VILNIUS UNIVERSITY
INSTITUTE OF BOTANY OF
NATURE RESEARCH CENTRE

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THE EXPRESSION *OF SACCHAROMYCES CEREVISIAE* K2 PREPROTOXIN GENE IN PLANT *NICOTIANA TABACUM* L. AND THE SEARCH OF TOXINS PRODUCING MICROORGANISMS AND THE ANALYSIS OF THEIR USE

Summary of doctoral dissertation
Biomedical sciences, biology (01 B)
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The dissertation will be defended at the public session held by the Council of Biology at 14 a. m. on May 25, 2011 at Institute of Botany of Nature Research Centre. Address: Žaliųjų Ežerų 49, LT-08406, Vilnius, Lithuania.

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VILNIAUS UNIVERSITETAS
GAMTOS TYRIMŲ CENTRO BOTANIKOS INSTITUTAS

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Saccharomyces cerevisiae K2 preprotoksino geno raiška *Nicotiana tabacum* L. augaluose bei naujų, toksinus produkuojančių mikroorganizmų paieška ir jų panaudojimo analizė

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INTRODUCTION

Many spieces of yeasts synthesize and export proteins or glycoproteins with toxic effects against sensitive yeasts, a phenomen called „killer system“ [Young, Yagiu, 1978; Tipper, Bostian, 1984; Magliani et al., 1997; Marquina et al., 2002]. The killer system was first described in *Saccharomyces cerevisiae* [Bevan, Makower, 1963] and soon after that this phenomenon was found in many other yeast genera such as *Candida, Cryptococcus, Debaromyces, Hanseniaspora, Hassenula, Kluyveromyces, Ustilago, Pichia,* etc. [Schmitt, Breinig, 2002]. The killer activity of yeast is detectable only when it is assayed against proper yeast as sensitive and is dependent on several factors, such as pH, saltinity and temperature. The genetic elements that encode for a killer phenotype may be double stranded RNA molecules (dsRNA) encapsulated in virus like particles (VLPs), linear double stranded DNA plasmid (dsDNA) or nuclear genes [Schmitt, Breinig, 2002]. The killer system of *Saccharomyces cerevisiae* has been best studied and has been classified in 3 groups (K1, K2, K28) according to their toxin properties and genetic determinants. Two classes of dsRNA with different molecules sizes and functions are responsible for the killer phenotype in that yeast: L dsRNA that encodes for a RNA polymerase and capside proteins and M dsRNA that encodes for the toxin and confer immunity.

The susceptibility to toxins varies greatly between yeast species and strains. Several experiments have been made to identify strains. The killer toxin was effective at preventing spoilage of higly salted food by yeasts. The biological activity of killer toxins are applied as antifungal agents [Magliani, 2004].

As the spectrum of action of some toxins has extended to microbial pathogens of clinical interest, killer toxins and/or killer toxin-like antibodies and mimotopes are of great relevance to medicine. Other toxins that exert a killing action on spoilage yeasts are applied in the fermentative and food industry where they are used as „natural“ food antimicrobials [De Ingeniis, 2008]. Previously antiphytopathogenic effect of different yeast species isolated from natural apple and grape habitats has been reported.

Plants are constantly exposed to a great variety of potentially pathogenic organism such as viruses, fungi, bacteria, protozoa, mycoplasma and nematodes, and can be affected by adverse environment conditions [Castro, Fontes, 2005]. Many different

genetic strategies have been proposed to engineer plant resistance to diseases including producing antibacterial or antifungal proteins of non-plant origin inhibiting microbial pathogenicity or virulence factors enhancing natural plant defense and artificially inducing programmed cell death at the site of infection (Mourgues et al., 1998).

Similarly, some bacteria, fungi or mammals synthesize a number of proteins and peptides with antiphytopathogenic properties [Selitrennikoff, 2001]. A numerous of yeast (*Saccharomyces cerevisiae*, *Ustilago maydis*, *Kluyveromyces lactis*) secreted proteins that are lethal to fungal cells [Magliani et al., 1997] or microbial-originated substances having antibiotic features [Melvydas et al., 2007; Mandryk et al., 2007] have been discovered.

In 1990 at Laboratory of Genetics of the Institute of Botany the copy DNA of the K2 virus genome M2 fragment was cloned and determined the nucleotides sequence of that fragment [Meškauskas, Čitavičius, 1992]. The features of the K2 killer preprotoxin gene were widely investigated [Gulbinienė, 2002; Servienė, Melvydas, 1999; Servienė ir kt., 2002] so it was decided to clone it into the plant *N. tabacum* and to check its expression in the transgenic plants. New microorganisms with wide fungal effect against pathogenic micromicetes were also searched for.

The aims of the scientific research were to investigate the newly found bacterial and yeast isolates killer, immune and fermentation features; to ascertain the influence on environment factors (pH, temperature) to secretion of toxins; to evaluate the possibilities of yeast *S. cerevisiae* K2 killer preprotoxin gene expression in plants.

The tasks of the work were as follows:

- ✓ To construct DNA plasmids with *S. cerevisiae* K2 killer gene suitable for plant transformation; to evaluate the possibility of expression of yeast killer K2 preprotoxin gene with different promoters in plants.

- ✓ To prepare spontanic fermentations from picked fruits and berries, to isolate and purify microorganisms from them; to carry out the analysis of killer isolates, immune and

fungicidal features, to compare newly found isolates secreting substances effects with the effects of *S. cerevisiae* K1, K2 and K28 killer toxins types.

- ✓ To optimise the conditions of cultivation of microorganisms, that ensure the ultimate toxins funcional display; to estimate the impact of pH and temperature to activity of secreting toxins.

- ✓ To investigate the effects of microorganisms with killer characteristics to some phytopathogenes species and animal and human pathogenes.

- ✓ To separate and evaluate killer yeast from purified spontanious fermentations from fruits and berries that has perspectives in production of wine and ethyl alcohol industry.

Novelty of the research:

- ✓ During the research K2 killer preprotoxin gene was first cloned successfully to plant *Nicotiana tabacum* L. and its expression was thoroughly analysed.

- ✓ Bacterial isolates that have wide spectrum killer and immune effect features and fungicidically impact numerous yeast and micromicetes species were found.

- ✓ The bacterial isolate (Tx) which has unusual autolysis and biological features that are suitable for the production of biopreparates and requirements resort was found.

- ✓ The bacterial isolate (Ux) that has enlarged toxin secretion in low pH levels was found.

- ✓ The strain of yeast 1M was used for natural apple wine semimanufacture production in the Joint-stock company „Vaisių sultys“ (Fruit Juice).

The defensive statements:

- ✓ The expression of the K2 killer preprotoxin gene in plant *N. tabacum* L. is possible.

- ✓ The production of the K2 killer preprotoxin gene in plant *N. tabacum* L. is of lower level than in yeast and the pH optimum of protein effect is different.

- ✓ Bacterial isolates from spontanious fruit and berry fermantations have unusual killer and fungicidal features, act in wide interval of pH and temperature and are superior than yeast *S. cerevisiae* standard killer strains.

- ✓ bacterial isolates from spontanious fruit and berry fermentations are able to act fungicidically on some plant, animal and human pathogenes.

- ✓ The new yeast *S. cerevisiae* strain 1M is economically useful for natural apple wine semimanufacture production.

Approbation of results. The dissertation material was reported at 2 international conferences and 3 conferences in Lithuania. The results of the research were presented in 5 scientific articles and 5 abstracts of conferences.

2. MATERIAL AND METHODS

The yeast expression plasmids pAD4 and pYEX12 and plant vectors pCGT [Jefferson et al., 1987], pGA482 [Proscevičius, Žukas, 1999] and pART27 were used for construction of recombinant plasmids pCGT/KillD, pCGT/KillR, pART/Kill K2 and pGA/Kill K2 (carrying *S. cerevisiae* K2 preprotoxin gene under control of *Cauliflower mosaic virus* CaMV and yeast ADH1 promoters) as well as yeast plasmids pAD/CGT-Kill K2D, pAD/CGT-Kill K2R and pART/Kill K2 (K2 under control of CaMV promoter). General procedures for the construction and analysis of recombinant DNAs were performed as described by Sambrook et al., 1989. All restriction enzymes (SalI, SmaI, XbaI, Ecl136II, EheI, SdaI), T4 DNA ligase, bacterial alkaline phosphatese,

Klenov fragment and DNA size marker (GeneRuler^{TM} DNA Ladder mix) were obtained from UAB „Thermo Fisher Scientific“ (Vilnius) and used according to the manufacturer’s recommendations.

The *S. cerevisiae* strain α’1, sensitive to all killers [Čitavičius et al., 1972], was transformed by plasmids of interest – according to [Gietz et al., 2002]. Transformants were selected by complementation of LEU2 auxotrophy. Killer phenotype selective indicative medium (MB) [Sherman et al., 1986] was used to test killer toxin production and the immunity of transformants. Transformants were checked for toxin production in a killing zone plateassay following replica-plating of transformants onto a lawn of the sensitive strain α’1. Immunity was tested by the streaking the standard *S. cerevisiae* K1, K2 and K28 killer strains (Rom-K100, wt, *HM/HM, kil-K2*; M437, wt, *HM/HM, kil-K2*; K7 *MATα, arg9, kil-K*; K28, wt, *HM/HM, kil-K28*) on the lawn of transformed cells. Stability of the Leu^+ and $K2^+$ phenotype of transformants was analysed growing cell colonies on non-selective media: minimal – in the case of LEU2; indicatory MB with the layer of α’1 – in the case of K2 preprotoxin gene.

Samples of spontaneous fermentations were prepared by grinding berries or fruits, diluted with a small amount of water and keeping them at room temperature for about 30 days. Yeasts and bacteria strains were isolated by multiple cloning from acid medium by the spread method on YEPD medium [Melvydas et al., 2005]. Selected colonies were streaked on the methylene blue agar seeded with a sensitive yeast *S. cerevisiae* strain α’1 to assay the killer activity. After incubation of plates at the temperature of 25 °C for 3 – 5 days clear zones of growth inhibition surrounding the killer cells were evaluated. The presented results were obtained from at least three independent experiments.

The bacterial and yeast strains were obtained from spontaneous fermentations:

Bacterial Killer Strains	Origin of Raw Material	Region
Ux	Hawthorn	Vilnius
Tx	Blueberry	Vievis
V8	Yellow flowered dogwood	Ignalina
B9	Cowberry	Ignalina
B18	Lily	Vievis
KB	Juniper	Vilnius

Yeast Killer Strains	Origin of Raw Material	Region
20K++	Red currants	Širvintos
4+	Cherry	Rokiškis
IIx31	Snowball-tree	Širvintos
III-2	Grapes	Šakiai
Cranberry	Cranberry	Vilnius
K+ob	Apples	Vilnius
1M	Apples	Anykščiai

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 YEPD medium without or with an appropriate ethanol concentration was used for the screening of yeast for ethanol tolerance. The identification of yeast strains was investigated at National Food and Veterinary Risk Assessment institute.

The identification of yeast strains was done at the Microbiological Laboratory of Lithuanian Public Health Centre. Automatised mini AP I 20 CAUX system for clinical yeast identification.

3. RESULTS AND DISCUSSION

The fruits and berries from different regions of Lithuania were collected and their spontaneous fermentations were prepared. About 230 yeast strains were isolated. All strains under investigation were first tested for their killer phenotype. Initial experiments were performed using methylene blue agar technique to determine the ability of various strains to kill sensitive *Saccharomyces cerevisiae* strain α'1 on media buffered at various pH values from 3,6 to 5,2. In addition, the microorganisms which formed lysis zones on sensitive *S. cerevisiae* strain and on standard killer yeasts lawn were found. Toxin properties possessing 10 (Tx, Ux, V8, B9I, B9II, KBI, KBII, B18I, B18II, B18III) microorganisms capable to kill some fungous agents of plant diseases were discovered. The strains mentioned exhibited different killer activity (Fig. 1).

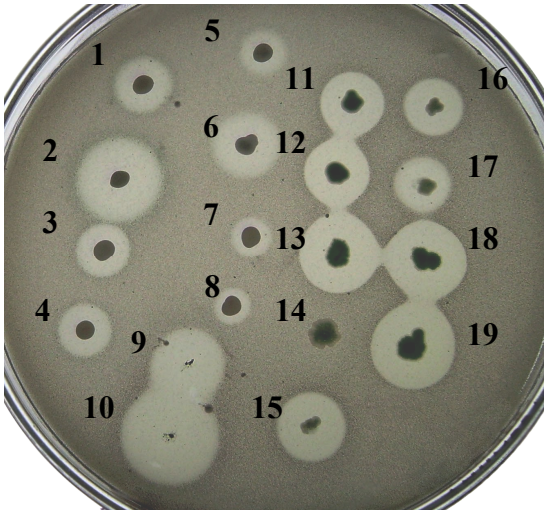

Fig. 1. Killer properties of bacterial strains. Control yeast killer srains: 1–RomK100, 2–M437, 3–K7, 4–DBY4975, 5–K28, 6–MS300, 7–B18I, 8–B18II, 9–Ux, 10–V8, 11–B9I, 12, 13–B9II, 14–TxI, 15–TxII, 16–TxIII, 17–TxIV, 18–KBI, 19–KBII, medium MB, pH 4,8, temperature 20 ºC, lawn – α'1.

It is believed that in the near future the microorganisms found might be used to create remedies against different plant, animal and human pathogens. The microorganisms analysed exhibited killer activity in the wide range of pH (3,0 – 7,0) at 20 – 37 °C (Fig. 2).

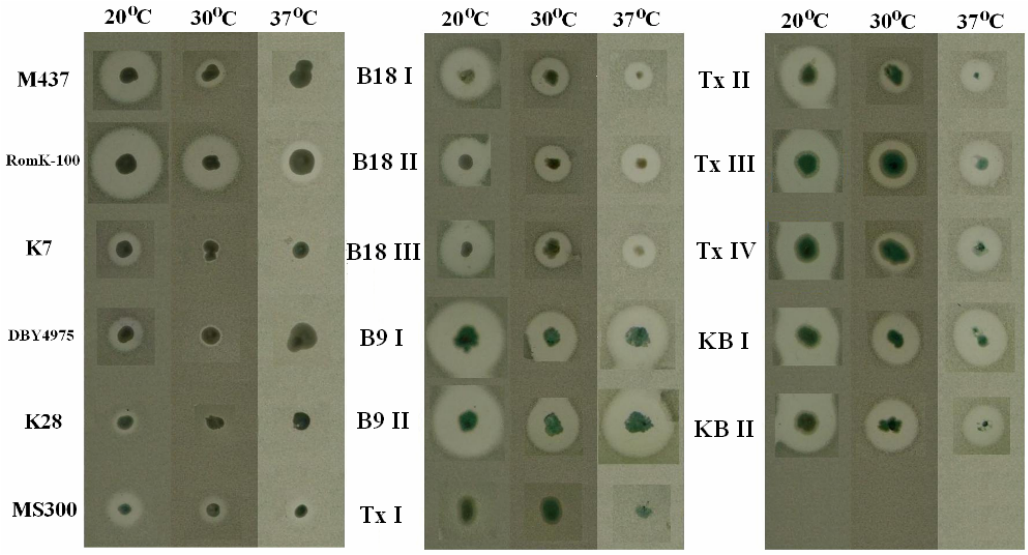

Fig. 2. Well test killer activity, medium MB, pH 4,4. Control yeast killer strains: M437, Rom K–100, K7, DBY4975, K28, MS300. Bacterial strains: B18 I, B18 II, B18 III, B9 I, B9 II, Tx I, Tx II, Tx III, Tx IV, KB I, KB I.

Increasing the temperature range the standard killer toxins in the cases mentioned above are less active and depend on the strain.

Ux and Tx bacterial isolates exhibited activity against dermatomycetes *Trichophyton rubrum*, *Microsporum canis*, yeast of the genera *Candida*, *Kluyveromyces* and against fungal disease agents of ornamental and woody plants such as *Verticillum albo-artrum*, *Venturia ineaqualis*, *Alternaria* and *Fusarium* (Fig. 3). The bacterial strain V8 is active against the microorganisms mentioned above. It is known that the killer activity is pH dependent and better expressed under low temperatures of 20 – 25 °C. Bacterial strains Ux and V8 probably produce different types of antimicrobial factors. However, resistance of the above mentioned bacteria to variable environmental conditions and the possibility of uncontrollable distribution limit its application in practice. It is believed that the microorganisms found is one of the most important results of this work and might have the commercial importance in the future. (Fig. 3). The strain Ux almost does not grow in the acid medium but secretes huge amount of the toxin. Biologically the growth is much more advantageous because other bacterias are killed under such conditions.

From biological viewpoint that growth is much more superior because other bacteria do not survive under such conditions. The strain tries to preserve the medium which allows greater multiplication. In salty environment isolate produces much more toxin, so it happens that after some time pH of the medium may become alkaline. The genes of Ux bacterial isolate that encodes toxin might be useful for the design of genetically modified organisms, while their toxins – for new generation of pharmaceuticals.

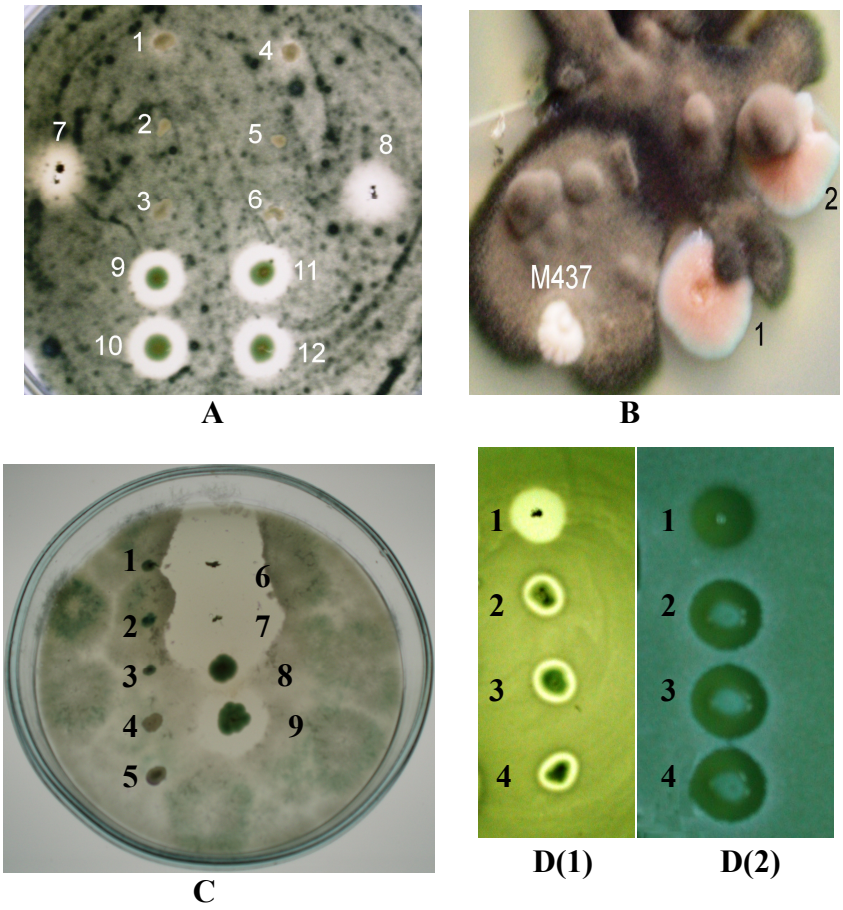

Fig. 3. A – Killer effect of bacterial strains for growing of *Verticillium albo-artum*: 1–6 control yeast killer strains, 7 – V8, 8 – Ux, 9, 10 – Tx, 11, 12 – KB. **B – fungistatic effect of bacterial strains for growing of *Venturia ineaqualis:*** 1 – KBI, 2 – Tx, M437 – control yeast killer strain**. C – killer properties of bacterial strains for growing of *Fusarium sp*., pH 4,8,** –1–5 – control yeast killer strains, 6 – Ux, 7 – V8, 8 – B9, 9 – Tx; **D – killer effect of bacterial strains for growing of *Candida glabrata* D(1) and *Candida tropicalis* D(2),** 1 – Ux, 2–4 – Tx.

It is important that killer factor produced by Tx degrade in three days at the temperature of 20ºC. The strain is also sensitive to environmental humidity. On the basis of this analysis this bacterial strain was chosen for the production of biopreparates (Fig. 4).

Tx bacterial isolates grow well on the medium YEPD and the supreme activities of secreting toxin occur on the medium MB in the pH range from 3,0 to 5,0. However, on the medium YEPD these strains stay badly because after some time the autolysis of the general line, but not sporulation (Fig. 4), begins.

Fig. 4. The autolysis of bacterial strain Tx: A – the start of autolysis, B – after autolysis.

After Tx identification analysis it was determined that isolate belongs to *Bacillus* sp.

The species are not announced because it might have a commercial importance and might be patented in the future.

Some functions of new discovered yeast strains from spontaneous fruit–berry fermentations were analyzed. The killer activity or strain sensitivity, immunity and dependence on killer type of selected strains were evaluated. We have analyzed the possibility to use the express-method for estimation of fermentation rates and glucose assimilation. It has been shown that using this method it is possible preliminarilly appreciate fermentation efficiency.

The occurrence of killer phenomenon among grape wine yeasts in a number of winemaking countries was studied. There is not much data about ethanol fermentable possibilities of killer yeast origin under northern climate conditions. The killer phenomenon of yeasts was investigated in naturally occurring yeast communities. The use of killer yeasts as starters in wine fermentation processes is important. 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 bring about the slowness of fermentation and ethanol productivity decrease. The killer system may become a way to avoid 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. To verify this

six killer yeast strains: 4+, 20K++, IIx31, Cranberry, 1M and two standard wine strains Rom K-100 and M437 were used in our experiment. It was established that the strains 20K++, 4+, IIx31 showed a powerful killer activity in comparison with *S. cerevisiae* killer standard strain M437. The first selection criteria for fermentable 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 yeast strain 1M showed higher rates of glucose assimilation and a better yield of fermentation than the standard RomK–100, M437 strain (Table 1).

Table 1. The results of analysis of apple juice fermentation efficiency

Strain	Visual fermentation rate (at ~20 ºC temperature)	Sugar quantity after a week		
		Glukose, mM	Glukose, mg/ml	Sugar, ºBlg
RomK100	+++	0,18	0,03	0.5
M437	++	0,42	0,08	2,0
4+	+++	0,19	0,03	0,5
20K++	+++	0,23	0,04	0,5
K+ob	+++	0,32	0,06	1,0
IIx31	+++	0,35	0,06	1,0
III2	++	0,49	0,09	2,5
Cranberry	+	0,26	0,05	1,0
1M	**++++**	**0,13**	**0,02**	**0,5**

Notes: ºBlg – Baling degree. 1 ºBlg conforms 10 g of sugar in 1 l of solution. CO_2 gass emission:: ++++ -very intensive, +++ - intensive, ++ - average, + - low.

The results could indicate a higher competitive advantage of the strains mentioned as the starter ethanol-making yeast against other yeasts when inoculated in a non-pasteurised juice medium. The central aspect of our research was the estimation of some quality parameters of apple juice fermentation with yeast killer strains selected from natural habitats. During fermentation sugars lead to the production of ethanol and carbon dioxid. Increasing the concentration of ethanol delays the growth of the yeast, which eventually stops the fermentation. It is important that the yeast strains used were 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 %. We estimated the yeast viability after the incubation of yeast cells in solutions with different ethanol concentration (from 12 to 23 %). The resistance to 18 % alcohol concentration was estimated for all yeast strains. There was a sharp decrease in cell viability with the yeast extracted from Cranberry (Fig. 5).

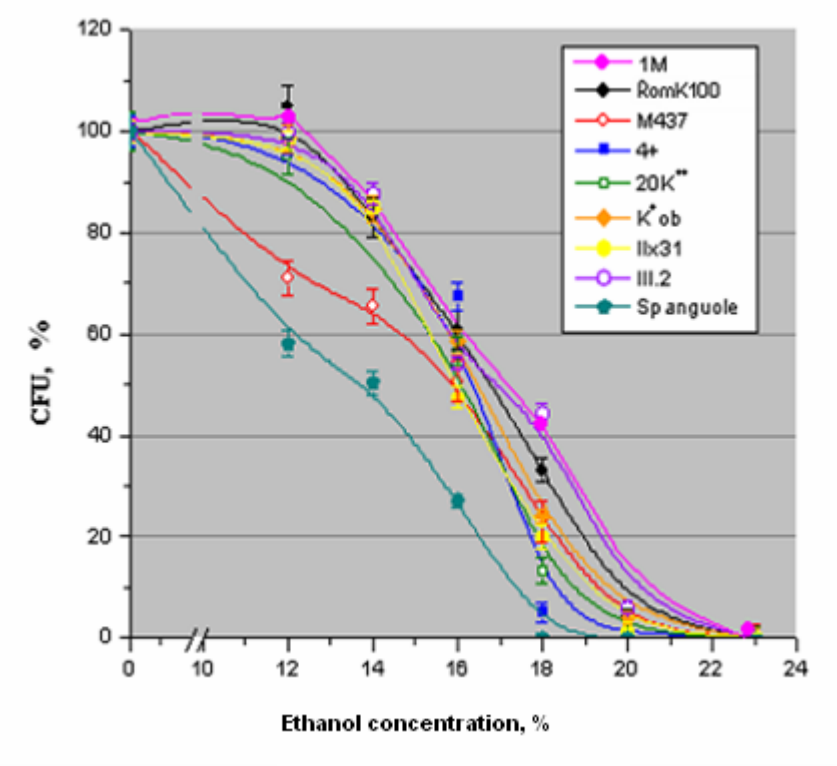

Fig. 5. Effect of ethanol concentration on yeast cell viability.

All selected yeast strains were able to ferment apple juice sugars and produce ethanol. The results presented in Tables 1 and 2 show RomK–100, 20K++, IIx31, M437, 4+, Cranberry produced ethanol the concentration of which was about 12 – 13 %. However, the highest concentration of ethanol (17,34 %) produced the yeast strain 1M. The identification of yeast strains was investigated at National Food and Veterinary Risk Assessment institute (Table 2).

Table 2. Characteristics of apple wine made with yeast strains Rom K-100, 20K++, 4+, IIx31, Cranberry, M437 after 10 days fermentation at 20 ^{0}C

No	Substance, parameters	Killer strains						
		RomK–100	**20K++**	**IIx31**	**M437**	**4+**	**1M**	**Cranberry**
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
	Alcohols							
1.	Ethanol, %	11,72	12,62	12,38	12,16	12,37	**17,34**	12,89
2.	Methanol, mg/dm^3	0,008	0,007	0,008	0,006	0,003	0,041	0,002
	Higher alcohols, mg/dm^3							
3.	2–methylbutanol, mg/dm^3	0,044	0,038	0,037	0,032	0,023	0,026	0,034
4.	3–methylbutanol, mg/dm^3	0,136	0,113	0,107	0,181	0,158	0,208	0,154
5.	Propanol, mg/dm^3	0,028	0,030	0,030	0,024	0,027	0,118	0,041
6.	Izobutanol, mg/dm^3	0,078	0,057	0,053	0,064	0,077	0,051	0,079
7.	2-butanol, mg/dm^3	<0,009	<0,009	<0,009	<0,009	<0,009	<0,009	<0,009
8.	n–butanol, mg/dm^3	<0,010	<0,010	<0,010	<0,010	<0,010	<0,010	<0,010
	Esters, mg/dm^3							
9.	Methyl acetate, mg/dm^3	< 0,009	< 0,009	< 0,009	< 0,009	< 0,009	< 0,009	< 0,009
10.	Ethyl acetate, mg/dm^3	0,002	0,006	0,001	0,021	0,019	0,082	0,094
	Aldehids, mg/dm^3							
11.	Etanal (acetaldehyde + acetal)	0,091	0,116	0,063	0,112	0,081	0,018	0,192

The strain *S. cerevisiae* 1M is optimal to the wine manufacture, because the peak of ethanol and other quality indicators were higher when compared to the standard. So the 3 independent fermentation trials were done under semi-industrial conditions. The results are presented in the table 3.

Table 3. Characteristics of apple wine made with yeast strain 1M after 10 days fermentation at 20 ^{0}C

No	Substance, parameters	Killer strains		
		1M	**1M**	**1M**
1	**2**	**3**	**4**	**5**
	Alcohols			
1.	Ethanol, tūrio %	**17,39**	**16,96**	**14,97**
2.	Methanol, mg/dm^3	0,228	0,246	0,487
	Higher alcohols, mg/dm^3			
3.	2–methylbutanol, mg/dm^3	0,300	0,205	0,483
4.	3–methylbutanol, mg/dm^3	2,565	1,349	2,794
5.	Propanol, mg/dm^3	0,160	0,706	0,250
6.	Izobutanol, mg/dm^3	0,635	0,314	0,845
7.	2-butanol, mg/dm^3	<0,009	<0,009	<0,009
8.	n–butanol, mg/dm^3	0,032	0,027	0,029
	Esters, mg/dm^3			
9.	Methyl acetate, mg/dm^3	< 0,009	< 0,009	<0,009
10.	Ethyl acetate, mg/dm^3	0,615	0,529	0,407
	Aldehids, mg/dm^3			
11.	Etanal (acetaldehyde + acetal)	0,176	0,116	0,479

As one of the most promising yeasts the strain 1M was introduced in apple wine production for natural half-finished products in Cooperative company "Vaisių sultys". The strain 1M exhibited the features needful for industrial wine yeast.

Identified strains are: 20K++, IIx31, K+ob and 1M – *S. cerevisiae,* III–2 – *Rodotorula* sp., Cranberry – *Kluyveromyces* sp.

After these experiments the possibility of expression of the yeast *Saccharomyces cerevisiae* K2 type preprotoxine killer gene in plants was checked. The aim was achieved by sorting out the conjugates of stems of neoncogenic agrobacteria (*Agrobacterium tumefaciens*) having the plant transformation vectors pART/Kill K2 and pGA/Kill K2 with yeast K2 type gene coding the killer preprotoxin by making the transformation of a natural tobacco plant (*Nicotiana tabacum* L.) with the help of the

above mentioned agrobacteria by making the analysis of transgenic plants and checking the expression of a killer gene in them.

In the previous study it was determined that cDNA of the *S. cerevisiae* K2 preprotoxin gene expressed under control of constitutive ADH1 promoter confer both the killer and the immunity phenotypes. We decided to investigate the expression of K2 gene controlled by ADH1 promoter in plant *Nicotiana tabacum*. For this purpose recombinant plasmid (K2 expression controlled by yeast ADH1 promoter) based on plant vector pGA482 was constructed (Fig. 6).

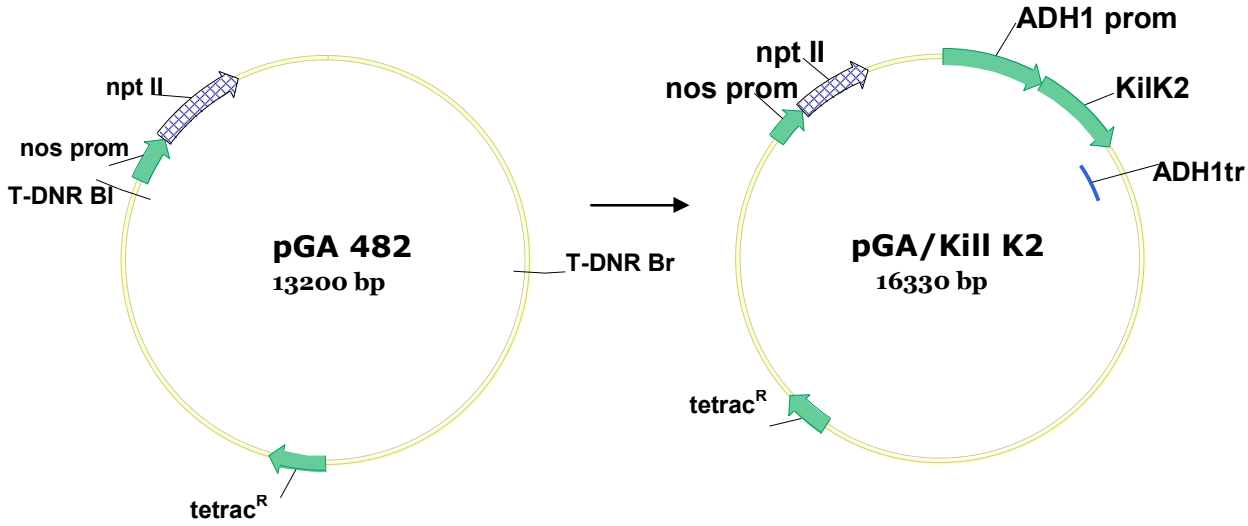

Fig. 6 Map of the plant plasmids pGA 482 and pGA/Kill K2: *npt II* – neomicinphosphotransferase gene, tetrac^R – gene, determing the resistance of *E. coli* to tetracyclin, KilK2 – K2 preprotoxin gene, ADH1 prom – alcoholdehydrogenase promoter, ADH1 tr – alcoholdehydrogenase terminator.

The plasmid pART/Kill K2 was constructed with the CaMV (*Cauliflower mosaic* virus CaMV 35S promoter) too. Also, different processing of protein in plant comparing to yeast can decrease killer production and activity. Therefore, we decided to investigate expression of K2 gene controlled by CaMV promoter in plant *N. tabacum*. Plant transformation vector pART/KillK2 (K2 under control of CaMV 35S promoter) was constructed (Fig. 7).

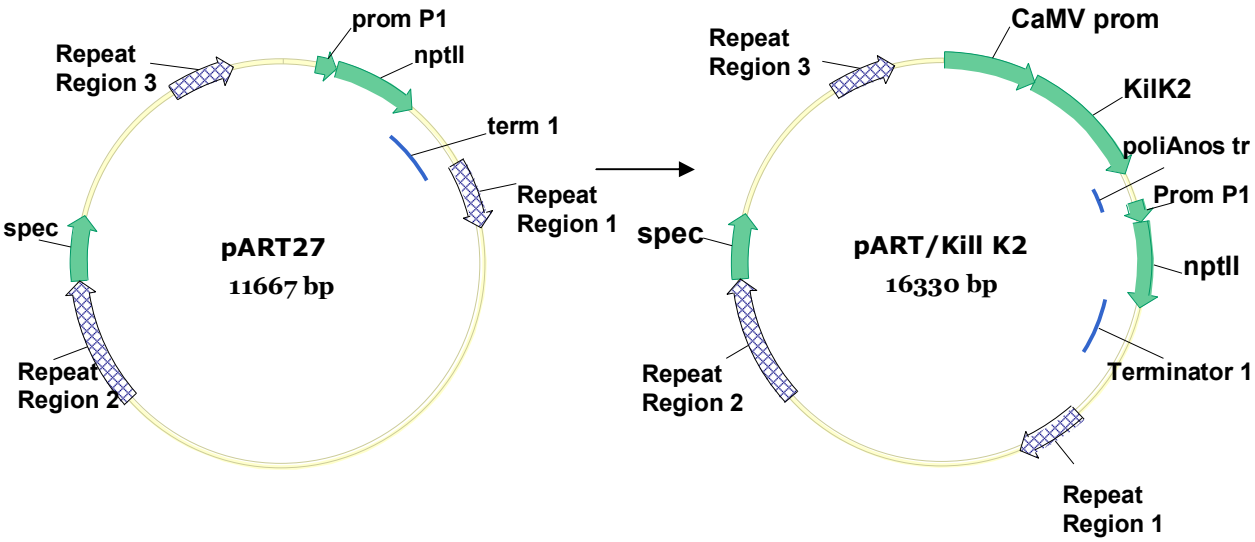

Fig. 7. Map of the plant plasmids pART27 and pART/Kill K2: KilK2 – K2 preprotoxin gene, CaMV prom – *Cauliflower mosaic virus* CaMV promoter, poliAnos – terminator.

Agrobacterium tumefaciens was conjugated with *E. coli* strain (it had killer plasmids pART27/K2 and pGA/Kill K2) and successfully transformed into the model plant *N. tabacum.*

The expression of *S. cerevisiae* K2 killer preprotoxin gene in transgenic plants (collaboration with the Laboratory of Cell Engineering) and the presence of gene was confirmed by PCR method (Fig. 8).

Plants, in which the gene coding killer preprotoxin of yeast had to be present, were low. It comparison with the control which was not transformed with plasmids started to root lately and rooted slowly.

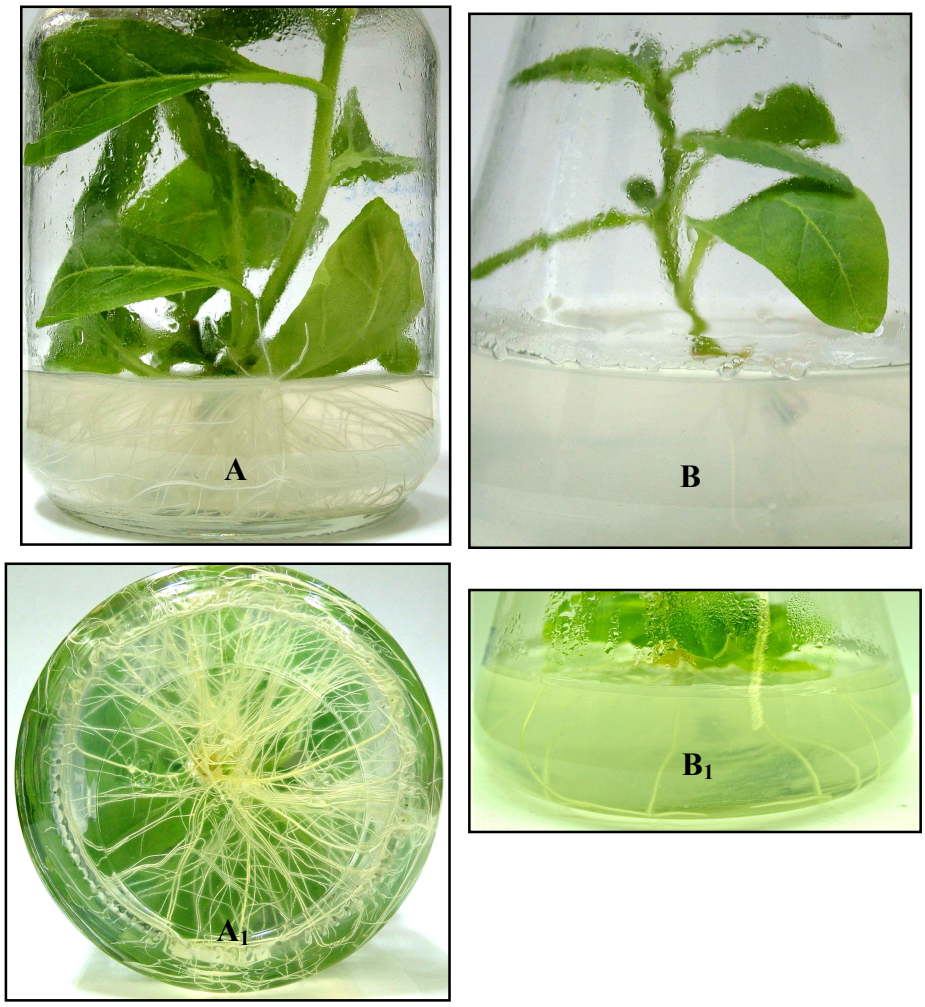

Fig. 8. Root systems of kanamycin-resistant transformants and regenerated control plants: A and A_1 – control kanamycin sensitive non-transformed shoots, B and B_1 – transformed with pGA/Kill K2 and regenerated shoots possessing dominant marker *nptII*.

Leaf explants were analysed for the production of active K2 toxin either by the placing small leafs directly onto yeast α'1 layer or after the grinding the tested leafs in liquid nitrogen and spotting of biomass on medium innoculated with yeast sensitive to killer toxin. Appearance of small clear lysis zones around leafs allowed to conclude that yeast ADH1 promoter is transcriptionally active in plant as well as preprotoxin can be preceded in plant cell (Fig. 9).

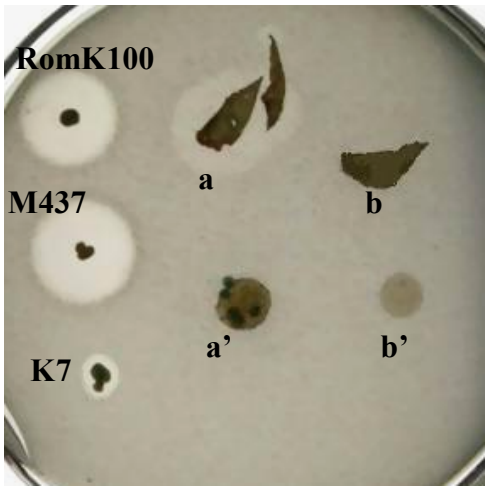

Fig. 9. Analysis of explants for production of active K2 toxin: a – transformed with pGA/Kill K2 plasmid leaf explants, a' – grinded biomass, b – non transformed leaf explant, , b' – biomass of control plants, control yeast killer strains – RomK100, M437, K7.

DNA was extracted from the leaves and PCR was done. PCR and DNA electrophoresis showed that K2 gene is incorporated into plant genome and there is weak expression of this gene (Fig. 10). However, the promoter of *Cauliflower mosaic virus* CaMV 35S upstream this gene was not found. In the previous studies it was considered that the expression of the transformed gene mentioned exists only when the transcription of it is directly regulated by alcoholdehidrogenase ADH1 promoter. However, after the investigation it was shown that promoter ADH1 is not connected to the beginning of the gene K2.

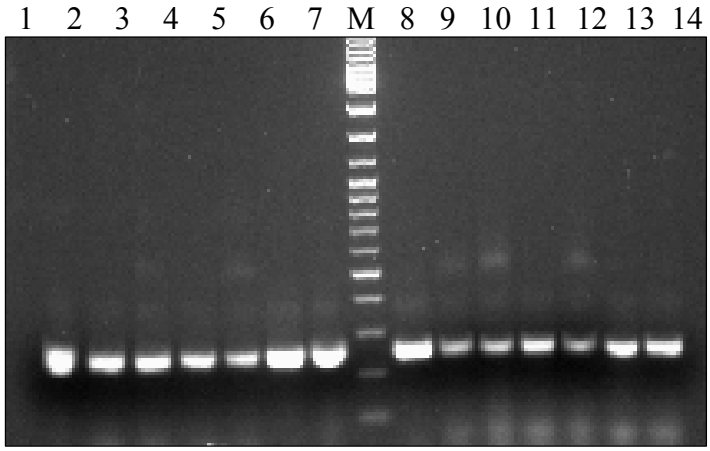

Fig. 10. Electrophoretic analysis of PCR products of the transformed plants with pGA/Kill K2 and pART/Kill K2 vector. 1-7 – Plant DNA transformed with pGA/KillK2 plasmid, 8-13 – Plant DNA transformed with pART/KillK2 plasmid; 14 – control (the pYEX12 plasmid), M – Marker (Gene Ruler^{TM} Ladder Mix).

The investigations of yeast killer toxins in medicine (for control of pathogenic yeast infections), food and vine industry are developing all over the world. Therefore,

searching for killer yeasts and micromycetes as well as their biochemical and genetic analysis have good prospects in the future.

In order to analyse expression of K2 preprotoxin gene controlled by CaMV promoter in plants recombinant plasmids pAD/CGT-Kill K2D and pAD/CGT-Kill K2R possessing the possibility to analyze this gene expression in yeast was created (Fig. 11).

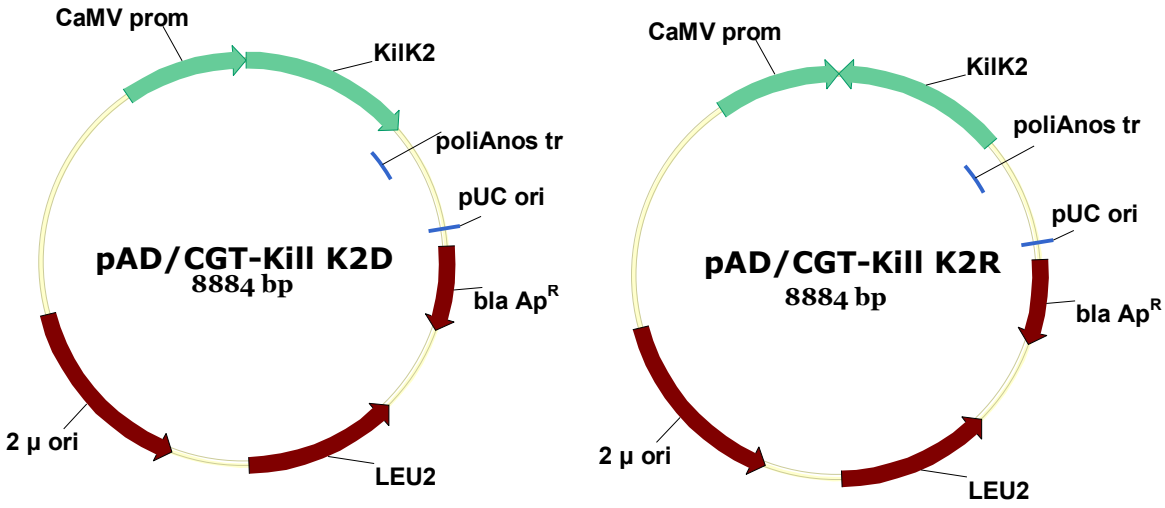

Fig. 11. Map of the yeast plasmids pAD/CGT-Kill K2D and pAD/CGT-Kill K2R: KilK2 – K2 preprotoxin gene, CaMV – *Cauliflower mosaic virus* CaMV promoter, poliAnos tr – terminator, 2µ ori – yeast replication origin, bla Ap^R – β-lactamase gene.

Constructs were transformed into yeast *S. cerevisiae* α'1. It was determined that the stability of these plasmids (monitored by maintaining the Leu$^+$K2$^+$ phenotype) under both non-selective and leucine-selecting conditions reached 72-75 %. It is important to point out that both α'1 (pAD/CGT-Kill K2D) and α'1 (pAD/CGT-Kill K2R) transformants (K2 gene placed in direct or reverse orientations to promoter sequence) showed weak killer activity and were sensitive not only to *wt* K2 toxins as well as to their own product (partial suicide phenotype). There is a possibility that both ADH1 and CaMV 35S promoters are remote from the beginning of K2 gene, or the iniciation is done by other regulators which are ineffective. Probably for this reason (with both pGA/Kill K2 and pART/Kill K2 vectors) weak and constitutive expression of the preprotoxin was observed in the transformed plants. Also the specifics of protein maturation in plants may have had effect. Killer preprotoxin maturation in plants (*N. tabacum*) and yeasts (*S. cerevisiae*) is very different because of the absence or the

existence of specific proteases or glycosylation. Even after a successful case of transformation, it is not known how the cells of a plant react to such toxin formation inside or secretion outside cell. Since plant cells are very different from the yeast cells, the functiality of killer toxin may have significant influence such as their viability. In order to fully answer these questions, a more detailed study of transformed plants is planned in the future.

CONCLUSIONS

1. Round 230 spontanic fermentations from fruits and berries were prepared and analysed 17 strains with the killer features were separated and their killer, immune and fungicidal features were evaluated comparing with yeast *S. cerevisiae* sensitive and killer strains.

2. The bacterial isolate with odd features called Ux was separated. These features might be used fighting with patogenic microorganisms; bacterial strain called Tx was purified, Tx strain characteristic an unusual autolysis and it might be suitable for biopreparation production.

3. The temperatures and pH were defined for the best expression of secreted toxins from separated killer yeast strains and bacterial isolates. It was shown that toxins secreting bacteria and yeasts are quite active in medium with pH 3,0 – 5,6, in temperature from 20 ºC to 37 ºC.

4. The effect of detected bacterial isolates was established to plant, animal and human pathogens: *Candida* spp., *Fusarium* spp., *Aspergillus* spp., *Penicillium* spp., *Verticillium* spp., *Venturia* spp.

5. After microbiological analysis of spontanic fermentations of fruit and berry microorganisms, new killer yeast strains (20K++, 4+, K+ob, IIx31, III-2,

Cranberry, 1M) were separated; individual isolates killer, immune features and their productive perspectives in wine and ethyl alcohol industry were established.

6. 1M yeast strain was found, which had features necessary for wine yeast and fermentated apple juice quality parameters, and was used for natural apple wine semimanufactures production in cooperative company „Vaisių sultys“.

7. The pART/KillK2 plasmid was constructed that codes yeast *S. cerevisiae* K2 killer preprotoxin gene, which works with cauliflower mosaic virus rRNA 35S CaMV promoter and polyA terminator. This plasmid is convenient for plant transformation; the pGA/KillK2 plasmid was constructed, that codes the K2 killer preprotoxin gene, which works with ADH1 promoter and ADH1 terminator, and is suitable for plant transformation.

8. After the molecular analysis of *N. tabacum* with the K2 killer preprotoxin gene vectors (pART/KillK2 and pGA/KillK2) it was shown that the killer gene exists; it was shown that transformed plants phenotype differs, the production of toxin is of low level, the pH optimum of the K2 killer protein in plants is varied.

9. Two plasmids were constructed (pAD/CGT-KillK2D ir pAD/CGT-KillK2R), coding yeast *S. cerevisiae* K2 killer preprotoxin gene, which works with cauliflower mosaic virus rRNA 35S CaMV promoter and polyA terminator. They are convenient for yeast cell transformation. The K2 killer preprotoxin gene with CaMV promoter expression was investigated in yeast.

The List of Original Publications by the Author

Articles and journals

Melvydas, V., Servienė, **V., Čapukoitienė**, **B.,** Petkūnienė, G., Lebionka, A. 2006. Toksinus produkuojančių mikroorganizmų paieška ir jų antipatogeninių savybių pradinis tyrimas. SODININKYSTĖ IR DARŽININKYSTĖ. **25(2)**: 91-98.

Melvydas, V., Gedminienė, G., Jarmalaitė, I., **Čapukoitienė, B.,** Nemceva, L. 2006. Initial analysis of highly competitive yeast strains promising for ethanol industry. BIOLOGIJA. **3**: 63-66.

Čapukoitienė, B., Karalius, V., Servienė, E., Proscevičius, J., Melvydas, V. 2008. Exspression of yeast *Saccharomyces cerevisiae* K2 preprotoxin gene in transgenic plants. SODININKYSTĖ IR DARŽININKYSTĖ. **27(2)**: 319-327.

Melvydas, V., Gedminienė, G., **Čapukoitienė**, B., Pilevičienė, S., Lebionka, A. 2009. Investigation of killer and adhesive properties of new microorganisms originated from Lithuania and Polar Ural. BOTANICA LITHUANICA. 15(3): 217-223.

Čapukoitienė, B., Gedminienė, G., Melvydas, V., Kondratienė, L., Levinskaitė, L. 2010. Influence of the temperature and pH medium on the killer features of the bacterial isolates from spontaneous fermentations of berries and fruits gathered on territory of Lithuania. Вести Национальной академии наук Беларуси. 4: 276-279.

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Čapukoitienė, B., Černyšova, O., Melvydas, V. Naujas mielių sekretuojamas X faktorius. Respublikinė konferencija „Lietuvos biologinė įvairovė: būklė, struktūra, apsauga“ skirta VPU Botanikos katedros 60-mečiui, Vilnius, 2005.

Čapukoitienė, B., Lebionka, A., Melvydas, V. Naujų kilerinių kamienų paieška gamtinėse populiacijose bei kolekcijose. IX-asis suvažiavimas-konferencija. „Biochemija: mokslas ir žinių visuomenė“. Lietuva, Tolieja (Molėtų raj.), 2006.

Čapukoitienė, B., Karalius, V., Servienė, E., Proscevičius, J., Melvydas, V. Exspression of yeast *Saccharomyces cerevisiae* K2 preprotoxin gene in transgenic plants. International scientific conference „Actualities in plant physiology“, Lithuanian Institute of Horticulture, Lithuanian University of Agriculture, Kaunas, 2008.

Melvydas, V., **Čapukoitienė, B.,** Gedminienė, G., Kurtinaitienė, B., Beniulytė, J. Ekspress metodas mielių rauginimo galimybėms įvertinti. X-asis suvažiavimas-konferencija „Biochemija ir sistemų biologija“. Lietuva, Tolieja (molėtų raj.), 2008.

Čapukoitienė, B., Gedminienė, G., Melvydas, V., Kondratienė, L., Levinskaitė, L. Influence of the temperature and pH medium on the killer features of the bacterial isolates from spontaneous fermentations of berries and fruits gathered on territory of Lithuania. Молодеж в науке – 2009, международная научная конференция молодых ученых. Национальноя академия наук Беларуси, Минск, 2009.

Saccharomyces cerevisiae K2 preprotoksino geno raiška *Nicotiana tabacum* L. augaluose bei naujų, toksinus produkuojančių mikroorganizmų paieška ir jų panaudojimo analizė

Santrauka

Naujos kartos plataus veikimo medžiagos, pasižyminčios bakteriocidinėmis ir fungicidinėmis savybėmis, gali būti aptinkamos tiek prokariotiniuose, tiek eukariotiniuose organizmuose. Šiuo metu tokių medžiagų ir jas produkuojančių mikroorganizmų kamienų paieška pasaulyje labai plečiasi. Galimybės surasti reikiamų naujų mikroorganizmų dar ilgą laiką bus neišnaudotos. Įvairių rūšių mielės ir bakterijos gali būti panaudotos kaip apsauginė priemonė nuo maisto gedimo pramonėje, medicinoje, genų inžinerijoje ir t. t. Mielės *S. cerevisiae* yra vienas plačiausiai naudojamų tyrimo objektų ir komercinių mikroorganizmų. Jos naudojamos maisto pramonėje, farmacijoje, medicinoje. Plačiausiai mielės naudojamos etilo spirito gamyboje. Mielės, pasižyminčios kilerinėmis savybėmis, turi privalumų konkurencinėje kovoje. GTC Botanikos instituto Genetikos laboratorijoje yra tiriama mielių *S. cerevisiae* kilerinė sistema. Pasiektas ištyrimo lygis leidžia šią sistemą pritaikyti maisto pramonėje, biotechnologijoje, medicinoje, bioetanolio gamyboje.

Šio darbo metu iš spontaninių vaisių-uogų raugų išskirti bakteriniai izoliatai, pasižymintys kilerinėmis savybėmis, dėl kurių gali būti panaudoti kovai su patogeniniais mikroorganizmais. Nustatytos išskirtų kilerinių bakterijų izoliatų sekretuojamų toksinų geriausios raiškos temperatūros ir pH sąlygos. Parodyta, kad bakterijų sekretuojami toksinai yra pakankamai aktyvūs terpėse, kurių pH 3,0 – 5,6, auginant jų producentus temperatūrose nuo 20 ºC iki 37 ºC. Nustatytas rastų bakterinių izoliatų poveikis augalų, gyvūnų bei žmonių patogenams: *Candida* spp., *Fusarium* spp., *Aspergillus* spp., *Penicillium* spp., *Verticillium* spp., *Venturia* spp.

Aptiktas mielių 1M kamienas, kuris pasižymėjo savybėmis, reikalingomis pramoninėms vynų mielėms ir fermentuotų obuolių sulčių kokybės parametrams ir buvo panaudotas obuolių vyno natūralaus pusgaminio gamyboje kooperatinėje bendrovėje „Vaisių sultys“.

Sukonstruota plazmidė pART/KillK2, koduojanti mielių *S. cerevisiae* K2 kilerinį preprotoksino geną, kurio veiklą užtikrina žiedinio kopūsto mozaikos viruso rRNR 35S CaMV promotorius bei poliAnos terminatorius, yra tinkama augalų transformacijai; sukonstruota plazmidė pGA/KillK2, koduojanti K2 kilerinį preprotoksino geną, kurio veiklą užtikrina ADH1 promotorius ir ADH1 terminatorius, tinkama trasformacijai į augalus.

Atlikus transformuoto augalo *N. tabacum*, nešančio K2 kilerinio preprotoksino geno vektorius (pART/KillK2 ir pGA/KillK2), molekulinę analizę, parodytas kilerinio geno buvimas; parodyta, kad transformuoti augalai skiriasi fenotipiškai, toksino produkcija – žemo lygio, yra pakitęs K2 kilerinio baltymo pH optimumas augaluose.

Sukonstruotos dvi plazmidės (pAD/CGT-KillK2D ir pAD/CGT-KillK2R), koduojančios mielių *S. cerevisiae* K2 kilerinį preprotoksino geną, kurio veiklą užtikrina žiedinio kopūsto mozaikos viruso rRNR 35S CaMV promotorius bei poliAnos terminatorius, tinkamos transformacijai į mielių ląsteles; įvertinta K2 kilerinio preprotoksino geno, reguliuojamo CaMV promotoriumi, raiška mielėse, nustatyta , kad toksino produkcija yra žemo lygio.

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