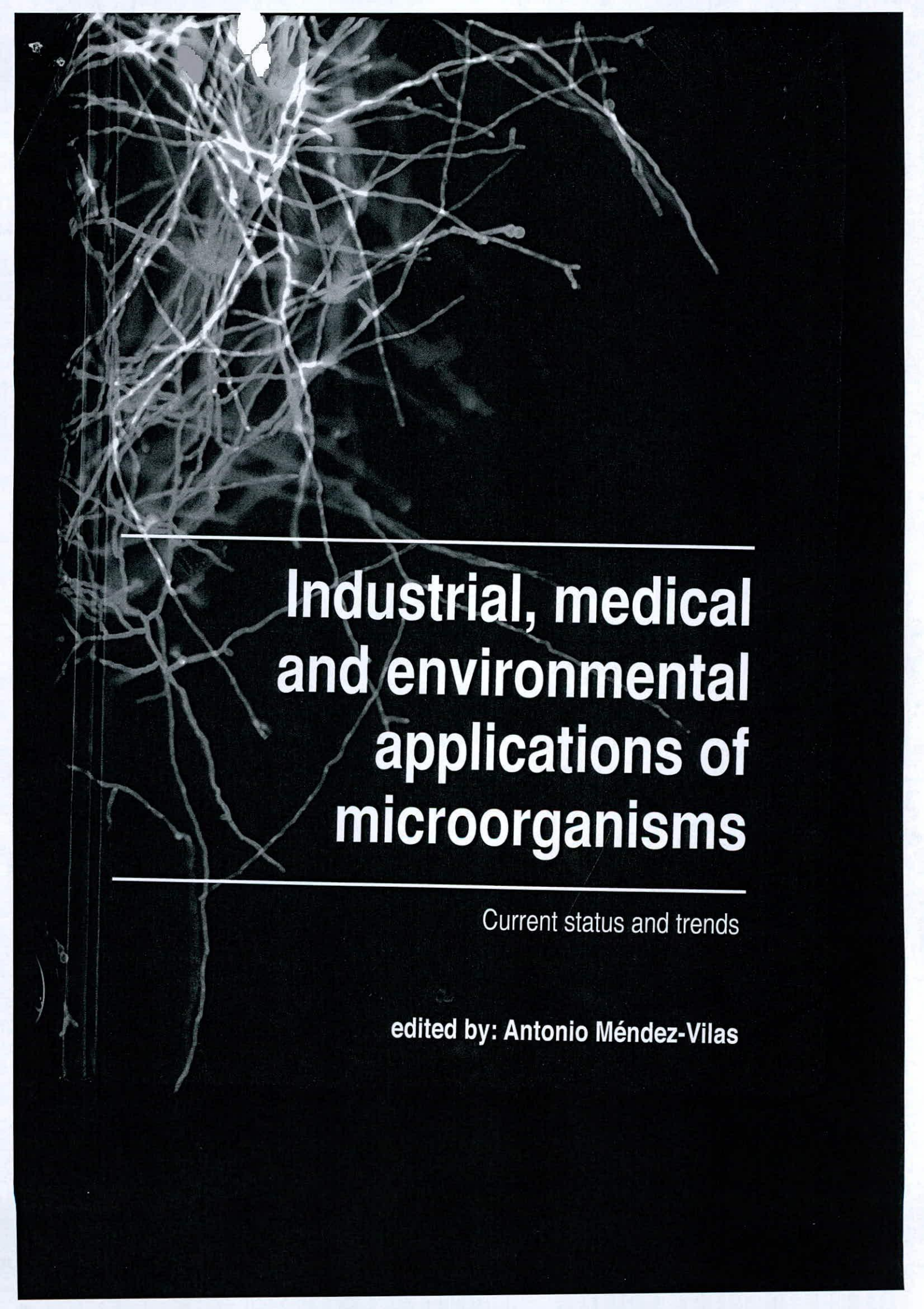

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Current status and trends

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Exploring the possibility of using *Kazachstania exigua* (ex. *Saccharomyces exiguus*) in wine production

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Abstract

Kazachstania exigua is a GRAS yeast isolated from different food sources such as sourdough, kefir grains and mezcal. In this work, the potential use of *K. exigua* in wine production was explored. After the determination of technological characteristics, the strains of *K. exigua* were tested in monosporial fermentation and, in multistarter fermentation, with *Saccharomyces cerevisiae* strain. The results show interesting properties of *K. exigua* when used in multistarter inoculation, regarding the production of higher amount of glycerol, the change of the aromatic profile and the reduction of the ethanol content in the wines obtained. These preliminary results represent the first technological survey of *K. exigua* and open interesting perspectives on the use of this specie in wine and in other alcoholic beverages industries and for production of reduced alcohol wines using a microbiological strategy.

Keywords: *Kazachstania exigua*; fermentation; glycerol; wine

1. Introduction

Since the discovery of yeasts as responsible for the alcoholic fermentation, the winemakers' attention has been focused on some species of the genus *Saccharomyces,* in particular *S. cerevisiae* and *S. bayanus,* because they can easily dominate the fermentation, ferment completely the sugars in grape must and they are able to product low amount of undesirable by product such as acetic acid. The introduction of selected yeasts spreaded the practice of guided fermentation, carried out by a single selected strain inoculated at a concentration high enough to guarantee dominance in fermentation. Nevertheless, in recent years, the interest is focusing on non- *Saccharomyces* species and their possible use during alcoholic fermentation. The reasons for this interest are due to the criticism that increasingly are moved to the guided fermentations, concerning the risk of leading to a excessive standardization of the sensory features of wines especially with regard to the olfactory ones. On the contrary, the spontaneous fermentation, through the alternation of various yeasts *Saccharomyces* and non-*Saccharomyces*, would provide greater organoleptic complexity due to the metabolic biodiversity of these species. Despite this, the practice of spontaneous fermentation raises doubts concerning the risks of incurring in organoleptic deviations, as many non-*Saccharomyces* yeasts produce high amount of secondary metabolites with negative impact on wine sensory properties. Moreover, sluggish or stuck fermentations can occur, especially in vintage characterized by high sugar contents with the development of high ethanol concentrations which represent a limiting factor for the development of most of the non-*Saccharomyces* species.

Several researchers have proposed a compromise between these two views through the introduction of multistarter fermentations. This practice consists in inoculate simultaneously or sequentially two or more strains of different species, one of which, belonging to *S. cerevisiae,* guarantee the conclusion of the fermentation while the strains non-*Saccharomyces* allow to obtain a given technology results or sensory characteristics. In this type of fermentation several yeasts combination have been suggested [1].

Among several issues in winemaking, reduce ethanol level in wine and, at the same time, preserving its quality represent an important theme in oenology, In fact, the raising of global temperature pose the problem of high sugar content in grape leading to an excessive ethanol content in wine, with a legal, healthy and technological drawbacks. Among the several approaches proposed for ethanol reduction, recently a microbiological way has been proposed concerning the use of non-*Saccharomyces* yeasts with a sugar respiratory metabolism in the first fermentation phase [2].

Kazachstania exigua Kurtzmann [3] (*ex Saccharomyces exiguus*) is a GRAS yeast isolated from different food sources such as sourdough [4,5], kefir grains [6] and mezcal [7].

To date, this yeast species has been poorly characterized from technological point of view [8] and no information is available on its potential use in fermentation industry, such in wine production or in other alcoholic beverage fermentations.

In this work, the potential use of *K. exigua* in wine production was investigated. In this aim, we firstly examined the technological features of some strains belonging to this specie in relation to the yeast *S. cerevisiae*, commonly used in winemaking. Afterwards, the yeasts were individually tested in fermentation. Finally, the most interesting strain was tested in fermentation in association with *S. cerevisiae* testing different multistarter inoculum methodologies.

2. Materials and methods

2.1 Strains and media

Five *K. exigua* strains (ISE 1451, ISE 1492, ISE 1493, ISE 1494, ISE 1495) belonging to the culture collection of CRA-Centro di Ricerca per l'Enologia (CRA-ENO) of Asti, Italy, and two *S. cerevisiae*, ISE 2 (CRA-ENO) and commercial strain Vitilevure DV10 (Lallemand Inc. Montreal, Canada), were used in this study.

The strains were conserved in glycerol stock at -80 °C and propagated in YPD for 24 h at 25 °C prior to the inoculation in must. Cortese grape musts was used in fermentation trials. The grape must composition was as follows: sugar 215 g/l, YAN (Yeast Assimilable Nitrogen) 190 mg/l, pH 3.45, total acidity 5.1 g/l. The must was previously heated at 70 °C for 20 min and this treatment was repeated for three days with the aim to eliminate the spontaneous microflora [9]

In monosporial fermentation sugar content was enriched to reach 240 g/l in order to test fermentative power of the strains. YAN was increased to 235 mg/l in order to avoid nitrogen limiting factor.

2.2 Enzymatic and technological screening

- Killer activity was evaluated using previously proposed assay [10], employing sensitive strain *S. cerevisiae* ISE 1 belonging to the CRA-ENO collection.
- Protease activity was monitored by streaking agar plate with medium containing casein as previously reported [11] observing the appearance of a clear zone after 5-6 days.
- Sulphite resistance was evaluated in microtiter plate using YPD buffered at pH 3.0 with increasing amount of K_2SO_5.
- β-glucosidase activity was monitored using 4-Nitrophenyl-β-D-glucopyranoside (Sigma-Aldrich, St. Louis, USA) as previously reported [12]
- H_2S production was determined using Biggy agar (Oxoid LTD, Hampshire, UK) incubated for 72 h at 26 °C evaluating the formation of brown colour of intensity proportional to a H_2S production.
- Sugar and ethanol tolerance were evaluated in microtiter plate whit increasing concentration of the relative compound. The concentration tested was from 2 to 50% w/v of sugar and from 4 to 20% v/v of ethanol.
- For every test a negative and positive control were included.

2.3 Monosporial fermentation

The yeasts were individually tested in fermentation with 300 ml of sugar enriched grape must in 500 ml Erlenmeyer flasks fitted with a Müller valve. The trial was carried out at 20 °C. During the alcoholic fermentation, the CO_2 loss was evaluated by weighing flasks while the cells population was monitored by absorbance measurement at 600 nm. Inoculum was carried out at concentration of 1×10^6 cells/ml. Each strain was tested in triplicate.

2.4 Multistarter fermentation

The most interesting *K. exigua* strain (ISE 1451) was employed for fermentation in association with commercial *S. cerevisiae* strain Vitilevure DV10. Trials were performed in natural grape must by sequential inoculation or co-inoculation. In the former experiment, *K. exigua* strain was inoculated at the concentration of 1×10^6 cells/ml. When the ethanol concentration in must reached 5% v/v, a second inoculum with 1×10^6 cells of *S. cerevisiae* strain was carried out in order to complete the fermentation.

In mixed inoculum (co-inoculation), the two strains were simultaneously inoculated using the previously reported concentration. Single monosporial fermentations employing the two strains were carried out as control tests. During fermentation, cells population were monitored using WL (Wallerstein Laboratory) Nutrient Agar (Oxoid), able to differentiate the two species on the basis of different colony morphologies. The trials were performed at 20 °C using 300 ml of grape must in 500 ml Erlenmeyer flasks fitted with a Müller valve. Each test was performed in triplicate.

2.5 Wine analysis

The wines obtained from the monosporial and multistarter fermentations were analysed in the principal parameter: ethanol, sugar, volatile acidity and glycerol using official methods [13].

GC MS analysis was performed on the wines obtained from multistarter fermentations and were carried out according to the method described by Ortega *et al.*[14]

3. Results and discussion

In the last decade researchers and winemakers have focused their attention on non-*Saccharomyces*, testing different combination of *Saccharomyces*/non-*Saccharomyces* species. Although previous studies have been carried out on *K. exigua* [4,5,6,7], the biotechnology and possible application in food technology is still largely unknown. To the best of our knowledge our work represents the first investigation on technological features applied to alcoholic beverages fermentation.

K. exigua showed interesting technological characteristics suitable in fermentation with a strain dependent variability (Table 1): osmotic tolerance around 30% of sugar, ethanol tolerance ranging from 10 to 12% v/v and sulphite resistance similar to *S. cerevisiae* strains. The killer toxin production against *S. cerevisiae* sensible strain and proteolitic activity were also revealed and depend on the strain. The *K. exigua* strains tested revealed a H_2S production while β-glucosidase activity was not found.

Monosporial fermentation (Table 2) showed fermentative powers of *K. exigua* strains ranging from 7.99 to 10,70% v/v ethanol with high production in glycerol reaching 100-110% greater than strains belonging to the *S. cerevisiae* species. With respect to *S. cerevisiae* strain, a greater acetic acid synthesis was measured: this production seems to be related to the glycerol content. As a consequence, the use of *K. exigua* as single fermentative specie is not feasible but a multistarter approach of fermentation with *S. cerevisiae* strain should be performed in grape fermentation, with the aim to ferment sugars completely and limiting acetic acid concentration in wines.

The multistarter trials were performed with co-inoculation (MIX) or sequential inoculation (SEQ) of *K. exigua* and *S. cerevisiae* strains. For these trials, the ISE 1451 was chosen among *K. exigua* strains, because of its major production in glycerol, while Vitilevure DV10 was used as commercial *S. cerevisiae* strain.

The single inoculum trials, performed as controls, confirmed the data obtained in monosporial fermentations: *S. cerevisiae* could quickly complete the alcoholic fermentation, while *K. exigua* was

Table 1. Technological characteristics of the strains studied.

Strain[1]	Killer activity	Proteolytic activity	β-glucosidase activity	H_2S production	Free SO_2 tolerance (mg/l)	Sugar tolerance (%w/v)
ISE 2 (S.c)	-	-	+	++	75	50
DV 10 (S.c)	+	-	+	+	75	50
ISE 1451 (K.e)	-	-	-	+++	50	30
ISE 1492 (K.e)	-	++	-	+++	50	30
ISE 1493 (K.e)	-	+	-	+++	50	30
ISE 1494 (K.e)	+	w	-	+++	75	30
ISE 1495 (K.e)	+	+	-	+++	75	30

[1] S.c: *Saccharomyces cerevisiae*; K.e: *Kazachstania exigua*.

Table 2. Main fermentative parameters in monosporial fermentations with natural grape must.

Strain[1]	Fermentation power (% vol.)	Fermentation rate (CO_2 after 3 days)	Volatile acidity (g/l acetic acid)	Glycerol (g/l)	Residual sugar (g/l)	Cell/ml max (10^6 cells)
ISE 2 (S.c)	14.25±0.13	4.05±0.05	0.40±0.04	6.85± 0.39	3.0±0.1	125.5±11.1
DV 10 (S.c)	14.36±0.13	4.42±0.02	0.30±0.04	7.21±0.58	traces	140.4±11.1
ISE 1451 (K.e)	10.31±0.36	2.41±0.26	1.70±0.05	14.87±0.73	50.3±5.1	90.3±7.4
ISE 1492 (K.e)	7.99±0.4	2.63±0.11	0.53±0.03	7.44±0.55	81.6±3.9	105.1±6.3
ISE 1493 (K.e)	9.12±0.23	2.65±0.24	1.30±0.03	10.90±0.55	62.3±3.5	84.1±5.4
ISE 1494 (K.e)	10.70±0.21	3.64±0.34	1.47±0.06	10.28±0.34	50.5±1.8	104.9±7.1
ISE 1495 (K.e)	10.55±0.42	3.23±0.41	1.08±0.06	12.08±0.33	54.56± 2.2	95.1±4.5

[1] S.c: *Saccharomyces cerevisiae*; K.e: *Kazachstania exigua*.

unable to ferment beyond 7.5% v/v ethanol content in the medium, leading to a stop in the fermentation process. The ethanol levels in single inoculum trials was lower in comparison to the ones obtained in monosporial fermentation: a lower content in YAN, which may represent a limiting factor for *K. exigua*, could be the basis to explain such result.

In multistarter inoculation, the fermentation kinetics (Fig. 1) showed differences in the two types of inoculum, revealing a faster fermentation in mixed inoculum with respect to the sequential one.

The analyses of the wines showed almost complete exhaustion of sugars in both MIX and SEQ inoculum. The two multistarter wines revealed glycerol concentration 70 and 117% higher than the fermentation carried out using *S. cerevisiae* alone. At the same time they showed a correspondent higher production of acetic acid too. MIX showed a lower level of acetate together with a lower concentration of glycerol in comparison to the SEQ test. The differences in the glycerol and acetic acid production between the two types of multistarter fermentation is probably due to a different concentration of *K. exigua* cells. In fact, it was lower in mixed fermentation respect to the sequential one during the whole fermentation process (data not shown).

As a consequence of differential production in glycerol, interesting differences in ethanol were found in the wines. Thanks to the diversion of glycolytic efflux toward the glycerol production, the wines fermented with *K. exigua*, in MIX and SEQ inoculum, ensured ethanol reduction by 3.7% (0.45 degrees

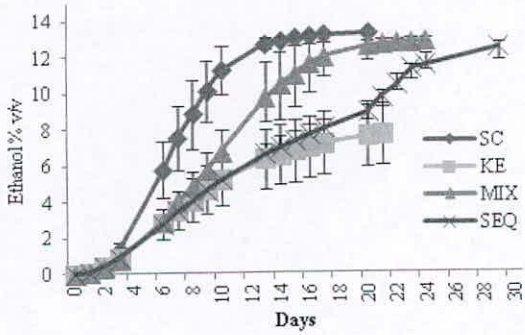

Fig. 1. Fermentative kinetics in fermentation tests with different types of inoculum, monitored by ethanol formation calculated by weight loss. SC: single inoculum with *Saccharomyces cerevisiae* Vitilevure DV10 strain; KE single inoculum with ISE 1451 *Kazachstania exigua* strain; MIX: co-inoculation of the two strain; SEQ: sequential inoculation of the two strains. Modality of inoculation is reported in Materials and methods. Error bars represent standard deviation (n=3).

of ethanol) and 7.2% (0.86 degrees) respectively, in comparison to the wine obtained from single *S. cerevisiae* inoculum which reached 12.73 alcohol degrees (Fig. 2).

The GC analysis of the wines revealed different aromatic profile compared to the ones obtained with single inoculation of *S. cerevisiae* and *K exigua* (Fig. 3). The multistarter trials were characterized by the highest concentration of acetates (statistically significant differences). In particular MIX trial showed the highest amount of isoamylacetate and 2-phenylethylacetate (data not reported) which are particularly important from the organoleptic point of view. The higher production of acetate esters in the multistarter trials, probably reflect an interaction effect of the two species as already found for other aromatic molecules [15]. As regard to the other groups of aromas the multistarter trials were characterized by intermediate concentrations among those seen in monosporial fermentations. Finally, it is noteworthy the highest concentration of FAEE produced by the monosporial KE fermentation.

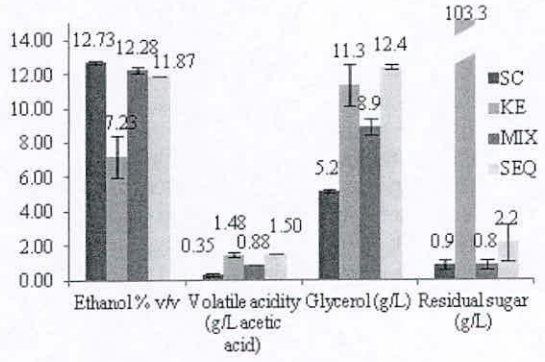

Fig. 2. Analysis of the wine obtained in fermentation tests. SC: single inoculum with *Saccharomyces cerevisiae* Vitilevure DV10 strain; KE single inoculum with ISE 1451 *Kazachstania exigua* strain; MIX: co-inoculation of the two strain; SEQ: sequential inoculation of the two strains. Modality of inoculation is reported in Materials and methods. Error bars represent standard deviation (n=3).

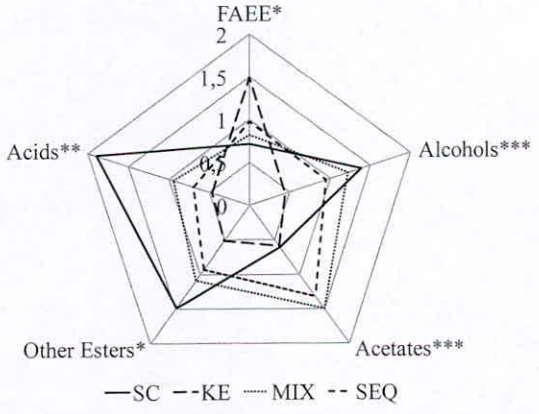

Fig. 3. Aromatic profiles of the major volatile compound groups in the wines obtained with multistarter fermentations, analysed by GC-MS. FAEE: Fatty acid ethyl esters: ethyl butyrate; ethyl hexanoate; ethyl octanoate; ethyl 3-hydroxybutyrate; ethyl decanoate; Alcohols: isobutanol; 1-butanol; isoamyl alcohol; 1-hexanol; cis-3-hexenol; methionol; benzyl alcohol; β-phenylethanol; Acetates: ethyl acetate; isoamyl acetate; hexyl acetate; phenylethyl acetate; Others esters: ethyl lactate; diethyl succinate; monoethyl succinates; Acids (organic and fatty acids): iso-butyric acid; butyric ac.; isovalerianic ac.; C6 ac.; C8 ac.; C10 ac. Data are normalized to the respective means. *=$P \leq 0.05$, **=$P \leq 0.01$, ***=$P \leq 0.001$.

In conclusion, these preliminary results open interesting perspectives on the use of *K. exigua* in wine and in other alcoholic beverages industries. In particular, it may represent a suitable tool for the production of reduced alcohol wines using a microbiological strategy. An extensive study is needed to explore the metabolic biodiversity of this species and test different inoculation methodologies. Although the *K. exigua* strains tested showed the drawback of high acetate production, a strain selection and an optimized procedure of inoculation may solve this negative aspect and permits to implement the use of this species at industrial level.

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