Assessing the enological potential of a *Kluyveromyces marxianus* yeast strain from the Linosa Island

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ABSTRACT

In this work we present the enological characterization of a *K. marxianus* isolate recovered on grapes (*cv* Inzolia) collected on the Linosa Island (Pelagie archipelago, Agrigento, Italy) in 2009. These were few plants, scattered around the island, not arranged in rows and whose plantation dated to about 30 years ago.

The isolate we studied was characterized from the molecular point of view (PCR-RFLP of the rDNA ITS region and sequencing of the D1/D2 26S rDNA region). Also its fermentative vigor, SO_2 tolerance, killer activity, H_2S production, β-glucosidase activity, as well as its ethanol, volatile acidity and glycerol production were assessed.

Finally after performing preliminary laboratory tests, we successfully used this isolate to ferment white must (cv Muscat of Alexandria) in a winery environment, in *K. marxianus*/*S. cerevisiae* mixed fermentations. Our results indicate that this isolate can be successfully utilized in enological applications.

RIASSUNTO

In questo lavoro presentiamo la caratterizzazione di un isolato di lievito *K. marxianus* ritrovato su uve Inzolia raccolte nel 2009 sull'isola di Linosa (arcipelago delle Pelagie, Agrigento). Si tratta di viti di vecchio impianto (risalenti ad almeno 30 anni fa) e che sull'isola si trovavano sparse e non organizzate in filari.

L'isolato è stato caratterizzato da un punto di vista molecolare (PCR-RFLP della regione ITS dell'rDNA e sequenziamento della regione D1/D2 dell'rDNA 26S). Sono stati anche determinati il vigore fermentativo, la resistenza alla solforosa, l'attività killer, la produzione di H_2S, l'attività β-glucosidasica; nonché la produzione di etanolo, di acidità volatile e di glicerolo.

Dopo aver effettuato dei test preliminari in laboratorio, il nostro ceppo è stato utilizzato con successo per produrre in cantina un vino bianco secco (varietà Moscato di Alessandria) in fermentazioni miste *K. marxianus/S. cerevisiae*.

I nostri risultati indicano che questo ceppo può essere usato con profitto in applicazioni enologiche.

INTRODUCTION

Over the last few years the demand of wine consumers has evolved: new niches have opened for products which promise novelty and which can deliver the taste experience of something

coming from a specific place in the world. As a consequence the search for "unusual" yeast species has been stimulated, with the attention put on non-*Saccharomyces* yeasts (e.g. (Ciani et al., 2010)) or "indigenous" *Saccharomyces* yeasts (Di Maio et al., 2012), the enological potential of which has not yet been fully exploited. This is becoming ever more difficult given the diffusion of industrial yeast strains and their ability to colonize wineries and vineyards (discussed in Bisson, 2012; Mendez-Vilas et al., 2010; Valero et al., 2005). Therefore we have devoted our attention to the yeast species found on a geographically isolated reality, the Linosa Island in the Mediterranean sea, where no wineries were present and the only commercial *S. cerevisiae* yeasts were those used for bread-making. Until 2008 the only vines were few scattered Inzolia plants (about 30 years old and used by the local families); that year two experimental fields of Muscat of Alexandria plants were established under the sponsorship of the IRVO.
In another OIV 2013 report (please see the full paper by Polizzotto *et al*. 2013) we provide an overview of the biodiversity of the yeast species recovered from the grapes of the new fields and from the old vines, focusing on the *Saccharomyces cerevisiae* strains that were found. In this report we explore the enological potential of the only *Kluyveromyces marxianus* isolate recovered from the Inzolia grapes. This yeast species is often part of the microbial flora during the tumultuous phase of must's fermentation; its utilization has been considered in biotechnological contexts, as a potential source of enzymatic activities (Fonseca et al., 2008). Here we show the results of winery *S. cerevisiae*/ *K. marxianus* mixed fermentations conducted during the 2012 vintage, which illustrate the enological potential of this yeast species.

MATERIALS AND METHODS

Yeast strains

The *S. cerevisiae* L404 belongs to the DIPROVAL collection (University of Bologna). The *Saccharomyces* strains Cpts1 33, Cpts1 25, A1-38 and A1-42, belong to the IRVO *Saccharomyces* yeasts collection. The *S. cerevisiae* BA11, ICV-k1, EC1118, ICV-D254 and RC212 are commercialized by Lallemand Inc. (Canada). The *S. cerevisiae* strain Zymaflore X-5 is commercialized by Laffort (France). The *Hanseniaspora uvarum* Hu03 and *Metschnikowia pulcherrima* 2003 yeast strains belong to the IRVO non-*Saccharomyces* yeasts collection. The *K. marxianus* yeast isolated utilized in this study was isolated as described in (Polizzotto et al., 2013).

Molecular analyses

A single *K. marxianus* yeast isolates was recovered and analyzed by PCR-RFLP (Granchi et al., 1999). Amplicons of the rDNA ITS sequence were digested with the appropriate restriction enzymes (*HinfI*, *HaeIII*; New England BioLabs, Hertfordshire, England). The D1/D2 region of the 26S rDNA and the rDNA ITS1 and ITS2 regions were amplified, and the DNA was sequenced and analyzed at the DBVPG (Industrial Yeasts Collection, University of Perugia- Department of Applied Biology) according to (Fell et al., 2000). PCR products were analyzed by agarose gel electrophoresis.

Technological characterization

Fermentative vigor and SO_2 tolerance were measured according to (Caridi et al., 2002). The L404 *S. cerevisiae* strain was used as positive control. Not inoculated must was used as negative control. B-glucosidase activity was assessed according to (Strauss et al., 2001); the *Hanseniaspora uvarum* Hp03 and *Metschnikowia pulcherrima* 2003 yeast strains were used

as positive controls; the L404 yeast strain as negative control. Killer activity of the isolates was measured as in (Regodón et al., 1997), by plating on Ba11 *S. cerevisiae* yeast cells and also *versus* a number of commercial yeast strains including Zymaflore X-5. The *S. cerevisiae* ICV-k1 and ECC118 strains were used as positive controls; the *S. cerevisiae* ICV-D254 and RC212 strains were used as negative controls. Production of H_2S was measured according to (Nickerson, 1953). The Cpts1 33, Cpts1 25, A1-38, A1-42 strains were used as references (from a very low to a high production).

Winemaking

Wines were produced during the 2012 vintage. Grapes were delivered to the IRVOS "G. Dalmasso" winery in Marsala (TP-Italy), de-stemmed and crushed. Approximately 300L of must were supplemented with 20 mg/L of SO_2 and pectolitic enzymes (Enartis 1000S). Cold static clarification was carried out at 5^{o}C for 24h. Microbiological and chemical analyses were performed. Two aliquots (90 liters each) of Muscat of Alexandria grapes were used. These were added with thiamine (60 mg/hL) and DAP (enough to reach a YAN concentration of 200 mg/L). After racking they were inoculated with Zymaflore X-5 yeast cells ($1.6x10^6$ CFU/mL); or with our *K. marxianus* isolate ($7.4 x10^6$ CFU/mL) and after 7 days with the Zymaflore X-5 yeast cells ($31x10^6$ CFU/mL). Crushed grapes were fermented at 18°C. Daily microbiological analyses were performed as well as controls to assess the amount of sugar and the temperature. Single and mixed fermentations took 12 days.

Samples were taken before and after malolactic fermentation (which occurred spontaneously in both wines) for downstream chemical and microbiological analyses. At the end of malolactic fermentation, samples were supplemented with 60 mg/L of potassium metabisulfite. Throughout fermentations, SO_2 levels were kept at 30 mg/L. After proteic and tartaric stabilization wines were bottled in April 2013.

Microbiological analyses

Everyday fermenting must samples were diluted in sterile peptone water (0,1% Bacteriological Peptone, Oxoid) and plated in duplicate on WL Nutrient Agar (Oxoid), Lysine Agar (Sigma). Further microbiological analyses were performed on WL Nutrient Agar and Agar-Lysine before bottling (Cavazza & Poznanski, 1998).

Chemical parameters

For the determination of wines' alcohol content, the OIV official method (OIV, 2006) was followed. Glucose, fructose, glycerol, acetic acid, malic acid, lactic acid, citric acid, tartaric acid concentrations were determined using a Enotech Steroglass apparatus (code SQRQ053586; Steroglass-Italy).

RESULTS

To assess the enological potential of our *K. marxianus* isolate, we first measured some of its technological parameters (some of which are reported in table 1).

Table 1
Technological an chemical parameters of the *K marxianus* isolate, compared to those of the L404 yeast strain and of the Blank . Standard deviations are indicated in parentheses.

Parameter	***K. marxianus***	**L404**	**Blank**
Fermentative Vigor 2 days	0.15 (0.02)	1.53 (0.05)	0.02 (0.02)
Fermentative Vigor 7 days	1.78 (0.12)	8.84 (0.02)	0.02 (0.02)
SO_2 tolerance 2 days	0.02 (0.02)	1.37 (0.14)	0.03 (0.02)
SO_2 tolerance 7 days	0.02 (0.02)	8.24 (0.13)	0.03 (0.00)
Ethanol[1] (% v/v)	3.76 (0.08)	11.49 (0.08)	0.04 (0.00)
Glycerol[1] (g/L)	1.84 (0.44)	7.25 (0.06)	0.00 (0.00)
Volatile Acidity[1] (g/L)	0.38 (0.11)	0.62 (0.01)	0.13 (0.01)

[1]Values measured after 13 days from the start of the fermentation.

Furthermore, the isolate was found to produce high levels of β-glucosidase and medium levels of H_2S. Its low fermentative vigor values, indicated that it could probably only be used in sequential inoculation with *S.cerevisiae* yeast strains. Given the low SO_2 tolerance values, several tests were performed to assess the ability of this strain to withstand decreasing concentrations of this chemical. As shown in fig 1 while 20 mg/L of SO_2 (purple curve) left unaffected the performance of the strain (identical to what seen at 0 mg/L SO_2), 40mg/L, 70 mg/L and 100mg/L SO_2 reduced or even abolished its fermentation capabilities.
Finally we assessed the killer activity of our isolate versus a number of commercial *S.cerevisiae* yeast strains, including ZymafloreX-5. No killer activity was observed toward this yeast strain.
Therefore we resolved to use this yeast strain to perform *K. marxianus/ S cerevisiae* mixed fermentations in the cellar. Because of the low SO_2 tolerance of our *K. marxianus* isolate, these were carried out in the presence of just 20 mg/L SO_2.
Figure 2 shows how the microbial populations evolved during the course of a single starter fermentation (top) and during a mixed fermentation (bottom). In both cases the growth of non-*Saccharomyces* yeasts (red curves) was maintained at low levels. Also in both cases sugar consumption was complete. Glycerol acetic acid and volatile acidity levels were higher in the mixed fermentation (table 2). Acidity values were of however within the prescribed range for these wines (Commission Regulation (EC) No 606, 2009).

Table 2
Some enochemical parameters of the single starter (X5) and sequential fermentation (*K. marxianus*+ X5)

Fermentation	**Glycerol**	**Acetic acid**	**Volatile acidity**
X5	5.90 ±0.14	0.25 ±0.00	0.39 ±0.01
K. marxianus+ X5	7.65 ±0.21	0.69±0.01	0.72±0,21

Malic and lactic acid levels showed that malolactic fermentation was carried on, probably because of the low concentration of SO_2 utilized during the fermentation (not shown).
Preliminary degustation tests gave a positive feedback. Therefore our results show that *K. marxianus* yeast species can be proficiently utilized in the winery to carry on *S. cerevisiae*/*K. marxianus* mixed fermentations. However further studies will be needed to define the appropriate conditions for the utilization of sulfites, in order to control the onset of malolactic fermentation.

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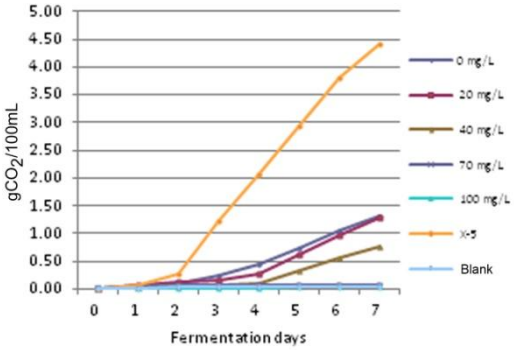
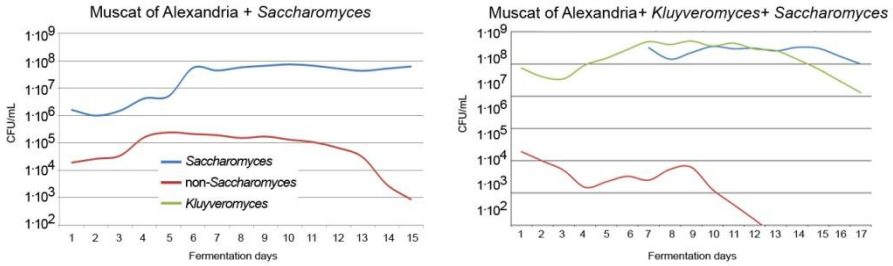
Figure 1



Testing the ability of our *K .marxianus* isolate to withstand different SO_2 concentrations. The performance of the strain is compared with that of the *S. cerevisiae* X5 yeast strain at 100 mg/L SO_2.

Figure 2



Evolution of the microbial populations during single (top) and mixed (bottom) fermentations