Journal of Basic Microbiology
Environment · Health · Techniques

Research Paper

Glycerol production by *Oenococcus oeni* during sequential and simultaneous cultures with wine yeast strains

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Growth and fermentation patterns of *Saccharomyces cerevisiae*, *Kloeckera apiculata*, and *Oenococcus oeni* strains cultured in grape juice medium were studied. In pure, sequential and simultaneous cultures, the strains reached the stationary growth phase between 2 and 3 days. Pure and mixed *K. apiculata* and *S. cerevisiae* cultures used mainly glucose, producing ethanol, organic acids, and 4.0 and 0.1 mM glycerol, respectively. In sequential cultures, *O. oeni* achieved about 1 log unit at 3 days using mainly fructose and L-malic acid. Highest sugars consumption was detected in *K. apiculata* supernatants, lactic acid being the major end-product. 8.0 mM glycerol was found in 6-day culture supernatants. In simultaneous cultures, total sugars and L-malic acid were used at 3 days and 98% of ethanol and glycerol were detected. This study represents the first report of the population dynamics and metabolic behavior of yeasts and *O. oeni* in sequential and simultaneous cultures and contributes to the selection of indigenous strains to design starter cultures for winemaking, also considering the inclusion of *K. apiculata*. The sequential inoculation of yeasts and *O. oeni* would enhance glycerol production, which confers desirable organoleptic characteristics to wines, while organic acids levels would not affect their sensory profile.

Keywords: Glycerol / Yeasts / *Oenococcus oeni* / Wine

Received: November 18, 2013; accepted: December 25, 2013

DOI 10.1002/jobm.201300934

Introduction

The fermentation of grape juice into wine represents a complex process that involves the sequential development of members of its indigenous microbiota, which is mainly constituted by yeasts and bacterial species [1]. Since grape must exerts a strong selective pressure on the microbiota because of its low pH and high sugar content, only a few species can proliferate, mainly under controlled conditions, *Saccharomyces cerevisiae*, *Kloeckera apiculata*, and *Oenococcus oeni* being the most representative ones. Sulfur dioxide addition, anaerobic conditions during winemaking, nutrients depletion, and high ethanol levels enhance the selective pressure [2–4].

Glycerol is a simple alcohol with many uses in the food, pharmaceutical, and textile industries [5]. Together with ethanol and carbon dioxide, this alcohol is the main product of fermentation by yeasts during wine and cider production [6, 7]. It contributes to smoothness and roundness on the palate and enhances the flavor components present in other fermented beverages; hence, its degradation has a negative effect on the sensorial quality of these products [8–10]. Glycerol dissimilation by homo- and heterofermentative lactic acid bacteria (LAB) from wine was reported to occur through different metabolic pathways [11–16]. However, it is interesting to point out that some LAB are able to produce glycerol to increase NAD^+ regeneration and ATP production in limiting nutrient media [5, 17–19].

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Oenococcus oeni is responsible for malolactic fermentation (MLF), which plays an important role in winemaking by diminishing acidity and contributing to both microbiological stability and complexity of flavor in wine [9, 10, 18, 20–22].

Yeast interactions have been found to affect glycerol metabolism dynamics since different microbial species can utilize the carbon sources available in the ecological niche [23] or they are able to produce antagonistic metabolites that inhibit the growth of starter cultures including potentially glycerol producing strains. Therefore, the final sensorial quality of wine can be modified [24, 25]. However, there is no information concerning glycerol production when *O. oeni* interacts with yeasts. Thus, the aim of this work was to carry out sequential and simultaneous cultures with indigenous wine yeast strains and *O. oeni* in grape juice medium to evaluate their growth kinetics, the modifications in glycerol production, and the carbon balances in order to select the best inoculation conditions.

Materials and methods

Microorganisms

Twenty-eight *Kloeckera apiculata* (apiculate yeast) strains and 32 *Saccharomyces cerevisiae* (elliptic yeast) strains isolated from Malbec grape must (northwest region of Argentina) [26], and 25 *Oenococcus oeni* isolated from an Argentinean wine [27] were used to select glycerol producing strains. All the microorganisms 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.

Media and culture conditions

Yeast strains were grown in YEPG medium (in g L^{-1}: yeast extract, 10; peptone, 20; glucose, 20), pH 5.5, while *O. oeni* X_2L was grown in MRS medium [28] supplemented with 150 ml L^{-1} of natural tomato juice (MRStj), pH 4.8. Tomato juice was added as a pantothenic acid source for *Oenococcus oeni* growth [20, 29].

To simulate winemaking conditions, the strains were sub-cultured in a medium formulated with natural grape juice (170 ml L^{-1}) and tween 80 (0.1 ml L^{-1}) (NGJ-T), pH 4.5. NGJ-T contains (in mM): glucose, 45.6; fructose, 43.97; pentoses, 12.5; and malic acid, 3.05. Solid media were prepared by adding 15 g L^{-1} agar to NGJ-T broth. Culture media were sterilized by autoclaving at 121 °C for 30 min, with the exception of NGJ-T, which was heated at 90 °C for 10 min to prevent thermal decomposition of the grape juice.

In all the assays, the yeast and LAB strains were grown at 28 and 30 °C, respectively, in microaerophilic conditions.

For sequential cultures assays, 50 ml NGJ-T medium was inoculated with a 14 h pre-culture of: (1) 10^6 cfu ml^{-1} *K. apicultata* mF; (2) 10^6 cfu ml^{-1} *S. cerevisiae* mc_2; (3) 10^6 cfu ml^{-1} *K. apiculata* mF and 10^6 cfu ml^{-1} *S. cerevisiae* mc_2. In all cases, the yeast strains were grown for 2 and 6 days under the above conditions. The cultures were centrifuged at 8000 × *g* for 10 min at 4 °C, filtered through a 0.22 μm pore-size membrane (Millipore) and kept at −20 °C until *O. oeni* X_2L inoculation (sequential cultures) and analytical determinations.

Bacterial cells were collected under the above conditions, resuspended in sterile distilled water and then inoculated into the yeast culture supernatants to achieve 10^6 cfu ml^{-1}. At different time intervals, samples were taken for both growth (each day) and analytical determinations (2 and 6 days).

The concentration of sugars and L-malic acid determined in the yeast culture supernatants at 2 and 6 days of fermentation are shown in Table 1.

For simultaneous cultures, the yeast strains and *O. oeni* X_2L were co-inoculated into 50 mL NGJ-T medium to reach 10^6 cfu ml^{-1} of each and incubated for 6 days at 30 °C in microaerophilia (unshaken capped tubes or flasks two-thirds full). Samples were taken for both

Table 1. Sugars and L-malic acid concentrations in yeast culture supernatants.

		Substrates (mM)			
Culture	**Time (days)**	**Glucose**	**Fructose**	**Pentoses**	**Malic acid**
K. apiculata	2	7.49 ± 0.15[a]	10.46 ± 0.21	5.44 ± 0.08	3.28 ± 0.03
	6	2.9 ± 0.09	5.69 ± 0.11	3.94 ± 0.16	3.04 ± 0.1
S. cerevisiae	2	1.03 ± 0.16	3.68 ± 0.16	1.69 ± 0.03	2.95 ± 0.06
	6	0	0	0	2.97 ± 0.18
Mixed	2	1.02 ± 0.23	3.99 ± 0.08	3.56 ± 0.27	2.99 ± 0.31
	6	0.29 ± 0.13	0.02 ± 0.05	0.00 ± 0.3	2.99 ± 0.17

[a]Data represents the mean of three consecutive trials ± SD.

growth (each day) and analytical determinations (3 and 6 days).

The components for culture media preparation were supplied by Britania laboratories (Buenos Aires, Argentina) and Sigma–Aldrich Chemical Co. (St. Louis, MO, USA).

Determination of microbial growth and differential cell enumeration

Sequential cultures. Growth was evaluated by determining the number of viable cells (cfu ml^{-1}) on YEPG agar and Optical Density (OD_{620nm}) by using a UV/vis WPA-biowave DNA spectrophotometer for the yeast strains while MRStj agar and determination of OD_{560nm} were used for *O. oeni* X_2L. The samples were evaluated every day for 6 days.

Simultaneous cultures. Growth was determined by measuring the number of viable cells. In order to discriminate between *Sacharomyces* and non-*Saccharomyces* yeasts from mixed cultures, samples were plated on YEPG supplemented with ethanol (120 ml L^{-1}), sodium metabisulfite (0.15 g L^{-1}), and chloramphenicol (1 g l^{-1}) for the elliptic yeast, while YEPG supplemented with cycloheximide (0.01% w/v) was used for the apiculate strain.

The samples were also plated on MRStj agar supplemented with cycloheximide (0.1% w/v) to evaluate *O. oeni* X_2L growth.

Analytical determinations

Cell-free supernatants were obtained from each experiment and stored at −20 °C for further analytical determinations. Glucose, ethanol, glycerol, and organic acids (total lactic, acetic, and malic) were quantified using kits supplied by Boehringer-Mannheim, Inc. (Germany). Fructose concentration was determined using the method of Roe [30] while total reducing sugars concentration was determined according to the technique of Somogyi–Nelson, which measures the number of reducing groups using D-galacturonic acid as a standard; after incubation, the enzymatic reaction was stopped by the Cu alkaline reagent [31] and reducing power was determined according to Nelson [32].

Chemicals used were purchased from Sigma–Aldrich Chemical Co.

Statistical analysis

The values shown correspond to the mean of three separate assays. One-way analysis of variance (ANOVA) was applied to the experimental data by using Student's *t*-test for multiple mean comparisons (95% confidence interval). Statistical treatments were performed using INFOSTAT software (2012 student version).

Results

Selection of *Kloeckera apiculata*, *Saccharomyces cerevisiae*, and *Oenococcus oeni* strains

K. apiculata mF was selected according to its ability to produce glycerol (32 mM) while *S. cerevisiae* mc_2, despite its low glycerol production (3.9 mM), was chosen because of its high fermentation power (2.68×10^{-3} mM/UFC × h) (data not shown). Only *O. oeni* X_2L was selected according to its capability of producing glycerol (9.8 mM).

Growth of yeast strains in pure and mixed cultures

Growth parameters of *K. apiculata* mF and *S. cerevisiae* mc_2 in pure and mixed cultures as well as in simultaneous cultures with *O. oeni* X_2L were evaluated.

K. apiculata mF in pure cultures reached a maximum of 1×10^{11} cfu ml^{-1} at 2 days (relative growth = 61.87%) and decreased below the initial value (13.35%) at 6 days. In mixed and simultaneous cultures, the strain reached the stationary phase at 3 days (~10^7 cfu ml^{-1}) and viable cell counts remained constant up to 6 days, relative growth being 30.54 and 27.87%, respectively.

In all culture conditions, *S. cerevisiae* mc_2 reached the stationary growth phase at 3 days. In pure cultures, the strain achieved 7×10^{11} cfu ml^{-1}, which represents a relative growth of 96.52%, and decreased up to 64.57% at 6 days. In mixed and simultaneous cultures, maximum growth values were about 10^8 cfu ml^{-1} (relative growth = 45.74 and 43.65%, respectively).

Growth of *Oenococcus oeni* X_2L in pure, sequential and simultaneous cultures

In pure cultures, *O. oeni* X_2L grew at a rate of 0.22 h^{-1} and reached the end of the exponential phase at 3 days (3.9×10^7 cfu ml^{-1}, relative growth = 28.91%). At 6 days, biomass increased up to 9.7×10^7 cfu ml^{-1} (relative growth = 35.71%) (Table 2).

When *O. oeni* was inoculated into 2- and 6-day *K. apiculata* mF culture supernatants (sequential cultures), the stationary phase was reached at approximately 2 days (1×10^7 cfu ml^{-1}). Cell populations increased until 3 days, mainly in 2-day culture supernatants, and decreased afterwards. At the end of the assay (6 days), the relative growth of *O. oeni* was 7.16 and 8.71% in 2- and 6-day yeast culture supernatants, respectively. However, when *O. oeni* X_2L was inoculated into *S. cerevisiae* mc_2 culture supernatants, the LAB strain reached the stationary phase after 3–4 days in 2-day culture supernatants (1×10^7 cfu ml^{-1}), which represents an increase of 1.9%. However, at 6 days a decrease of 4.73% (0.95 cell viability) below the initial cells

Table 2. Growth parameters of *Oenococcus oeni* X_2L.

Cultures		Growth parameters	Time (days)						
			0	1	2	3	4	5	6
Pure culture		A	5.88	5.89	5.95	7.58	7.75	7.91	7.98
		B	–	0.17	1.19	28.91	31.80	34.52	35.71
		C	–	1.00	1.01	1.29	1.32	1.35	1.36
KA culture supernatant	2 Days	A	5.59	6.72	7.83	7.93	6.98	6.55	5.99
		B	–	20.21	40.07	41.86	24.87	17.17	7.16
		C	–	1.20	1.40	1.42	1.25	1.17	1.07
	6 Days	A	5.51	6.51	6.99	7.00	6.97	6.65	5.99
		B	–	18.15	26.86	27.04	26.50	20.69	8.71
		C	–	1.18	1.27	1.27	1.26	1.21	1.09
SC culture supernatant	2 Days	A	5.28	5.94	6.12	6.91	7.01	6.87	5.03
		B	–	12.50	15.91	30.87	32.77	30.11	−4.73
		C	–	1.13	1.16	1.31	1.33	1.30	0.95
	6 Days	A	5.89	0	0	0	0	0	0
		B	–	0	0	0	0	0	0
		C	–	0	0	0	0	0	0
MY culture supernatant	2 Days	A	5.99	6.69	6.99	7.00	7.53	7.54	7.53
		B	–	11.69	16.69	16.86	25.71	25.88	25.71
		C	–	1.12	1.17	1.17	1.26	1.26	1.26
	6 Days	A	5.99	5.99	6.00	6.00	5.96	5.96	5.95
		B	–	0	0.17	0.17	−0.50	−0.50	−0.67
		C	–	1.00	1.00	1.00	0.99	1.00	1.00
Simultaneous culture		A	5.99	6.75	6.99	7.06	7.17	7.2	7.2
		B	–	12.69	16.69	17.86	19.70	20.20	20.20
		C	–	1.13	1.04	1.01	1.02	1.00	1.00

KA, *K. apiculata* mF; SC, *S. cerevisiae* mc_2; MY, mixed yeast. (A) Cells number ($\log$ cfu ml^{-1}); (B) relative growth (%) $= (N_t - N_0/N_0) \times 100$; (C) cell viability $= \log N_t/\log N_0$. N_0, initial viable cell number; N_t, viable cell number.

number was observed. Moreover, the LAB strain did not grow in 6-day *S. cerevisiae* culture supernatants (Table 2).

When *O. oeni* X_2L was inoculated into supernatants of mixed cultures of both yeasts, maximum population (3.4×10^7 cfu ml^{-1}) was detected at 4 days (relative growth = 25.71%) in 2-day yeast culture supernatants, while in 6-day culture supernatants the initial population remained constant up to 3 days and then decreased slightly at the end of assay (relative growth = −0.67%). In these sequential culture conditions, growth rates were lower than in pure cultures.

In simultaneous cultures, the growth rate of *O. oeni* was 0.1 h^{-1}, the stationary phase started at 2 days (9.9×10^6 cfu ml^{-1}) and cell counts remained constant up to 6 days (Table 2).

Substrates consumption and end-products formation

Kloeckera apiculata mF and Saccharomyces cerevisiae mc_2 in pure and mixed cultures. Substrates consumption and products formation by yeast strains in pure and mixed cultures were quantified (Table 3).

In pure cultures, both strains consumed mainly glucose and fructose. *K. apiculata* mF consumed 76 and 87% of total sugars at 2 and 6 days of culture, respectively, while *S. cerevisiae* mc_2 utilized 94% at 2 days.

The major end-product for both yeast strains was ethanol, *S. cerevisiae* mc_2 being the higher producer strain, reaching 198 mM at 2 days of growth, without significant differences ($p \leq 0.05$) at 6 days. By this time, *K. apiculata* only produced 139 mM ethanol.

In mixed cultures, at 2 days, 91.3% of the total sugars were consumed and 95% of ethanol production (153 mM) was detected. At 6 days of culture, ethanol production was 159.15 mM.

With respect to organic acids, the highest lactic acid production was observed in pure cultures of *K. apiculata* mF in 2- and 6-day culture supernatants (2.66 and 4.33 mM, respectively), while in mixed cultures a higher production was detected (3.51 and 4.92 mM, respectively). *S. cerevisiae* mc_2 produced 0.33 mM of lactic acid at 6 days.

With respect to acetic acid, pure cultures of *K. apiculata* mF produced 17.31 and 13.67 mM at 2 and 6 days, respectively. In the same conditions, acetic acid production by *S. cerevisiae* was lower. Thus, the organic acid concentration found in mixed cultures was synthesized by the apiculate yeast.

Table 3. Substrates consumption and products formation by *S. cerevisiae* mc_2 and *K. apiculata* mF in pure and mixed cultures.

		Substrates consumption (mM)				Products (mM)					
Time	Culture	Glucose	Fructose	Pentoses	Malic acid	Lactic acid	Acetic acid	Ethanol	Glycerol	CO_2*	% CR
2 Days	KA pure	$37.89 \pm 0.16^{\#a}$	10.06 ± 0.11^{a}	7.10 ± 0.20^{a}	0.05 ± 0.02^{a}	2.66 ± 0.41^{a}	17.31 ± 0.14^{a}	123.58 ± 0.58^{a}	3.91 ± 0.04^{a}	140.9	97
	SC pure	45.88 ± 0.23^{a}	41.84 ± 0.6^{a}	9.33 ± 0.15^{a}	0.04 ± 0.01^{a}	0.18 ± 0.03^{a}	2.02 ± 0.01^{a}	198.53 ± 0.78^{a}	0.05 ± 0.02^{a}	200.6	105.1
	MY	42.95 ± 0.43^{a}	40.13 ± 0.78^{a}	6.91 ± 0.17^{a}	0.03 ± 0.02^{a}	3.51 ± 0.12^{a}	17.5 ± 0.12^{a}	152.84 ± 0.96^{a}	2.89 ± 0.05^{a}	170.3	99.43
6 Days	KA pure	42.49 ± 0.24^{b}	36.87 ± 0.20^{b}	8.60 ± 0.04^{b}	0.33 ± 0.24^{a}	4.33 ± 0.40^{b}	13.67 ± 0.17^{b}	139.52 ± 1.08^{b}	4.38 ± 0.02^{b}	153.5	93.37
	SC pure	46.87 ± 0.16^{a}	45.52 ± 0.12^{b}	11.02 ± 0.38^{b}	0.02 ± 0.01^{a}	0.33 ± 0.04^{b}	2.84 ± 0.13^{b}	199.12 ± 0.67^{a}	0.10 ± 0.01^{b}	202	99.63
	MY	43.69 ± 0.36^{a}	44.09 ± 0.28^{b}	10.48 ± 0.1^{b}	0.02 ± 0.01^{a}	4.92 ± 0.05^{b}	19.86 ± 0.18^{b}	159.15 ± 0.98^{b}	3.56 ± 0.02^{b}	179.03	97.12

KA, *K. apiculata* mF; SC, *S. cerevisiae* mc_2; MY, mixed yeast. #Data represents the mean of three consecutive trials ± SD. [a,b]Indicate significant differences in substrates consumption and products formation between 2 and 6 days of fermentation for each condition tested ($p \leq 0.05$). *CO_2 concentration was calculated considering its generation by pyruvate dehydrogenase: 1 mol of CO_2/mol of acetate, 1 mol of CO_2/mol of ethanol, and 1 mol/mol of hexose catabolized. % CR, carbon recovery percentage.

Table 4. Substrates consumption and products formation by *O. oeni* X_2L in cell-free supernatants of *S. cerevisiae* mc_2 and *K. apiculata* mF.

	2-Day fermented supernatants									
	Substrates consumption (mM)				Products (mM)					
Culture	Glucose	Fructose	Pentoses	Malic acid	Lactic acid	Acetic acid	Ethanol	Glycerol	CO_2*	% CR
KA supernatant	$4.0 \pm 0.12^{a\#}$	10.06 ± 0.11^{a}	3.79 ± 0.12^{a}	3.02 ± 0.2^{a}	14.1 ± 0.13^{a}	8.26 ± 0.21^{a}	10.9 ± 0.32^{a}	4.8 ± 0.02^{a}	22.18	98.82
SC supernatant	0.73 ± 0.02^{a}	1.75 ± 0.07^{a}	1.01 ± 0.02^{a}	2.95 ± 0.84^{a}	3.43 ± 0.07^{a}	3.01 ± 0.02^{a}	2.88 ± 0.14^{a}	0.23 ± 0.02^{a}	8.16	97.44
MY supernatant	0.67 ± 0.08^{a}	3.11 ± 0.05^{a}	3.32 ± 0.01^{a}	2.9 ± 0.2^{a}	4.86 ± 0.02^{a}	3.49 ± 0.42^{a}	5.0 ± 0.05^{a}	1.05 ± 0.04^{a}	11.38	90.5
	6-Day fermented supernatants									
KA supernatant	2.88 ± 0.45^{b}	5.49 ± 0.05^{b}	3.93 ± 0.09^{a}	3.24 ± 0.2^{a}	8.5 ± 0.1^{b}	5.45 ± 0.02^{b}	3.99 ± 0.14^{b}	8.0 ± 0.04^{b}	12.68	97.86
SC supernatant	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0	0
MY supernatant	0.28 ± 0.12^{b}	0.02 ± 0.01^{b}	0^{b}	2.96 ± 0.17^{a}	2.86 ± 0.08^{b}	0.12 ± 0.01^{b}	0.12 ± 0.01^{b}	0.05 ± 0.01^{b}	3.2	90.4

KA, *K. apiculata* mF; SC, *S. cerevisiae* mc_2; MY, mixed yeast. #Data represents the mean of three consecutive trials ± SD. [a,b]Indicate significant differences in substrates consumption and products formation between 2 and 6 days of fermentation for each condition tested ($p \leq 0.05$). *CO_2 concentration was calculated considering its generation by pyruvate dehydrogenase: 1 mol of CO_2/mol of acetate, 1 mol of CO_2/mol of ethanol, and 1 mol/mol of hexose catabolized. % CR, carbon recovery percentage.

With respect to glycerol, in pure cultures *K. apiculata* mF produced 3.91 and 4.38 mM at 2 and 6 days, respectively, while *S. cerevisiae* mc_2 failed to show significant production ($\leq$0.1 mM). In mixed cultures, glycerol production was 2.89 and 3.56 mM at 2 and 6 days, respectively.

In all these cultures, the yeasts did not use L-malic acid and carbon recovery was above 93%. The final pH of the cultures ranged from 4 to 4.2 at 2 days and 3.9 at 6 days.

Sequential cultures

Substrates consumption and end-products formation by *O. oeni* X_2L inoculated in supernatants from pure and mixed yeast cultures were determined (Table 4).

The LAB strain consumed all sugars, especially fructose. The highest sugar consumption values were detected in cell-free supernatants of pure cultures of *K. apiculata* mF at 2 and 6 days. The lowest values of sugars consumption in 2-day supernatants of pure cultures of *S. cerevisiae* mc_2 are related to the low concentration of sugars in this medium (Table 1). Thus, *O. oeni* did not grow in 6-day supernatants of this elliptic strain (Table 4). When the LAB strain was inoculated into mixed yeast culture supernatants, consumption of sugars was also low due to their diminished availability after yeasts growth. L-Malic acid was completely consumed by *O. oeni* X_2L during the first 2 days ($p \leq 0.05$).

Overall, lactic acid was the major end-product. The highest values were detected in *K. apiculata* mF supernatants (14.1 and 8.5 mM at 2 and 6 days, respectively) while in 2-day *S. cerevisiae* mc_2 supernatants the organic acid production was 3.43 mM. The low lactic acid concentration detected in 6-day supernatants of yeasts mixed cultures is directly related to L-malic acid consumption because the availability of sugars was low.

The highest acetic acid (8.26–5.45 mM) and ethanol (10.9–3.99 mM) synthesis was detected in *K. apiculata* mF supernatants at 2 and 6 days, respectively. In *S. cerevisiae* culture supernatants the LAB strain produced 3.01 mM acetate and 2.88 mM ethanol at 2 days.

In 2-day mixed culture supernatants, both acetic acid and ethanol production were lower than in *K. apiculata* culture supernatants but higher than in *S. cerevisiae* culture supernatants.

The *O. oeni* strain produced 4.8 and 8.0 mM glycerol in *K. apiculata* supernatants at 2 and 6 days, respectively. Lower glycerol concentrations were detected in supernatants from pure cultures of *S. cerevisiae* and mixed cultures at 2 days. In these supernatants, no glycerol production was detected at 6 days.

Carbon recovery was between 90.4 and 98.8% at 2 and 6 days.

Simultaneous cultures

Substrates consumption and end-products formation by *O. oeni* X_2L inoculated simultaneously with *S. cerevisiae* and *K. apiculata* mF were determined (Table 5).

In these culture conditions, 96% (96.1 mM) of total sugars and 65.9% of malic acid (1.91 mM) were consumed at 3 days of culture (Table 5). At this time, ethanol was the major end-product (98.8%, 157 mM). 96.8% acetate (19.7 mM) and 65.2% of total lactic acid (7.7 mM) were also detected. Moreover, glycerol production reached 2.54 mM, representing 98% of the total production. At 6 days, substrate consumption and end-products formation were lower. Carbon recovery was 98.7 and 93.5% at 3 and 6 days, respectively.

Discussion

Nowadays there is a tendency to use modified microorganisms to enhance metabolic pathways in solid and liquid cultures by using different substrates [33, 34]. In winemaking, these genetic tools are used to increase glycerol production; however, ethanol synthesis is

Table 5. Substrates consumption and products formation in simultaneous cultures of *S. cerevisiae* mc_2, *K. apiculata* mF and *O. oeni* X_2L.

3 Days of fermentation									
Substrates consumption (mM)				Products (mM)					
Glucose	Fructose	Pentoses	Malic acid	Lactic acid	Acetic acid	Ethanol	Glycerol	CO_2*	% CR
$43.6 \pm 0.14^{\#a}$	39.7 ± 0.3^{a}	12.8 ± 0.09^{a}	1.91 ± 0.01^{a}	7.7 ± 0.08^{a}	19.7 ± 0.12^{a}	157 ± 0.45^{a}	2.54 ± 0.06^{a}	179	98.7
6 days of fermentation									
1.23 ± 0.06^{b}	2.11 ± 0.03^{b}	0.10 ± 0.01^{b}	0.99 ± 0.07^{b}	4.11 ± 0.07^{b}	0.64 ± 0.04^{b}	1.88 ± 0.08^{b}	0.05 ± 0.02^{b}	5.41	93.5

$^{\#}$Data shown represents the mean of three consecutive trials $\pm$ SD. ^{a,b}Indicate significant differences in substrates consumption and products formation between 3 and 6 days of fermentation for each condition tested ($p \leq 0.05$). *CO_2 concentration was calculated considering its generation by pyruvate dehydrogenase: 1 mol of CO_2/mol of acetate, 1 mol of CO_2/mol of ethanol, and 1 mol/mol of hexose catabolized. % CR, carbon recovery percentage.

affected, so wines with low ethanol contents are obtained [35]. Therefore, the utilization of starter cultures designed with autochthonous microbial consortia that include yeasts (*Saccharomyces cerevisiae* and *Kloeckera apiculata*) and a lactic acid bacterium (*Oenococcus oeni*) would ensure the regular development of both alcoholic and malolactic fermentation of grape must and thus the wine flavor. The synthesis of end-products would be conditioned by the expression of metabolic pathways, microbial interactions and inoculation strategies of the grape must [18, 36]. Several works have described the sequential and simultaneous inoculation of *O. oeni* and *S. cerevisiae* to improve the final characteristics of wines [35–38]. Consequently, the aim of this work was to select indigenous yeasts and lactic acid bacteria (LAB) strains so as to further knowledge of microbial population dynamics, metabolic behavior, and inoculation strategies in order to increase glycerol production, which confers desirable features to wines by enhancing sweetness, smoothness, and overall body [8–10, 35].

K. apiculata mF and *S. cerevisiae* mc_2 were selected because of their ability to produce glycerol and ethanol, respectively, while *O. oeni* X_2L was chosen for its capacity to decarboxylate malic to lactic acid when cultured in grape juice medium.

In pure and mixed cultures, *S. cerevisiae* mc_2 exhibited growth rates higher than *K. apiculata* mF, but both strains reached the stationary phase at 2–3 days and maintained cell viability until 6 days. Previous studies [38] indicated that mixed cultures of *S. cerevisiae* mc_2 and another *K. apiculata* strain (mc_1) in the same culture medium used in our work had lower growth rates, but cells remained viable during a longer time period than in pure cultures. Our results are in agreement with Moreira *et al*. [39], who reported a similar growth rate for pure and mixed cultures of *S. cerevisiae* PYCC 3507 and *K. apiculata* PYCC 4193T in YM medium. However, Pérez-Nevado *et al*. [40] demonstrated that in mixed cultures of *K. apiculata* PYCC 4193T and another *S. cerevisiae* strain in synthetic grape juice media the apiculate yeast was unable to grow and cell viability decreased after 24 h. The authors attributed this effect to the physical presence of *S. cerevisiae* and/or to the toxic metabolites produced.

Substrates consumption and products formation were also determined (Table 3). Overall, both yeast strains consumed 80–90% of total sugars at 2 and 6 days, respectively. Mendoza *et al*. [38] reported that *S. cerevisiae* mc_2 and another *K. apiculata* strain consumed 200 mM of total sugars by producing 249.13 and 238.59 mM ethanol, respectively in pure cultures and 210.21 mM in mixed cultures. However, we observed a higher relationship between ethanol production and sugars consumption because the culture media used in our experimental conditions had half the sugars concentration than that used by these authors.

With respect to lactic and acetic acids, high production levels were detected in *K. apiculate* mF pure supernatants being the highest in yeasts mixed cultures, especially at 6 days (Table 3). This finding indicates that the apiculate strain is responsible for organic acids production in mixed cultures. Ethanol levels were intermediate between those observed in the pure cultures of each strain. A previous study [38] showed that *K. apiculata* mc_1 produced higher organic acids concentrations when grown in pure cultures, which was related to lower ethanol synthesis.

Although *S. cerevisiae* synthesizes acetic acid during sugar utilization in a synthetic medium [18, 35], high production level is a common pattern among apiculate yeasts and therefore they are considered as spoilage microorganisms [41, 42]. In our experimental conditions, the acetic acid concentrations detected ($<$20 mM) were within the allowed limits for wine organoleptic quality (Table 3).

On the other hand, high glycerol synthesis was detected in *K. apiculata* mF supernatants (Table 3), as demonstrated for other apiculate strains [37, 43]. However, the diminution observed in mixed cultures could be related to the decrease in *K. apiculata* mF growth in the presence of *S. cerevisiae*.

O. oeni X_2L growth and metabolic behavior were evaluated only when the strain was inoculated in cell-free supernatants of pure and mixed cultures of *S. cerevisiae* mc_2 and *K. apiculata* mF (sequential cultures). In our experimental conditions, *O. oeni* grew or maintained both viability and metabolic activity in all yeast supernatants, with the exception of 6-day *S. cerevisiae* mc_2 supernatants (Table 2). This inhibition in *O. oeni* X_2L growth would not be unusual since *S. cerevisiae* was reported to produce peptide-like compounds against this species [36, 37]. However, a decrease in inhibition was observed when the strain was grown in mixed yeast supernatants.

With respect to sugars consumption, *O. oeni* X_2L used mainly fructose, as reported by Rodriguez and Manca de Nadra [44], when the strain was grown in commercial grape juice medium supplemented with yeast extract. In all culture conditions studied in this work, lactic acid production by *O. oeni* was related to both sugars and L-malic acid utilization, the latter equaling or exceeding the 1:1 substrates/products ratio ($\sim$3 mM) (Table 4), which would indicate that MLF was successful.

In 2- and 6-day *K. apiculata* culture supernatants (sequential cultures), the LAB strain produced the highest

acetate, ethanol, and glycerol concentrations, which may be associated with the higher substrates availability. This fact would allow the maintenance of *O. oeni* growth and viability because of its ability to obtain reducing power and ATP, as reported for other strains of this species [16, 17, 19]. However, in 2-day *S. cerevisiae* and mixed yeast cultures supernatants the sugars availability was lower, with the exception of pentoses in 2-day mixed cultures. This could provide a carbon source and therefore, both higher *O. oeni* growth and acetate, ethanol and glycerol production compared to 6-day culture supernatants. The metabolic behavior of *O. oeni* X_2L was also observed for other strains of this species that produce acetate and erythritol during growth as an alternative way to regenerate reducing power [17, 19, 29].

In simultaneous cultures, the growth of each microbial strain showed a differential behavior with respect to their pure cultures. Moreover, substrates consumption and products formation were higher than 80% at 72 h of growth. Since no significant growth was observed after this time (stationary growth phase), the low sugars concentration was only used to maintain cell viability (Table 2). These results are in agreement with Nehme *et al*. [45], who reported that sugar consumption and MLF were faster in simultaneous cultures of *O. oeni* X and *S. cerevisiae* D than in sequential inoculation in synthetic grape medium. Moreover, Jussier *et al*. [46] reported that *O. oeni* EQ54 and Alpha in simultaneous cultures with *S. cerevisiae* CY 307 used all the available sugars and the MLF was performed faster than in sequential inoculation of Chardonnay musts. However, the end-products concentrations were similar in both inoculation conditions.

In all cases, carbon recovery was above 97% with the exception of *O. oeni* X_2L when inoculated in mixed yeast supernatant (90%). This decrease could be attributed to a lower compounds synthesis that was not dealt with in this work.

The inoculation strategies used in this study (sequential and simultaneous cultures) allowed us to compare the population dynamics and metabolic behavior between *O. oeni* and yeast strains cultured in grape juice medium. With respect to glycerol production, the best inoculation condition would be the sequential inoculation of *O. oeni* on 2- and 6- day *K. apiculata* supernatants. Although the yeast strain produced high amounts of ethanol, *S. cerevisiae* would ensure an effective alcoholic fermentation. Thus, we propose that the optimal condition for successful alcoholic and malolactic fermentations is the mixed inoculation of 10^6 cfu ml^{-1} *S. cerevisiae* $mc_2 + 10^6$ cfu ml^{-1} *K. apiculata* mF followed by the sequential inoculation of *O. oeni* X_2L (10^6 cfu ml^{-1}) after 6 days. In these conditions, *O. oeni* enhances glycerol production and the levels of other end-products would not affect the sensory profile of the wine.

The studies carried out in this work contribute to adopt a strategy of inoculation during winemaking, also considering the inclusion of a non-*Saccharomyces* strain.

Finally, this work represents the first report of glycerol production by *O. oeni* X_2L in mixed cultures with wine-related yeast strains which would enhance the organoleptic characteristics of wines.

Acknowledgments

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 statement

The authors declare that there is no conflict of interests.

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