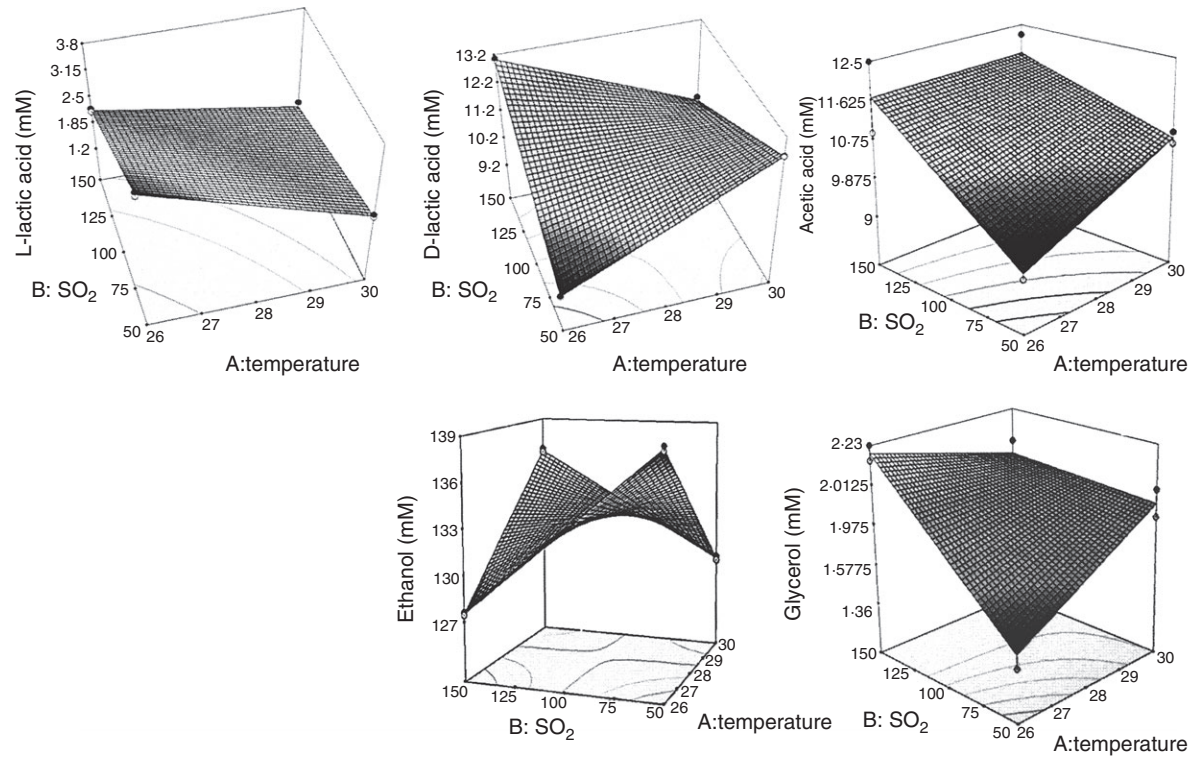

Figure 3 Response surface method of products formation by simultaneous cultures of wine micro-organisms. SO_2 (mg l^{-1}), temperature (°C), initial pH = 5·5 (fixed factor).

(≤1 g l^{-1}), 15 potential results using RSM were found (Table 4). As our aim was to study the increase in glycerol production in a mixed culture system of yeasts and LAB and to obtain lactic and acetic acid concentrations supporting the desirable organoleptic quality, condition 8 (26°C, 60·24 mg l^{-1} SO_2, pH 5·5—Table 4) was selected. In this condition, substrates consumption and products formation (in mmol l^{-1}) were as follows: glucose, 35·97; fructose, 37·56; pentoses, 6·77; malic acid, 2·60; D-lactic acid, 9·66; L-lactic acid, 3·51; acetic acid, 9·40; ethanol, 137·51; and glycerol, 1·50.

To evaluate condition 8 at laboratory scale, NGJ medium was supplemented with 60·24 mg l^{-1} SO_2, pH 5·5, inoculated with 10^6 CFU ml^{-1} of each micro-organism and incubated at 26°C for 6 days.

The results obtained were statistically studied using confidence intervals (99%) to evaluate the approximation of condition 8 to the experimental data (Table 4). All of them were within confidence intervals with the exception of glucose, which exceeded the corresponding interval right end by about 0·2 mmol l^{-1}.

Microbial growth in the selected condition was similar to the pattern shown in section 3·1. Yeasts were the predominant populations at 6 days, reaching a maximum of approx. 10^9 CFU ml^{-1}, *S. cerevisiae* mc_2 being the main strain. *Oenococcus oeni* X_2L population was approximately 10^7 CFU ml^{-1} and remained around this value until the end of the assay (data not shown).

Discussion

In this study, a statistical inferential technique was used to study the simultaneous influence of different winemaking factors on one or more responses by mixed cultures of *S. cerevisiae* mc_2, *K. apiculata* mF and *O. oeni* X_2L. The resulting response surfaces showed the effect of temperature, pH and SO_2 concentration on growth, substrates consumption and products formation.

The design of starter cultures for winemaking usually includes *Saccharomyces* yeasts and *O. oeni* strains inoculated in a sequential way (Alexandre *et al.* 2004; Comitini *et al.* 2005), although the use of apiculate strains represents a novel trend to improve wine flavour (de Benedictis *et al.* 2011; Jolly *et al.* 2013). In our work, the effect of physicochemical factors on the growth and metabolic behaviour of cocultured wine micro-organisms was evaluated to optimize fermentation aimed at improving the organoleptic characteristics of wine, especially those related to glycerol production. Thus, *S. cerevisiae* mc_2,

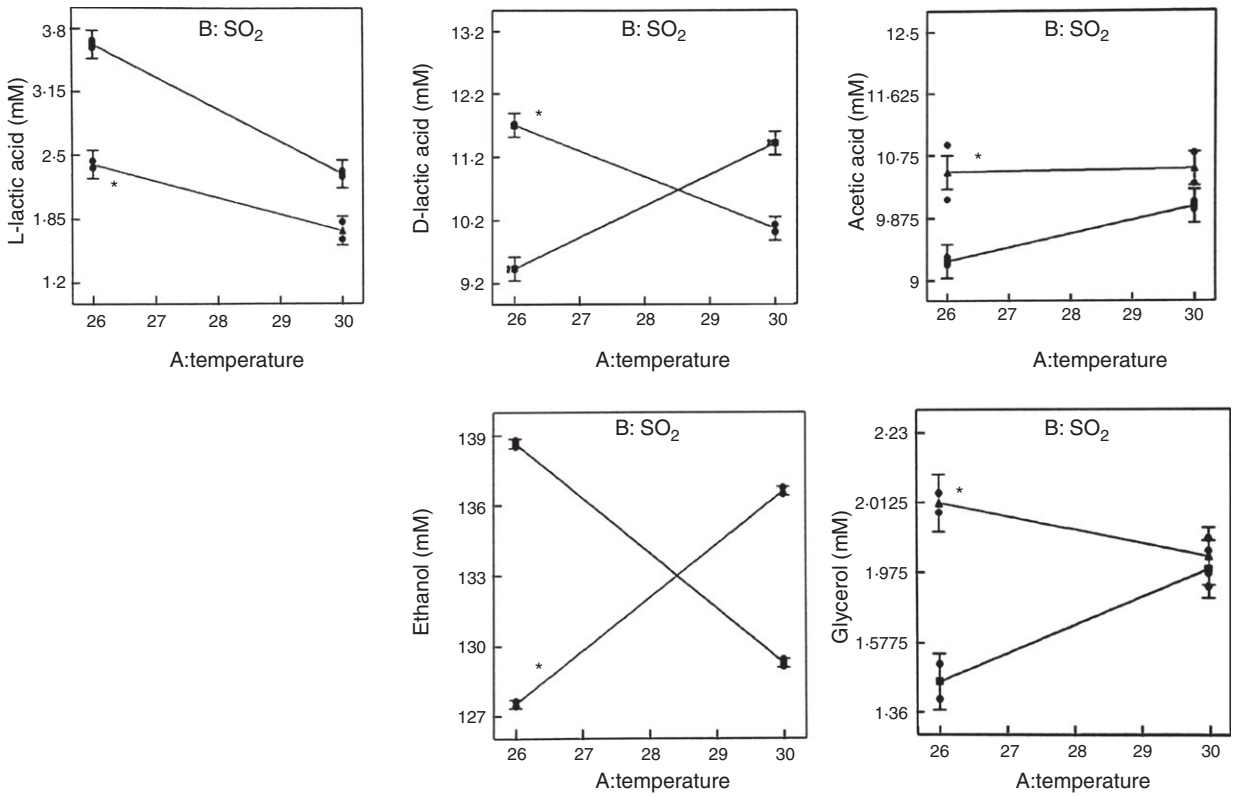

Figure 4 SO_2–temperature interaction on products formation during simultaneous cultures of wine micro-organisms. [—] 50 mg l^{-1} SO_2, [*—] 150 mg l^{-1} SO_2, initial pH = 5·5 (fixed factor).

Table 4 Optimal estimated solutions obtained with the response surface method for substrates consumption and products formation by *Saccharomyces cerevisiae* mc_2, *Kloeckera apiculata* mF and *Oenococcus oeni* X_2L in simultaneous cultures

				Substrates consumption (mmol l^{-1})				Products formation (mmol l^{-1})					
Solution	Temperature (°C)	SO_2 (mg l^{-1})	pH	Glucose	Fructose	Pentoses	Malic acid	D-lactic acid	L-lactic acid	Acetic acid	Ethanol	Glycerol	Desirability
1	26·00	50·00	5·50	35·88	37·64	6·38	2·64	9·43	3·64	9·27	138·65	1·48	0·757
2	26·00	50·78	5·50	35·89	37·64	6·41	2·65	9·45	3·63	9·28	138·56	1·47	0·756
3	26·00	51·14	5·50	35·89	37·64	6·42	2·64	9·46	3·63	9·28	138·52	1·48	0·755
4	26·00	50·22	5·48	35·88	37·64	6·42	2·64	9·44	3·62	9·28	138·59	1·48	0·753
5	26·07	50·00	5·50	35·93	37·65	6·47	2·65	9·47	3·61	9·28	138·47	1·48	0·747
6	26·00	50·00	5·43	35·88	37·65	6·51	2·67	9·43	3·58	9·32	138·51	1·47	0·741
7	26·17	50·00	5·50	35·88	37·66	6·58	2·66	9·51	3·58	9·30	138·24	1·47	0·734
8	26·00	60·24	5·50	35·96	37·56	6·77	2·60	9·66	3·51	9·40	137·51	1·50	0·734
9	26·19	50·00	5·50	36·00	37·66	6·61	2·62	9·53	3·58	9·31	138·20	1·48	0·731
10	26·00	67·12	5·50	36·04	37·51	7·04	2·64	9·82	3·43	9·48	136·75	1·50	0·716
11	26·00	75·02	5·50	36·11	37·44	7·34	2·65	9·99	3·33	9·58	135·87	1·47	0·693
12	26·00	56·21	5·22	35·93	37·61	7·12	2·58	9·56	3·33	9·55	137·50	1·57	0·677
13	26·00	150·00	4·11	36·71	36·90	8·89	2·55	10·06	2·87	11·84	130·60	1·60	0·454
14	26·00	150·00	4·19	36·71	36·90	8·97	2·51	10·15	2·84	11·76	130·42	1·56	0·453
15	26·00	150·00	4·02	36·71	36·90	8·81	2·51	9·96	2·90	11·92	130·78	1·49	0·453

K. apiculata mF and *O. oeni* X_2L were selected according to their ability to produce glycerol in NGJ medium and sequential cultures were found to be the best way of inoculation (Ale *et al.* 2014), but when the performance of both simultaneous and sequential cultures under standard winemaking conditions (NGJ adjusted at pH = 3·8,

supplemented with 125 mg l^{-1} SO_2 and incubated at 28°C) was previously evaluated by our research group, highest glycerol synthesis was found in simultaneous cultures and the statistical analysis of main effects revealed that these cultures performed at initial pH 5·5 had the best response, considering high final glycerol concentration, low acetate production and appropriate AF and MLF. Although pH 5·5 do not correspond with standard winemaking processes, it was selected according to the predictive model (RSM) to obtain high glycerol levels and low acetate production and represents only the initial pH value of the simultaneous cultures as this parameter was not controlled during the assay (6 days), so at the end of the microbial exponential growth phase, the cultures reached pH < 4·0 (data not shown). Thus, pH 5·5 would favour the non-*Saccharomyces* yeast strain growth by increasing glycerol production.

The design of the experiments carried out in the present study provided information about the effect of some variables of great impact on winemaking. Therefore, the influence of temperature, pH and SO_2 concentrations on substrates consumption and products formation was evaluated using RSM, and the data obtained were studied. Although many studies report the influence of different physicochemical factors on wine production, they were focused on the analysis of each factor in successive experiments (Henick-Kling *et al.* 1998; Hansem *et al.* 2001; Gawel *et al.* 2007; Yalcin and Ozbas 2008).

The growth and persistence of autochthonous *S. cerevisiae* mc_2, *K. apiculata* mF and *O. oeni* X_2L were also evaluated. When the simultaneous cultures were carried out in the experimental conditions specified by the RSM, all the micro-organisms showed high viability values. In a previous work, *S. cerevisiae* mc_2, *K. apiculata* mc_1 and *O. oeni* X_2L remained viable when inoculated in Malbec musts under winemaking conditions (Mendoza *et al.* 2011) in sequential and simultaneous cultures, reaching maximum population values of approx. 10^7 CFU ml^{-1}. However, *K. apiculata* mc_1 remained viable for a longer time period than *S. cerevisiae* mc_2 (Mendoza *et al.* 2009, 2011).

When the microbial system used in the present work was grown in NGJ without modifications (Ale *et al.* 2014) and varying SO_2 concentrations, pH and temperature of incubation, both yeasts and *O. oeni* X_2L remained viable until 6 days and no modifications in their growth patterns were observed. The increase in growth rate with temperature would indicate that this is the main factor involved in microbial growth kinetics (Table 3).

Microbial metabolic behaviour depends on temperature and pH changes as it was previously observed for both simple and mixed yeast cultures (Yalcin and Ozbas 2008). The simultaneous cultures carried out in this work achieved total malic acid consumption in all incubation conditions, which would indicate that MLF was performed successfully and the total sugar consumption was 75–85% (Figs 1 and 3). Mendoza *et al.* (2011) reported that simultaneous cultures with *S. cerevisiae* mc_2, *O. oeni* X_2L and *K. apiculata* mc_1 in Malbec musts under common winemaking conditions showed a similar behaviour considering that initial sugars concentration was 2·5 times higher than those used in our work (NGJ medium).

Ethanol content in wine mainly affects perceived hotness, body and viscosity, with a smaller effect on sweetness, acidity, aroma, flavour intensity and textural properties (Gawel *et al.* 2007). Its production by yeasts depends on the culture system (single or mixed), carbon source availability and microbial genes implicated on its utilization as well as temperature and osmotic stress conditions (Jackson 2000; Mendoza *et al.* 2007; Rossouw *et al.* 2013; Tilloy *et al.* 2014). In the present work, ethanol was the main metabolic product (127·83–138·80 mmol l^{-1}) and its production depended on the incubation temperature. As total ethanol production resulted from a mixed system containing three different micro-organisms, the values obtained were lower than those observed by Rossouw *et al.* (2012), in which the mixed system reached about 2000 mmol l^{-1} at 7 days culture in a synthetic medium inoculated with *S. cerevisiae–O. oeni.* In other mixed systems (*S. cerevisiae* mc_2–*K. apiculata* mc_1), incubation temperature affected ethanol production when cultures were grown in NGJ supplemented with yeast extract. In that case, ethanol concentration was 212·6–226·74 mmol l^{-1}, highest values being obtained at 30°C (Mendoza *et al.* 2009).

Reduction on ethanol content in wine is a novel trend. Nowadays, the consumption market requires wines with low alcohol because high ethanol concentrations reduce the perception of flavours and aroma and have negative effects on economy and human health (Contreras *et al.* 2014).

Glycerol synthesis by yeasts can be affected by microbial growth parameters and environmental factors (Albers *et al.* 1996; Remize *et al.* 2000). In our experimental conditions, glycerol production increased when temperature increased from 26 to 30°C, with low SO_2 concentrations. The opposite effect was observed when the antimicrobial compound was supplemented at high concentration, which could be related to changes in the reducing compounds levels. Thus, micro-organisms exhibited a different behaviour as glycerol production is directly related to this phenomenon. Few studies have shown that variations in temperature or osmotic conditions resulted in higher glycerol production (Remize *et al.* 2000; Tilloy *et al.* 2014). Differences in glycerol synthesis associated with

pH and temperature modifications by two *S. cerevisiae* strains in single cultures were reported (Yalcin and Ozbas 2008; Tilloy *et al.* 2014). Mendoza *et al.* (2009) observed maximum glycerol production (17·33 mmol l^{-1}) at 30°C when *S. cerevisiae* and *K. apiculata* (single and mixed cultures) strains were inoculated in NGJ medium supplemented with yeast extract and approx. 100 mg l^{-1} SO_2. A similar behaviour was reported by Rossouw *et al.* (2012), who demonstrated an increase in glycerol production by a mixed culture of *S. cerevisiae–O. oeni* strains in a synthetic grape medium at pH 5·5 without SO_2 supplementation and incubated at 30°C.

Volatile acids (mainly acetic acid) content in wines should not exceed 1·0–1·5 g l^{-1}, depending on the country (Eglinton and Henschke 1999). In our work conditions, acetic acid production did not exceed 0·72 g l^{-1} (approx. 12 mmol l^{-1}). This value is under the organoleptic quality limit, and therefore, the non-*Saccharomyces* strain does not act as a spoilage micro-organism, thus supporting its inclusion in a mixed starter culture.

L- and D-lactic acid production was under of 3·5 and 13 mmol l^{-1}, respectively. As major production of both isomers was found at opposite ends of the temperature/SO_2 combinations (Fig. 3), total lactic acid did not reach 15 mmol l^{-1} in any condition. Thus, total acidity due to D- and L-lactic and acetic acids would not affect the organoleptic quality of wines.

The challenge of the RSM using the experimental data is to find a condition that ensures low volatile acidity, high glycerol and ethanol concentrations and an effective MLF to obtain a high-quality wine. The results obtained in the present work demonstrate that maximum glycerol and ethanol values were obtained in opposite conditions (Fig. 3). However, 15 potential solutions by the RSM prediction were found (Table 4). Taking into account a desirability factor above 0·7, the predictive solution 8 (desirability = 0·734) would guarantee the proposed requirements. The results showed that the formulated medium together with the selected incubation condition could optimize the metabolic behaviour (substrates consumption and products formation) of the selected strains without affecting the oenological sensory profile. Thus, the best conditions for high glycerol (approx. 1·5 mmol l^{-1}) and low (<9·5 mmol l^{-1}) acetate concentrations were 26°C, approx. 60 mg l^{-1} SO_2 and initial pH 5·5. Low SO_2 content ensures the maintenance of the quality of the final product and is in keeping with the novel trend to diminish SO_2 concentrations and use alternative microbial controls (Santos *et al.* 2013). When these parameters were experimentally tested, confidence intervals showed that predictive model values were reproducible in most of the responses evaluated, confirming the condition selected for the simultaneous cultures of *S. cerevisiae* mc_2, *O. oeni* X_2L and *K. apiculata* mF. The results allowed optimizing the culture conditions to design mixed starter culture for winemaking with autochthonous micro-organisms to obtain products with high glycerol, low acetate contents and regional characteristics. However, further studies are necessary to validate these results in real winemaking conditions.

Acknowledgements

This research was supported by grants from Consejo de Investigaciones de la Universidad Nacional de Tucumán (26/D 436), Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 320) and Agencia Nacional de Promoción Científica y Tecnológica (PICT 847).

Conflict of Interest

The authors declare that there is no conflict of interests.

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