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PHENOTYPES INVESTIGATION IN THE YEAST *SACCHAROMYCES CEREVISIAE* ISOLATED FROM DIFFERENT GRAPE CULTIVARS FOLLOWIG FERMENTATION

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Micobiological investigation was carried out on *Saccharomyces cerevisiae* yeast cultures, which were isolated from different varieties of vintage grape harvested from the "Koblevo" winery, Nikolaev region of Ukraine. It was determined that wild yeast cultures tend to be of one of three different phenotypes. For comparison and reference, investigation of test cultures was performed with previously known phenotypes and yeast cultures *Saccharomyces cerevisiae* used in wine industry. It was noted that the most desirable yeast cultures had a killer phenotype. These are able to generate toxins that inhibit the growth of other yeast cultures that are present in the same substrate and compete for habitat. It was observed that yeast a neutral phenotype can also be can also utilized in wine industry biotechnology. Yeast cultures with sensitive phenotype, however, die in the substrate (grape musts) in the presence of yeast culture killer phenotypes that secrete killer toxins harmful to these sensitive phenotypes.

Key words: yeast, Saccharomyces cerevisiae, phenotype, killer, neutral, sensitive.

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ИССЛЕДОВАНИЕ ФЕНОТИПОВ У ДРОЖЖЕЙ *SACCHAROMYCES CEREVISIAE*, ВЫДЕЛЕННЫЕ ИЗ РАЗЛИЧНЫХ СОРТОВ ВИНОГРАДА ПОСЛЕ БРОЖЕНИЯ

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В работе установлены фенотипы дрожжей на основании данных микробиологических исследований культур дрожжей *Saccharomyces cerevisiae*, выделенных от различных сортов винограда, собранного в период массового сбора урожая с полей винзавода "Коблево", Николаевской области Украины. Установлена принадлежность диких дрожжевых культур к одному из трех фенотипов. Для сравнения исследовались тест-культуры с заведомо известными фенотипами и дрожжевые культуры, используемые в промышленном виноделии. Отмечено, что наиболее желателен киллерный фенотип, как способный вырабатывать токсины, подавляющие рост других дрожжевых культур в одном субстрате и их способность конкурировать за среду обитания. Определено, что нейтральный фенотип также может быть использован в биотехнологии виноделия. Культуры же чувствительного фенотипа погибают в субстрате в присутствии киллерных фенотипов дрожжевых культур, выделяющие киллерные токсины, которые губительные для чувствительных фенотипов дрожжей.

Ключевые слова: дрожжи, Saccharomyces cerevisiae, фенотип, киллерный, нейтральный, чувствительный.

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ДОСЛІДЖЕННЯ ФЕНОТИПУ У ДРІЖДЖІВ *SACCHAROMYCES CEREVISIAE*, ВИДІЛЕНІХ З РІЗНИХ СОРТІВ ВИНОГРАДУ ПІСЛЯ БРОДІННЯ

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В роботі на підставі даних мікробіологічних досліджень культур дріжджів *Saccharomyces cerevisiae*, виділених від різних сортів винограду зібраного в період масового збору з полів винзаводу "Коблево", Миколаївської області, України, встановлені фенотипи дріжджів. Встановлена приналежність у диких дріжджових культур до одного з трьох фенотипів. В якості

порівняння досліджувалися тест культури з завідомо відомими фенотипами і дріжджові культури яки використовуються в промисловому виноробстві. Відзначено, що найбільш бажаний кілерний фенотип, як здатний виробляти токсини яки пригнічують ріст інших дріжджових культур в одному тому ж субстраті і конкурують за місце існування. Визначено, що нейтральний фенотип також може бути використаний в біотехнології виноробства. Культури ж чутливого фенотипу гинуть в субстраті в присутності кілерних фенотипів дріжджових культур яки виділяють кілерні токсини згубні для чутливих фенотипів дріжджів.

Ключові слова: дріжджі, Saccharomyces cerevisiae, фенотип, кілерних, нейтральний, чутливий.

Dry or liquid yeast cultures *Saccharomyces cerevisiae* are widely used in the wine industry, and require resilient strains. Ideally, these culture populations must be able to neutralize or kill other competitive strains that grow in the same media. In 1963, Makower (Makower at al) observed antagonism between yeast cultures *Saccharomyces cerevisiae*, wherein some of the cultures were able to produce substances that killed other yeasts. These yeast killing substances were collectively termed "killer factor", and yeast with this killer factor have been assigned to the killer phenotype ("the killers"). Killer factor consists of protein and polysaccharide (Drotschmann, 1999; Glassner, 1998; Musmanno, 1999).

Among the different competitive mechanisms that microorganisms can use to multiply and survive in natural communities, the production of antimicrobial toxins represents a common, efficient and specific ecological way to eliminate competitor strains / species from the same habitat. By killing or severely reducing the fitness of sensitive strains, toxin-producing microorganisms which are self-immune can be selected and so dominate in specific environments (Aguilera, 1994; Bussey, 1990). In particular, the production of killer toxins by yeasts has aroused great interest, because of its implications, technological transfer and intriguing perspectives for wine industry (Camarasa, 2011; Govender, 2010; Salinas, 2010).

A phenotype determination method, based on the reaction of a sensitive strain being transferred to medium pre-fermented by a killer strain, is proposed for the quantitative determination of the killer activity in fermentation media by *Saccharomyces cerevisiae* K1. This technique enables killer activity to be closely monitored throughout the duration of batch fermentation. The killer activity in the culture medium is represented by the percentage decrease in viable biomass in comparison with the viable biomass of a reference culture (Bussey, 1999; Portugal, 1994).

Phenotyping of the yeast species *Saccharomyces cerevisiae* is of significant interest to wine industry biotechnology as the use of pure cultures for the fermentation of grape musts potencially allows for the removal of random variations inherent in wild yeast. Fermentation of carbohydrates is uncontrolled and proceeds spontaneously, which often breaks the smooth flow of alcoholic fermentation. Pure yeast culture also allows identifying the best taste of the final product from each grape variety (Duarte, 2009; Golubev, 2006; Jelier, 2011).

During selection of pure cultures of the yeast *Saccharomyces cerevisiae*, their belonging to a particular phenotype is considered: killer, neutral, or sensitive yeast cultures (Camarasa, 2011; Duarte, 2009; Glassner, 1998). Yeast cultures producing killer toxins are referred to as the killer phenotype. Neutral phenotypes observed in yeast cultures, which do not die under the action of yeast killer toxins, do not suppress sensitive yeast cultures. Yeast culture, which die when exposed to killer toxins produced by the killer yeast cultures are of the sensitive phenotype. The phenotypes preferred for wine industry yeast biotechnology are yeast cultures with either a killer or neutral phenotype.

During anaerobic alcoholic fermentation, if the yeast culture belongs to a sensitive phenotype, it will be replaced by natural, wild yeasts which are predominantly of the killer phenotype. These will, therefore, dominate in the process of anaerobic fermentation. The use of pure yeast cultures with a specific phenotype identified prior to selection allows for control of culture purity in the substrate, in which other yeast culture/s may be present.

The aim of this study was to determine the phenotype of each isolated yeast culture, *Saccharomyces cerevisiae*, from different French, Hungarian, Georgian, Moldovan, Ukrainian (Toirov`s Research Institute) grape cultivars following fermentation. To achieve this goal it was necessary to address the following objectives:

- Isolation of yeast cultures from fermented grape musts taken from different grape varieties;
- Identification and selection for further investigation of the yeast culture species *Saccharomyces cerevisiae*, isolated after fermentation process;
- Identification of the phenotype for each isolated culture *Saccharomyces cerevisiae* in wort agar with the addition of 0.3% methylene blue;
- Preservation of yeast cultures with the killer phenotype for further use;

The advantages of use of pure cultures of yeast are as follows:

- Fermentation proceeds smoothly, without delays and strong foaming gives a complete and thorough fermentation of sugar;
- The grape should ferment predictably and swiftly according to those yeast strain properties, which are known and predictable;
- Predictable fermentation of grape must results in the formation of 0.6 to 0.8% ethanol, which is comparable to wild yeast;
- Wines fermented due to pure yeast cultures contain less volatile acids and volatile esters, become transparent more quickly, and accelerate the formation of sediment on the bottom of the tank or barrel;
- Wines have predictable, improved taste and flavor, and are less inclined to wine diseases, in comparison with a wine obtained as a result of spontaneous fermentation. All of aforementioned yeast conditions provide the wine industry with the potential ability to have selected yeast cultures with new properties after selection.

MATERIALS AND METHODS

Samples from different industrial grape species were collected during the vintage season from the vineyard of the Koblevo Company winery, located in the Nikolaev region of Ukraine. In total, 15 species were selected for the research. The following industrial grape species were selected: Aligote, Bastardo, Cabernet Sauvignon, Chardonnay, Irshai Oliver, Isabella, Merlot, Muscat Ottonel, Hamburg`s Muscat, Odessa`s black, Riesling Rhenish, Rkatsiteli, Sauvignon, Traminer, Fetyaska. All isolated yeast cultures were investigated for phenotype.

Isolated yeast cultures were cultivated on Wort Agar (Becton Dickinson Company, USA). To determine the yeast culture phenotype, special selective media was prepared: Sterile grape must – 100 ml., Agar 3% - 100 ml., Methylene blue – 0.5% - 0.5 ml. When temperature was between 60 to 70 degrees Celsius, the prepared sterile 3% agar was diluted with sterile grape must (1:1). For improved contrast of growth zones, 0.5 ml - 3% methylene blue was added to sensitive yeast cultures in the molten medium.

Each colony of yeast, for which it was necessary to determine phenotype, was taken from agar on Petri dish and placed into the test tube with 0.5 ml of distilled water, where they were suspended. For determination of phenotype, a culture suspension of the sensitive

phenotype (Kahuri-7 strain of *Saccharomyces cerevisiae*) was uniformly inoculated (as a microbial lawn) onto prepared agar-containing Petri dishes. To study the yeast culture phenotypes the following industrial yeast cultures were selected:

1. *Saccharomyces cerevisiae* VKPM – 1236; DMVP-12-4-20 – test culture to determine killer activity in yeast.
2. *Saccharomyces cerevisiae* VKPM – 1173; NCYC-1006 - test culture to determine killer activity in yeast. Strain has sensitive phenotype (S).
3. *Saccharomyces cerevisiae* VKPM – 2177; extra sensitive test culture to the killer toxin K-2.
4. *Saccharomyces cerevisiae* (*Saccharomyces cerevisiae bayanus*) Lalvin QA23 – has killer phenotype. An industrial yeast culture originally isolated at Tras os Montes e Alto Douro (UTAD) University in Portugal.
5. *Saccharomyces cerevisiae* VIN 13 - has killer phenotype. An industrial yeast culture developed using hybridisation in the Department of Microbiology, Institute of Biotechnology of Wine, the University of Stellenbosch, South Africa.
6. *Saccharomyces cerevisiae* VIN 2000 - has killer phenotype. An industrial yeast culture again developed using hybridisation in the Department of Microbiology, Institute of Biotechnology of Wine, the University of Stellenbosch, South Africa.
7. *Saccharomyces cerevisiae* Lalvin ICV D47 - has killer phenotype. An industrial yeast culture, originally isolated in Institute of Wine Montpellier, France.
8. *Saccharomyces cerevisiae* Lalvin ICV D254 - has neutral phenotype. An industrial yeast culture, originally isolated in Institute of Wine, ICV, France.
9. *Saccharomyces cerevisiae* Uvaferm CS2 - has killer phenotype. An industrial yeast culture, originally isolated in Alsace, France, and the future selected for use by INRA, France.

RESULTS AND DISCUSSION

After 48 h incubation at 26-28 degrees Celsius, phenotyping of the yeast cultures was complete. If the colonies were formed around the zone growth inhibition on a microbial lawn consisting of the sensitive henotype yeast culture strain, it was identified as the killer (K) phenotype - Fig.1. Strains of yeast on the microbial lawn on which killer culture forms the zones are of the sensitive (S) phenotype. All other yeast cultures that do not belong to the killer or to sensitive phenotypes, as highlighted above, are of the neutral (N) phenotype.

Fig.1 and Fig.2 illustrates the zones of inhibition or growth around disks on the wort agar microbial lawn of five cultures with the killer phenotype and two cultures with the neutral phenotype.

This investigation established the phenotype for each yeast culture, isolated after spontaneous fermentation from different grape varieties: French, Moldovan, Georgian, Ukrainian (Tairov`s) selections.

It was apparent during the identification process that the killer phenotype was identified most frequently in yeast cultures *Saccharomyces cerevisiae*, which allows for the selection yeast of cultures for further use in the biotechnology of winemaking.

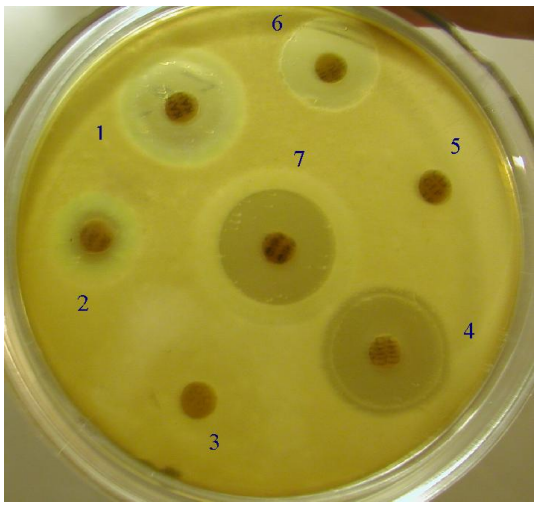

Fig.1. Killer phenotype in yeast *Saccharomyces cerevisiae*:
Samples 1, 2, 4, 6 and 7 have killer phenotype. Samples 3 and 5 have neutral phenotype.

Table 1

Phenotypes of yeast cultures isolated from different grape cultivars

Grape cultivar	**Phenotype**	**Collection number (USRCB)**
Aligote	Killer phenotype (K)	Y-3479
Bastardo	Killer phenotype (K)	Y-3486
Cabernet Sauvignon	Killer phenotype (K)	Y-3487
Chardonnay	Killer phenotype (K)	Y-3480
Irshai Oliver	Killer phenotype (K)	Y-3488
Isabella	Killer phenotype (K)	Y-3489
Merlot	Neutral phenotype (N)	Y-3490
Muscat Ottonel	Killer phenotype (K)	Y-3491
Odessa`s Black	Killer phenotype (K)	Y-3492
Hamburg`s Muscat	Killer phenotype (K)	Y-3493
Riesling Rhenish	Killer phenotype (K)	Y-3481
Rkatsiteli	Killer phenotype (K)	Y-3482
Sauvignon	Neutral phenotype (N)	Y-3483
Traminer	Killer phenotype (K)	Y-3484
Fetyaska	Killer phenotype (K)	Y-3485

Most of isolated wild yeast cultures of *Saccharomyces cerevisiae* have killer phenotype.

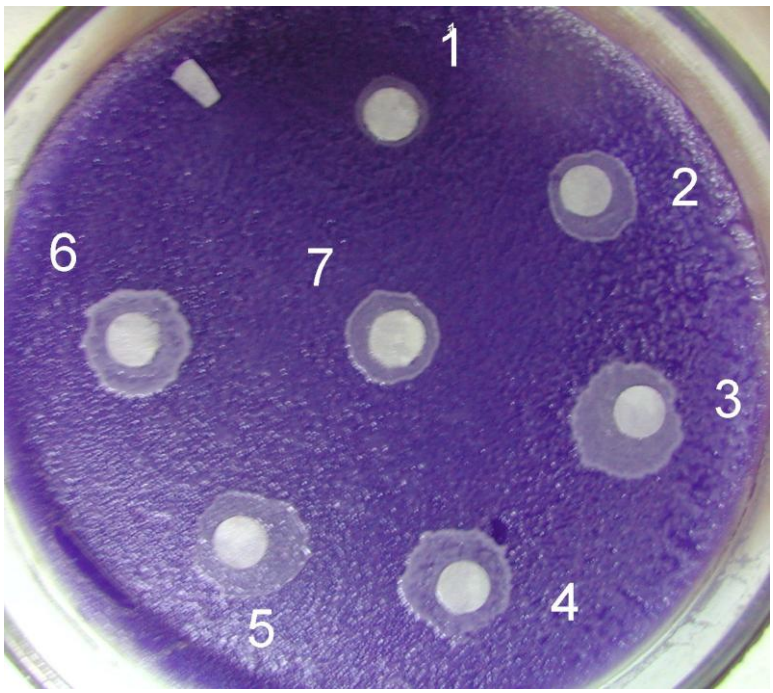

Fig. 2. Killer phenotype in yeast *Saccharomyces cerevisiae*:
Samples 2, 3, 4, 5, 6 and 7 have killer phenotype. Sample 1 have neutral phenotype.
Growth of yeast cultures *Saccharomyces cerevisiae* on microbial lawn around each disk confirm killer phenotype.

Table 2

Phenotype of industrial and standard yeast cultures *Saccharomyces cerevisiae* isolated from different grape cultivars

Yeast culture *Saccharomyces cerevisiae*	**Phenotype**	**Collection number (USRCB)**
Kahuri-7	Sensitive phenotype (S)	Y-3494
VKPM – 1236	Killer phenotype (K)	Y-3147
VKPM – 1173	Sensitive phenotype (S)	Y-3145
VKPM – 2177	Killer phenotype (K)	Y-3150
QA-23	Killer phenotype (K)	Y-3458
VIN 13	Killer phenotype (K)	Y-3460
VIN 2000	Killer phenotype (K)	Y-3461
Lalvin ICV D-47	Killer phenotype (K)	Y-3456
Lalvin ICV D-254	Neutral phenotype (N)	Y-3457
Uvaferm CS2	Killer phenotype (K)	Y-3459

Tables 1 and 2 show that yeast cultures *Saccharomyces cerevisiae* are predominantly of the killer phenotype. This is expected for wild yeast cultures. It is necessary, therefore, to include in the grape must more than one phenotype, such as killer (K) or neutral (N),

concurrently. The combination of yeast culture phenotypes for grape must fermentation gives faster and more complete fermentation of carbohydrates than is achieved with each individual type separately. Combined cultivation of a few cultures increases alcohol forming ability and formation of volatile acids in comparison with the original yeast culture *Saccharomyces cerevisiae*. Numerous researchers have suggested that fermentation with the grape must yeast complex obtains better results than fermentation with a single culture.

CONCLUSIONS

Phenotypes of yeast cultures, *Saccharomyces cerevisiae,* isolated from different cultivars from the "Koblevo" winerd, Nikolaev region of Ukraine were identified and specified.

Most yeast cultures, *Saccharomyces cerevisiae*, isolated from the studied grape cultivars belong to the killer (K) phenotypes – 10 cultures. 5 cultures with neutral (N) phenotype were also identified.

Given its sensitivity to killer toxins, Kahuri-7 was selected as a basic culture for the microbial lawn on which all of the tested yeast cultures were inoculated.

Isolation of regional yeast cultures *Saccharomyces cerevisiae* from the grape and identification of their phenotypes are important and advantageous for the ongoing selection of yeast cultures for wine industry biotechnology.

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