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Initial analysis of highly competitive yeast strains promising for ethanol industry

V. Melvydas[1],

G. Gedminienė[2*],

I. Jarmalaitė[3],

B. Čapukoitienė[1],

L. Nemceva[2]

[1] *Institute of Botany, Vilnius, Lithuania*

[2] *Gediminas Technical University, Vilnius, Lithuania*

[3] *National Veterinary Laboratory, Vilnius, Lithuania*

We have analyzed *S. cerevisiae* Rom K-100, M437, 4+, 18M, 20K^{++}, K^{+}ob and the newly discovered IIx31, III.2, Spanguolė yeast strains which are promising to use in alimentary (wine) industry, their efficiency of secrete killer toxin K2, and the reference strains. We studied the enzymatic potential of these strains and their influence on apple juice fermentation. We found that 20K^{++}, IIx31 strains were more promising in alimentary industry and strains 4+, III.2, Spanguolė could be used in the production of ethanol in other industries. The other strains require more detailed study. The Rom K-100, III.2, K^{+}ob, 4+, IIx31 and 20K^{++} strains had a good resistance (18%) to ethanol.

Key words: *Saccharomyces cerevisiae*, yeast, killer strain, fermentation, ethanol tolerance, competitive ability

INTRODUCTION

The budding yeast *Saccharomyces cerevisiae* has a long history in the fermentation industry. Owing to its efficiency in producing alcohol, *S. cerevisiae* is the most important commercial microorganism with GRAS (Generally Regarded As Safe) status [1]. Mankind's oldest domesticated organism is used for brewing beer and other alcoholic beverages. Though in our days the use of yeast extended in modern molecular genetics, ethanol produced by yeast fermentation still remains in the first place. Ethanol is an important industrial chemical with emerging potential as a biofuel to replace vanishing fossil fuels [2]. All indications show that dramatic changes in energy supply will occur in the 21th century, particularly relating to oil [1].

So, the demand of yeast strains with a good fermentative efficiency and increased alcohol tolerance remains topical. Particular attention has been paid to the use of killer yeasts as selected starters in fermentation processes. Killer strains guarantee competitive advantages to the starter ethanol making yeast. Research on killer yeasts for industrial application is relatively new. Many of fermentative processes use non-pasteurised medium which can allow the predominance of wild yeast strains coming from the raw material outnumbering the starter yeast. Those contaminations can retard fermentation and decrease ethanol productivity. The use of a yeast killer system may be a way to avoid the effects caused by undesirable yeasts in the fermentative processes. Some species with known genetic characteristics and killer phenotype have been adopted in wine industry [3]. On the other hand, the more strains from nature are screened for their range of toxic activity, the better their complex genetics, regulating mechanisms, compatibility, and the level of toxin production will be understood [4].

The goal of our work was to perform the primary analysis of some yeast strains selected from naturally occurring communities of berries, apples and grapes in Lithuania and in some cold climate Russian regions.

MATERIALS AND METHODS

Yeast strains. The *Saccharomyces cerevisiae* strains Rom-K100 (wild type, (wt, *HM/HM [kil-K2]*); M437 (wild type, (wt, *HM/HM [kil-K2]*), *α'l ((MATa, leu2-2 [kil-0])*, sensitive to all killers were used for testing the activity of killer toxin [5]. The yeast strains signified as 20K^{++}, 4+, K^{+}ob, III.2, IIx31 were obtained from spontaneous fermentation of grapes, apples, cloudberries, black-berries and described as killer yeasts [6].

The identification of yeast strains IIIxI, VII.2.1, 20K^{++}, 4+, and VII.1 was done at the Microbiological Laborato-

* Corresponding author. E-mail: Genovaite.Gedminiene@fm.vtu.lt

ry of the Lithuanian Public Health Centre. An automatised mini API 20 C AUX system for clinical yeast identification was used, applying the methods and reagents of the BioMerieux Foudation (France).

Media, plate test and test of killer activity were determined as in [5, 6].

Fermentative efficiency of selected killer strains. The initial fermentative tests were accomplished using selected killer strains 20K+, 4+, K+ob, II-31, III-2 and two standards, Rom-K100 and M437. The inoculum was prepared by cell loop transfers to flasks with 250 ml of apple juice supplemented with vitamins and ammonium chloride as recommended by the producer – the cooperative society "Vaisių sultys". The flasks were cultivated at 30 ˚C on a rotary shaker at 150 rpm. After 20 h of growth, 40 or 50 ml of night culture was used to inoculate 2 l of apple juice and to achieve the starting cell density 0.4 O.D. Fermentative efficiency tests were accomplished in 3 l flasks containing 2 l of apple juice (sugar concentration 9%), vitamins (biotin and thiamin), ammonium chloride (1 g/l), pH 3.4, at 20 ˚C and 26 ˚C.

Reducing sugars. Reducing sugar analysis in the cell-free filtrates was accomplished by 3.5 dinitrossalicilic acid [7].

Levels of ethanol, methanol, higher alcohols, esters and aldehides were determined by gas chromatography at the National Veterinary Laboratory according to LST EN ISO/IEC requirements.

Ethanol tolerance. The YEPD medium without or with an appropriate ethanol concentration was used for the screening of yeast for ethanol tolerance. Enough of absolute ethanol was added to different flasks of the same medium to constitute the varying percentages of ethanol differing by 2% (v/v) from one flask to another. The media were triplicated and inoculated with different yeast strains. Samples were incubated for 4 h at 30 ˚C with shaking at 150 rpm. The viable cells as CFU were established by plating an appropriately diluted sample on solid YEPD.

RESULTS AND DISCUSSION

Preliminary evaluation of the fermentative capability of killer yeast strains

We investigated the possibility of yeast killer strains 4+, $20K^{++}$, K^{+} ob, IIx31, III.2, Cranberry and two standard wine strains Rom K-100, M437 to ferment apple juice. Apple wine is a fermented beverage made from apple juice. It has had a long tradition in Europe and has taken an important place in the fruit wine industry [8]. Apple juice contains many sugars, including fructose and glucose.

The primary selection criteria for fermetable industrial yeasts are good conversion of sugar to alcohol and carbon dioxide; total time and low temperature of fermentation, and rather high ethanol tolerance.

The occurrence of killer phenomenon among grape wine yeasts in a number of winemaking countries was studied [3]. Data on ethanol fermentable possibilities of killer yeast origin under northern climatic conditions are not numerous [4]. The killer phenomenon of yeasts was investigated in naturally occurring yeast communities. The use of killer yeasts as starters in wine fermentation processes has been reported to be important [9]. Many of fermentative processes use non-pasteurised medium which can allow the predominance of wild yeast strains coming from the raw material outnumbering the starter yeast. Those contaminations can hamper fermentation and decrease ethanol productivity. The killer system may be a way to awoid the effects caused by undesirable yeasts in the fermentative processes. The nature of the yeast killer phenomenon implies a potential role for competition, considering that yeast killer toxins may prevent antagonistic microorganisms from gaining access to resourses that would provide a selective advantage during the early phases of microbial growth [4]. Yeast killer toxins are produced optimally by growing cells and are exquisitely active against cells in the same stage when nutrients are available and pH is low. The probability that a killer toxin produced by yeast may kill certain susceptible yeasts would also depend on ecological characteristics such as the region, the host plant and the habitat from which the killer yeasts were collected [4].

To verify this, six killer yeast strains (4+, $20K^{++}$, K^{+}ob, IIx31, III.2, Cranberry and two standard wine strains Rom K-100 and M437) were used in our experiment. It was established that the strains 20 K^{++}, 4+, IIx31 showed a powerful killer activity incomparable with that of *S. cerevisiae* killer standart strain M437. These results could indicate a higher competitive advantage of those mentioned strains as the starter ethanol-making yeast against other yeasts when inoculated in a non-pasteurised juice medium. The central aspect of our research was estimation of some quality parameters of apple juice fermentation with yeast killer strains selected from natural habi-

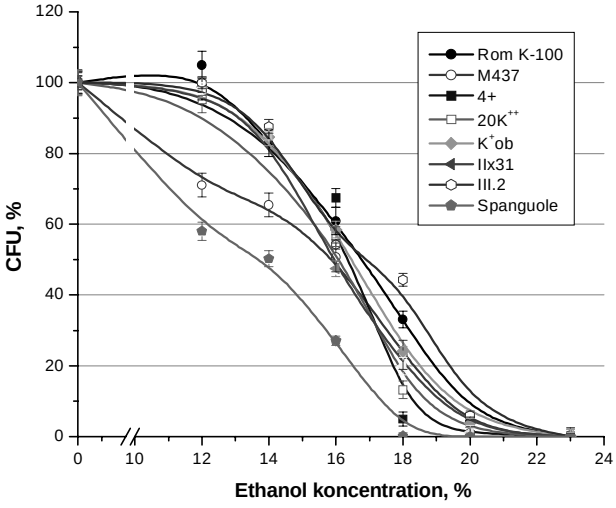

Figure. Effect of ethanol concentration on yeast cell viability. Each curve corresponds to a strain: Rom K-100, M437, 4+, $20K^{++}$ and K^{+} ob, IIx31, III.2, Cranberry (Spanguolė). Cell viability, %, (Ordinate axis). Ethanol concentrations, % are indicated on the abscissa.

Table 1. **Characteristics of apple wine made with yeast strains Rom K-100, 20K^{++}, 4+, IIx31, III.2, Cranberry, M437 after 10 days fermentation at 20 ˚C**

Substance, parameters	Apple juice fermentation with killer strains						
	RomK-100	20K^{++}	4^{+}	IIx31	III.2	Cranberry	M437
Alcohols							
Ethanol, %, v/v	5.99	6.07	6.11	6.08	6.26	2.27	5.75
Methanol, mg/dm^3	2.3	2.1	1.2	1.3	11.6	11.5	1.8
Higher alcohols							
2-methylbutanol, mg/dm^3	12.2	8.6	12.8	10.8	12.2	11.2	13.4
3-methylbutanol, mg/dm^3	97.2	68.2	109.0	101.2	110.8	23.9	136.3
Propanol, mg/dm^3	13.5	11.0	15.3	14.7	26.9	< 0.6	11.9
Isobutanol, mg/dm^3	37.8	19.2	29.4	21.5	24.8	21.5	29.0
2-butanol, mg/dm^3	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6
n-butanol, mg/dm^3	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6
Esters							
Methyl acetate, mg/dm^3	< 10.0	< 10.0	< 10.0	< 10.0	< 10.0	< 10.0	< 10.0
Ethyl acetate, mg/dm^3	15.5	11.8	17.8	18.9	22.2	139.2	19.7
Aldehides							
Etanal (acetaldehyde + acetal)	25.5	33.9	28.2	38.3	38.5	99.4	20.6

Table 2. **Characteristics of apple wine made with yeast strains Rom K-100, 20K^{++}, 4+, IIx31, III.2, Cranberry, M437 after 10 days at 20 ˚C and 6 days of fermentation at 26 ˚C**

Substance, parameters	Apple juice fermentation with killer strains						
	RomK-100	20K^{++}	4^{+}	IIx31	III.2	Cranberry	M437
Alcohols							
Ethanol, %, v/v	6.09	6.16	6.54	6.23	6.26	6.27	6.32
Methanol, mg/dm^3	2.6	1.9	12.2	1.4	11.5	11.8	1.8
Higher alcohols							
2-methylbutanol, mg/dm^3	9.6	7.9	18.0	11.5	12.4	17.3	13.6
3-methylbutanol, mg/dm^3	83.6	66.6	129.5	100.4	110.9	91.1	132.1
Propanol, mg/dm^3	11.4	11.8	29.3	16.6	27.4	39.1	13.2
Isobutanol, mg/dm^3	30.0	19.5	40.4	23.3	25.3	36.4	32.2
2-butanol, mg/dm^3	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6
n-butanol, mg/dm^3	< 0.6	< 0.6	5.9	< 0.6	< 0.6	6.2	< 0.6
Esters							
Methyl acetate, mg/dm^3	< 10.0	< 10.0	< 10.0	< 10.0	< 10.0	< 10.0	< 10.0
Ethyl acetate, mg/dm^3	10.0	9.6	16.4	17.3	19.6	135.7	16.0
Aldehides							
Etanal (acetaldehyde + acetal)	57.2	33.9	154.5	53.6	95.4	50.2	82.7

tats from cold climat regions. The results obtained in the preliminary evaluation of the killer strain fermentative capability are shown in Table 1 as compared to pressed industrial yeast Rom K100 and M437.

All selected yeast strains were able to ferment apple juice sugars and produce ethanol. The results presented in Tables 1 and 2 show that strains 4+, 20K^{++}, K^{+}ob, IIx31, III.2 produced ethanol whose concentration was about 6% (5.75% for M437 and 5.99% for Rom K100 (standards wine-making strains) and 6.26% for strain III-2 at a temperature of 20 ˚C. The Cranberry strain was an exception – production of ethanol reached only 2.27%. The level of 6.27% of ethanol was produced by Cranberry strain only at a prolonged fermentation at 26 ˚C. The highest concentration of ethanol was achieved with the yeast strain 4+, but it produced a high level of methanol

(26 °C, Table 2) in comparison with standard strains Rom K100 and M437 (2.6 and 1.8, respectively). On the basis of the results presented in Tables 1 and 2 we conclude that the most perspective strains for ethanol production in apple juice are $20K^{++}$ and IIx31.

Ethanol tolerance. During fermentation, sugars lead to the production of ethanol and carbon dioxide. Increasing the concentration of ethanol delays the growth of the yeast, which eventually stops the fermentation (10). It is important that the yeast strain used be able to survive the highest ethanol concentration produced. For beer these concentrations range within 3–9%, for grape wine 11–15% and for honey wine to 17% [11, 12]. Alcohol tolerance is particularly important for alcoholic fermentation while producing ethanol for fuel. We need to have yeast with a tolerance more than 25%. The results of our experiment are presented in Figure. We estimated the yeast viability after incubation of yeast cells in solutions with different ethanol concentration (from 12 to 23% v/v). A cell suspension ($1.97–2.1 \cdot 10^8$ cell/ml) was incubated at 20 °C under agitation (150 rpm) for 2 h in different ethanol concentrations. The effect of ethanol toxicity on yeast cells is presented in Figure. The resistance to 18% v/v alcohol concentration was estimated for yeast strains 4+, $20K^{++}$, K^{+}ob. There was a sharp decrease in cell viability with yeast extracted from cranberry.

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ETANOLO PRAMONEI PERSPEKTYVIŲ MIELIŲ, PASIŽYMINČIŲ PADIDINTU KONKURENCINGUMU, PRADINĖ ANALIZĖ

Santrauka

Šiame darbe tirtos etanolio pramonei perspektyvių mielių *S.cerevisiae* Rom K-100, M437, 4+, 18M, $20K^{++}$, K^{+}ob ir naujai paieškos būdu atrastų IIx31, III.2, „Spanguolė“ kamienų rauginimo galimybės bei poveikis obuolių sulčių fermentacijos proceso kokybei. Nustatyta, kad ypač perspektyvūs obuolių sulčių rauginimui yra $20K^{++}$, IIx31, kurie galėtų būti naudojami vyno pramonėje; 4+, III.2 ir „Spanguolė“ kamienai galėtų tikti etanolio, skirto ne maisto reikmėms, gamybai. Didžiausia alkoholio tolerancija (18%) pasižymi Rom K-100, III.2, K^{+}ob, 4+, IIx31 ir $20K^{++}$ mielių kamienai.