Selection of Indigenous *Saccharomyces cerevisiae* Strains from Kutjevo Wine Growing Area at the Laboratoy Scale

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SUMMARY

The use of selected yeasts for winemaking has clear advantages over traditional spontaneous fermentation. Selection of wine yeasts is usually carried out within the *Saccharomyces cerevisiae* species. Yeast strains produce different amount of secondary compounds that impart specific characteristics to the wines. This suggests that it is necessary to isolate naturally occuring autochthone strains, which exhibit a metabolic profile that corresponds to each wine. Twenty two strains of *S.cerevisiae*, isolated from the Kutjevo region (Gornji and Donji Hrnjevec, Mitrovac), Graševina grapes, were tested for: fermentation vigor, ethanol resistance, volatile acidity, H_2S production and β-glucosidase, polygalacturonase, and killer activity. From the results of this investigation we are able to select two yeast strains (RO 1272 and RO 1284) for more detailed fermentation trials and possible use as a starter culture in production of typical wines

KEY WORDS

strain selection, wine, *Saccharomyces*, Graševina

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Received: April 27, 2005

ACKNOWLEDGEMENTS
This work was supported by grant 0178047 from the Croatian Ministry of Science, Education and Sports.

INTRODUCTION

Wine fermentation was traditionally carried out by indigenous yeasts associated with grapes and cellar equipment. Today majority of wine production is based on use of active dried yeast which ensures rapid and reliable fermentation and reduces the risk of sluggish or stuck fermentation and microbial contamination. Most commercial wine yeast strains available today have been selected in the vineyard for oenological traits. The selection of yeasts for winemaking consists of identifying specific cultures, mainly *Saccharomyces* spp., which can ferment grape juice effectively and can produce good quality wines. The criteria for selection of yeast strains assist in the choice of yeasts that are able to improve the quality and consistency of wine. The selection process of yeast strains depends on their oenological characteristics, such as fermentative rate, tolerance to ethanol and SO_2, flocculent characteristics, the presence of killer factors, acetic acid production, H_2S, malic acid metabolism, higher alcohol production, alcohol yield, glycerol production, and extra cellular enzyme production (Rainieri, S., Pretorius, I.S., 2000). These and other technological developments have contributed to an improvement in the quality of wine, and have enhanced the ability of winemakers to control the fermentation process and achieve specific outcomes.

Commercially available dried yeast strain starter culture of *S.cerevisae* could be inoculated into the grape juice in order to establish a high population and accomplish well-controlled must fermentation. However, the use of local, indigenous, selected strains of *S.cerevisiae* as a starter culture are preferable (Romano, 1997), since these yeasts are better acclimated to micro area conditions of the wine producing region (Querol et al. 1992; Romano et al., 1998; Comi et al., 2000; Fundira et al., 2002; Romano et al., 2003) and can dominate the natural flora easier. Moreover, such treatment, among others, could assure maintenance of the typical sensory properties and a characteristic profile of local wines.

In the Kutjevo region there is a long grape growing tradition especially of growing Graševina grapes. Graševina from this location always gives high quality wines, recognizable for their aroma and flavour profile.

The aim of our research was to select *Saccharomyces cerevisiae* strains that are starters for the production of Graševina. We examined oenological differences, affinities and suitability of yeasts for wine production at a laboratory scale for possible future starter production.

MATERIAL AND METHODS

Sample collection and yeast isolation

Vineyards in which the samples were collected are situated near the Kutjevo wine region (location Donji and Gornji Hrnjevac, Mitrovac), which is located in the eastern region of Croatia. The isolation of *Saccharomyces* yeasts from Graševina grapes was done essentially as described by Martini *et al.* (1980) and Van der Westhuizen *et al.* (2000). Whole clusters (3-4 kg bunches) were gathered aseptically and dropped directly into sterile plastic bags. The tightly sealed plastic bags were transported to the laboratory in cooled containers. At the laboratory, grapes were crushed by hand in still tightly sealed plastic bags. After thorough shaking, the bags were opened and juice (500 ml) poured into sterile 750 ml bottles, which were immediately sealed by affixing sterile fermentation caps. The bottles were then placed in a dark, temperature-controlled room (15 °C). The progress of fermentation was determined by measuring mass loss. Successful fermentations were sampled when residual fermentable sugar was less than 4 g/l. Before withdrawing the samples, the bottles were shaken to thoroughly mix the contents and get all of microbial cells in suspension. Each of these samples was streaked on 10 Petri dishes containing YPD agar medium (1% yeast extract, 2% peptone, 2% glucose and 1.5% agar) and incubated at 30°C for 3 days to allow colony formation. A total of 50 colonies were selected randomly. Cultures were then stored at 4°C until further analysis.

Identification of *Saccharomyces* spp.

Identification of *Saccharomyces* spp. was carried out in accordance with Cavazza (1992) using WL nutrient agar (Oxoid, UK) and Lysine medium (Oxoid, UK).

Determination of killer activity

Strains 1080 (non-killer strain) and 1079 (a K_1 killer strain) were obtained from G.A. Farris (Sassari, Italy) and used as negative and positive indicator strains to determine the killer activity of the isolated strains. YEPG agar plates, buffered at pH 4.5, were used to detect zones of growth inhibition caused by the K_1 toxin secreted by the killer yeast. The non-killer strain 1080 was used as a sensitive lawn on the indicator agar plates, while the killer strain 1079 was used as a positive control. The plates were incubated for 48 h at 25°C and then examined to note killer activity.

The production of hydrogen sulphide

The production of H_2S was determined by the colour formation of the yeast on BIGGY agar (Difco) plates. The degree of browning associated with yeast growth on BIGGY agar plates was scored using the following scale: 1=white, 2=cream, 3=light brown, 4=brown, 5=dark-brown, 6=black.

Fermentation tests

Fermentation trials were conducted according the protocol of Redzepovic et al. (2002) and Romano (1998) with some modification. Each 300 ml Erlenmeyer flasks cantained 100 ml of Graševina grape juice (with a sugar content of 190 g/L, a total acidity of 6 g/L and malic acid content 1,2 g/L). Pasteurised must (95 ml) was inoculated with 5 ml of actively fermenting *Saccharomyces cerevisiae* strains (2 x 10^5 CFU/mL), covered with sterile Vaseline and incubated at 18°C. The fermentation process was followed by quantifying weight loss caused by CO_2 production. The quantity (in grams) of CO_2 produced was used to express strain fermentation vigour after 3 days (Δp3) and 7 days (Δp7). Fermentation was considered to be complete when the weight remained constant. The samples were refrigerated for 2 days at 3°C, then racked and stored at -20°C until further analysis. Standard methods for wine analysis (Ough and Amerine, 1988) were used to determine the ethanol content and levels of volatile acidity. Glycerol and malic acid content were determined enzymaticaly (Boehringer, Mannheim, Germany).

Extracellular enzyme production

Extracellular enzyme production was carried out by the methods proposed by Strauss et al. (2001)

RESULTS AND DISCUSSION

A total of 23 yeast strains were identified as *Saccharomyces cerevisiae* from Kutjevo winegrowing region and were tested for their fermentation ability, H_2S production, killer activity, and extracellular enzyme production in order to carry out yeast strain selection at a laboratory scale.

The results of the fermentation trials with the isolated yeasts are shown in Table 1. Ethanol together with the CO_2 is a major product of alcoholic fermentation. The concentration on ethanol depends of the yeast strain and on the initial concentration of sugar in must. Strain RO 1280 from Mitrovac produced the highest level of ethanol (11.3 %), and RO 1277 (Gornji Hrnjevac) the lowest. Four yeast strains (17.4%) synthesize more then 10 % of ethanol. We have also found that the *S. cerevisiae* isolates generally fermented vigorously, and that all the tested yeast strain produced more then 2.0 g/L CO_2/day (Regodon et al., 1997). The highest Δp3 and Δp7 values were strongly correlated with the highest ethanol production.

The values of volatile acidity were between 0.22 (RO 1264 and RO 1267) and 1.20g/L (RO 1268). The lowest volatile acidity producers (RO 1264, 1267 and 1269) were isolated form Donji Hrnjevac area. Formation of volatile acidity is highly undesiderable and levels at which acetic acid is normally produced by *S.cerevisiae* are 0.3-0.8 g/L (Fleet and Heard, 1993)

Glycerol is one of the most important secondary metabolite of alcoholic fermentation. Typically it is produced at levels ranging from 4 to 10 g/L (Rainieri, Pretorius, 2000). This major component of wine improves quality by affecting sweetness (threshold level 5.2 g/L) (Noble and Bursick, 1984), fullness and smoothness. It is well established fact that differences exist in the amount of glycerol formed by various yeast strains during fermentation (Rainieri, Pretorius, 2000). The majority of the tested yeast strains (47, 8%) synthesize glycerol between 4.5 and 5.5 g/L. The yeast strain with the highest glycerol production (5.7g/L) was isolated form the Gornji Hrnjevac (RO 1272).

Malic acid is one of the main organic acids in grapes and sometimes it is necessary to remove it from wine in order to improve their organic qualities and to ensure biological stability (Redzepovic et al., 2003). The ability of *S. cerevisiae* to use malic acid during fermentation has been known for many years. Generally, depending on the strain, they are known to metabolize from 3 % up to 50% of malic acid which is never completely consumed (Usseglio-Tomasset, L., 1989).Tested yeast strains showed different degradation ability of malic acid. The degradation range was from 10% to 25% of majority of the strains, with the highest value of 58% (RO1273). Good capacity of RO1273 strain to decompose malic acid can be considered positive or negative in accordance with different technological applications.

The formation of hydrogen sulphide (H_2S) by yeasts during the fermentation of grape juice is longstanding and serious problem in winemaking process (Mendes Ferreira et al., 2002). Yeast strains differ widely in their ability to produce H_2S. From a winemaking point of view, it was therefore encouraging to find out that all of the isolates have produced low concentrations of H_2S (Table 1).

Presence of killer yeasts is particularly important in wine fermentations conducted by inoculation with selected strains of *S.cerevisiae*. Wild wine yeasts with killer phenotype are wide-spread in many wine regions of the world (Vagnoli et al., 1993; Hidalgo, Flores, 1994). With respect to the possible production of killer toxins that may interfere with the growth of *Saccharomyces* yeasts during the process of winemaking, we have found that none of the isolates produced any zymocidal peptide that could inhibit the growth of the indicator strain, 1080 (Table 1). Presence of non *Saccharomyces* killer strains especially at the early stage of fermentation could provoke spoilage and lead to stuck fermentation or production of undesirable aroma compounds (Da Silva, 1996).

Enzymes play a definite role in wine production, which could be seen as the product of enzymatic transformation of grape juice. Two major groups of

Table 1. Results of the fermentation and enzymatic trials

	Ethanol	Volatile acidity	Malic acid (g/L)	Gylcerol (g/L)	Δp3	Δp7	Pectinolytic activity	β-glucosidase	Location
RO 1264	9.8	0.22	1.1 (10%)	4.5	1.75	1.58	–	–	Donji Hrnjevac
RO 1265	9.0	0.85	0.8 (34%)	4.9	1.63	1.53	–	–	Donji Hrnjevac
RO 1266	8.8	0.72	0.9 (25%)	4.8	2.43	1.62	++	–	Donji Hrnjevac
RO 1267	8.5	0.22	0.8 (34%)	1.5	2.07	1.60	+++	+	Donji Hrnjevac
RO 1268	10.4	1.20	0.9 (25%)	4.9	2.54	1.88	–	–	Donji Hrnjevac
RO 1269	9.2	0.24	0.9 (25%)	2.0	1.71	1.59	–	–	Donji Hrnjevac
RO 1270	9.0	0.44	1.1 (10%)	4.1	2.54	1.77	–	–	Donji Hrnjevac
RO 1271	8.8	0.84	1.0 (17%)	5.3	2.17	1.62	–	–	Gornji Hrnjevac
RO 1272	9.3	0.76	0.8 (34%)	5.7	2.71	1.83	++	–	Gornji Hrnjevac
RO 1273	11.0	0.79	0.5 (58%)	5.6	2.56	1.97	–	–	Gornji Hrnjevac
RO 1274	9.3	0.70	0.7 (41%)	4.8	2.32	1.66	+	–	Gornji Hrnjevac
RO 1275	6.2	0.64	0.6 (50%)	2.5	1.78	1.10	++	–	Gornji Hrnjevac
RO 1276	8.1	0.58	0.9 (25%)	1.0	1.88	1.07	++	–	Gornji Hrnjevac
RO 1277	5.9	0.65	0.9 (25%)	4.2	2.44	1.50	+++	+	Gornji Hrnjevac
RO 1278	8.5	0.77	0.8 (34%)	5.1	2.37	1.57	+++	–	Gornji Hrnjevac
RO 1279	8.8	0.73	0.9 (25%)	4.3	1.89	1.52	–	–	Mitrovac
RO 1280	11.3	0.80	0.7 (41%)	4.0	2.56	1.99	++	–	Mitrovac
RO 1281	9.0	0.52	0.7 (41%)	4.1	1.93	1.48	–	–	Mitrovac
RO 1282	8.8	0.68	0.9 (25%)	2.9	2.00	1.45	+	–	Mitrovac
RO 1283	8.5	0.90	0.9 (25%)	3.9	1.97	1.57	–	–	Mitrovac
RO 1284	11.2	0.70	0.9 (25%)	4.2	2.64	1.90	++	–	Mitrovac
RO 1285	8.6	0.68	1.1 (10%)	3.5	2.37	1.55	+++	–	Mitrovac
RO 1286	8.8	0.49	0.7 (41%)	3.8	2.29	1.61	–	–	Mitrovac

extracellular enzymes are presented by â-glucosidase and pectinase. Only two yeast strains (RO 1267 and RO 1277) showed weak β-glucosidase activity (Table 1). In a previous study Hernandez et al. (2003) from the 60 *Saccharomyces* strains tested, selected only one strain that showed very high â-glucosidase activity in grape must and this activity was expressed at high pH level and in the aerobic conditions of winemaking. In contrast, Rosi et al. (1994) found only one strain with β-glucosidase activity among 153 *S.cerevisiae* analyzed.

The results of pectinolytic activity presented in Table 2 indicat that 12 (52%) isolated yeast strains showed extra cellular activity. The highest activity was observed in RO 1285, RO 1278, RO 1277, and RO 1267. These results are in agreement with results of Ubeda Iranzo et al. (1998), who found that 33% of the 74 selected strains were capable of hydrolysing galacturnonic acid. In contrast, Charoenchai et al. (1997) did not detect pectinolytic activity in any of the wine yeasts from the genus *Debaromyces, Torulaspora, Metschnikowia, Candia, Pichia, Kloeckera* and *Saccharomyces* spp.

CONCLUSION

Use of selected yeast cultures as startes for wine fermentation has led to the production of more consistent wines. Oenological industry is always directed towards the selection of new wine yeast strains which will improve the fermentative process in such a way as to impart particular characterisitcs of taste and aroma to wine (Comi et al., 1997).

This study represents a preliminary selection phase of *S. cerevisiae* strains isolated form Kutjevo winegrowing region in order to verify if they are suitable for use in wine production. During the microfermentation (100 mL) all the tested yeasts grew correctly, with typical fermentation curves. The analytical results (Table 1) of the produced wine were used for selection according to the following criteria: volatile acidity less than 0.8 g/L, ethanol concentration higher than 10%, glycerol content from 4 to 5g/L and with no or little of malic acid degradation. We selected two yeast strains (RO 1272 and RO 1284) for more detailed fermentation trials. In conclusion, this study was useful in to obtaining interesting information about the oenological characteristics of *Saccharomyces* tested, which is a necessary first step in strain selection.

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