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ANALYSIS OF THE 2-PHENYLETHANOL PRODUCTION BY YEASTS GENUS *KLUYVEROMYCES*

The present study is focused on the determination of the 2-phenylethanol production by yeasts genus Kluyveromyces. The most productive strain was K. marxianus UCM Y-305. It can be concluded that 2-phenylethanol production by K. marxianus UCM Y-305 can be improved from 0.0268 g/l to 0.37 g/l by selecting the suitable cultivating conditions.

K e y w o r d s: Kluyveromyces marxianus, producer, 2-phenylethanol, conditions.

Many yeasts have been found to produce *de novo* odours compounds with fruity or floral flavours [1—5, 7]. The yeasts of the genus *Kluyveromyces* can produce significant amounts of important flavor compound with the rose-like odor 2-phenylethanol (2-PE) and it can achieve high product yields; this depends on different cultivation conditions. Previously five strains of *K. marxianus* and one strain of *K. lactis* by Etschmann et al. (2003) [2], *K. marxianus* ATCC 10022 by Medeiros et al. (2001) [8], *K. marxianus* CBS 5670 by Wittmann et al. (2002) [7], twenty-one yeast strains of *K. marxianus* by Fabre et al. (1997) [9] were tested for 2-PE production with high product yields. From this yeasts *K. marxianus* are receiving an increasing interest for the development of biotechnological production processes for 2-phenylethanol [2, 7]. It is one of the best producers of 2-PE described in the literature [1—3, 7]. Also, *K. marxianus* is Crabtree-negative which is an advantage for future production processes, because the ethanol formation as a toxic by-product under aerobic conditions can be avoided [10].

The purpose of the present study is to determine the levels of the 2-phenylethanol production by yeasts genus *Kluyveromyces* under the influence of different conditions.

Materials and methods

The 20 yeast strains *Kluyveromyces marxianus, K. lactis, K. africanus, K. lactis var. lactis, K. lactis var. drosophilarum, K. thermotolerans, K. wickerhamii* from the collection of yeasts (UCM) of the Industrial Microorganisms Physiology Department, Institute of Microbiology and Virology of National Academy of Sciences of Ukraine, Kiev were used in this study.

The yeasts inoculums (10^6—10^7 cells/ml) were grown in media for initial screening (medium № 1), containing (g/l): 80.0 sucrose, 7.0 L-phenylalanine, 22.8

$Na_2HPO_4 \cdot 2H_2O$, 10.3 citric acid, 0.5 $MgSO_4 \cdot 7H_2O$, 0.17 Bacto Yeast Nitrogen Base without amino acids and $(NH_4)_2SO_4$, adjusted to pH 5.0 [2] and medium № 2, containing (g/l): 77.0 glucose, 7.0 L-phenylalanine, 0.5 $MgSO_4 \cdot 7H_2O$, 0.17 Bacto Yeast Nitrogen Base without amino acids, additionally amended 1.25 KH_2PO_4 and 0.16 K_2HPO_4, pH 5.0 [7].

Sucrose was determined by cleavage with invertase and measuring glucose concentration with by the dinitrosalicylic acid method [6].

1 ml aliquot of sell suspension (10^6—10^7 cell/ml) was transferred to a test-tube containing 9 ml of medium: 1—8% carbon sources, 0.05—0.25% L-phenylalanine, and 0.25—1.25% yeast extract. The effect of carbon sources (glucose, sucrose, maltose, trehalose, rafinose, and ethanol), cultivating temperatures (12—14 °C, 20—22 °C, 28—30 °C, and 42—48 °C), pH value (2, 4, 5, 7, and 9), medium amount (50, 100 and 150 ml) on the production of 2-phenylethanol and biomass production were determined. The effect of the various concentration of sucrose in medium from 1% to 8% on growth of yeast biomass and a level of synthesis 2-phenylethanol were investigated.

The growth of yeast biomass after cultivation was measured as optical density at 540 nm and recalculated as biomass dry weight. Medium pH was determined directly with pH meter (Model pH-150MA, Antex, Byelorussia). All data were standardized and carried out in triple frequency.

The concentrations of 2-phenylethanol in the medium were measured after filtrations by GC/MS analyses. Samples were filtered through 0.2 μm filters prior to GC/MS analyses (Agilent Technologies, USA). GC/MS analyses were performed on Agilent 6890N/5973 inert chromatograph/mass spectrometer (Agilent Technologies, USA) equipped with DB-FFAP capillary column (30 m x 0.25 mm x 0.25 μm, J&W Scientific, USA). Helium was used as the carrier gas at a flow rate of 1 ml/min. The temperature program was as follows: 60 °C for 1 min and then increased to 220 °C at a rate 20 °C/min and held for 10 min. The temperature of injector was maintained at 250 °C. Detection was followed at SCAN rate. 2-phenylethanol was identified by NIST 02 mass spectrum database and 2-phenylethanol standard solution (Merck, Germany).

Results and discussion

It was determined that the presence of the 2-phenylethanol in the medium reaches the maximal values in the beginning of stationary growth phase (18—24 h, strain specific). Among the twenty yeast strains tested, nine strains *K. marxianus* produced 2-phenylethanol. These strains were isolated previously from wine and beer making industries. *K. marxianus* UCM Y-305 gained the highest yield at 0.0268 g/l after 18 h of cultivation and reached biomass yield 1.54 ± 0.16 g/l DWB (tab.). The strains *K. lactis, K. africanus, K. lactis var. lactis, K. lactis var. drosophilarum, K. thermotolerans, K. wickerhamii* did not exhibit any 2-phenylethanol production.

High concentration of precursor of carbon source (sucrose or glucose), L-phenylalanine provide shift of biochemical reactions for 2-phenylethanol syntheses that is more typical for *Kluyveromyces* yeasts [2, 3, 5, 7]. Different carbon sources in the culture media also resulted in the changes of the qualitative and quantitative production of aromatic compounds by microorganisms [4].

Table

The initial analysis of *K. marxianus* yeast strains ability to synthesize 2-phenylethanol

K. marxianus UCM Y-	Medium № 1 (Etschmann M.M.W. et al., 2003)		Medium № 2 (Wittmann C. et al., 2002)	
	2-PE concentration, g/l	Biomass, g/l CDW	2-PE concentration, g/l	Biomass, g/l CDW
12	0.0189	1.69 ± 0.11	0.0203	1.78 ± 0.10
13	0.0112	1.78 ± 0.10	0.0145	1.50 ± 0.13
17	0.0151	1.57 ± 0.10	0.0141	1.62 ± 0.15
301	0.0242	1.67 ± 0.15	0.0261	1.36 ± 0.10
305	0.0268	1.54 ± 0.16	0.0271	1.50 ± 0.10
320	0.0175	1.66 ± 0.10	0.0177	1.42 ± 0.12
2096	0.0098	1.58 ± 0.20	0.0099	1.66 ± 0.20
2098	0.0127	1.79 ± 0.14	0.0143	1.58 ± 0.10
2387	0.0080	1.54 ± 0.16	0.0086	1.67 ± 0.12

Obtained results revealed that among six carbon sources tested, sucrose was suitable carbon sources for *K. marxianus* UCM Y-305 resulting in production high amounts of 2-phenylethanol (Fig. 1). In the medium containing 1–8% sucrose, the production of biomass increased, but the production of 2-phenylethanol was found to be the highest at 8% (Fig. 2). The same results were obtained after studying the ranges of the L-phenylalanine and yeast extract. It was established that the highest level of biomass and 2-PE synthesized at 1.25% yeast extract and 0.25% L-phenylalanine in cultivating medium. This phenomenon may be caused by high sucrose concentration like inhibitory factor (increasing osmotic pressure due to increasing sucrose concentration) for 2-phenylethanol and biomass production [4]. We can note that the yeast extract demonstrated its ability to affect 2-phenylethanol syntheses. This could be explained by the fact that yeast extract in addition to providing adequate nitrogen contains various vitamins increased the yeast strains the growth. The received results completely coincided with the results of other authors [4, 11]. Previously, we received such results for *S. cerevisiae* UCM Y-514 and UCM Y-524 which confirmed this statement [12]. The further increasing of the sucrose concentrations (up to 15%, data are not shown) in the media of not reduced amounts of the 2-phenylethanol.

Medium contained 0.25% yeast extract, 0.05% L-phenylalanine and 5.0% carbon source.

The final pH ranged from 5.0 to 3.2–4.8 in media with different carbon sources. This is probably due to the production of 1–2% acidic acid by tested yeasts strains under these conditions (Fig. 1B). Significantly higher amount of 2-phenylethanol was detected in medium containing sucrose than in media containing other carbon sources (Fig. 1).

The temperature is one of the most important parameters for the development of alcoholic fermentation since it can affect both the kinetics of the process in terms of duration and rate of fermentation and the production of metabolites and the fi-

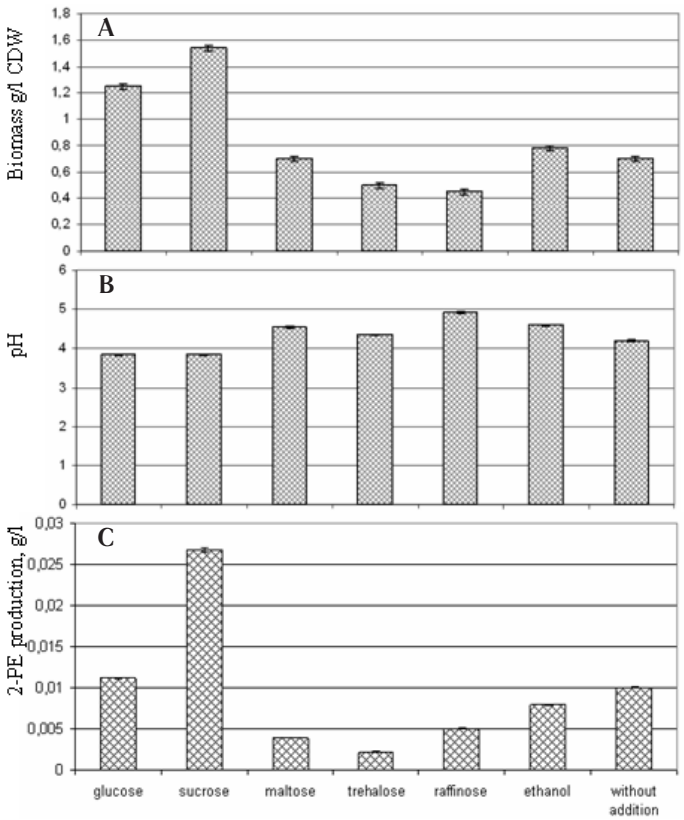

Fig. 1. The growth (A), final pH (B), and production of 2-PE (C) by *K. marxianus* UCM Y-305 in medium containing various carbon sources

nal quality of wine, for example. As expected, the strain *K. marxianus* UCM Y-305 gained the highest yield at 1.6 g/l biomass CDW after 18 h at temperature 28–30 °C, respectively.

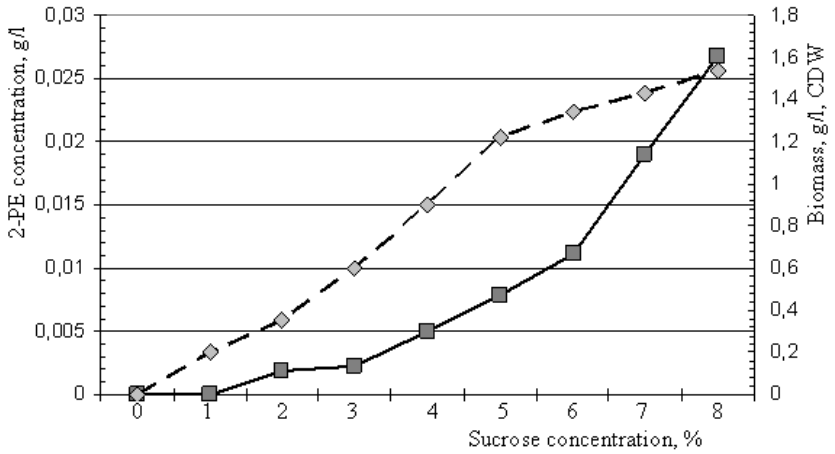

Fig. 2. Production of biomass and 2-phenylethanol by *K. marxianus* UCM Y-305 in medium with different range of sucrose

Data are means derived from three replicated determinations. The straight line — SEM $\leqslant$ 0.1 for biomass yield; the dash line — SEM $\leqslant$ 0.001 for 2-PE production.

Laboratory fermentations in 750 ml Erlenmeyer flasks have some characteristic properties. Limited aeration and thus limitation of oxygen can be a strong challenge for yeast due to its necessity for 2-phenylethanol production. The effects of the medium value, from 25 to 150 ml, and as sequent aeration levels changing were investigated. The data showed that with reduction of cultivation medium's quantity, the level of oxygen saturation increased; at that biomass yields (Fig. 3) and 2-phenylethanol production for *K. marxianus* UCM Y-305 increased too.

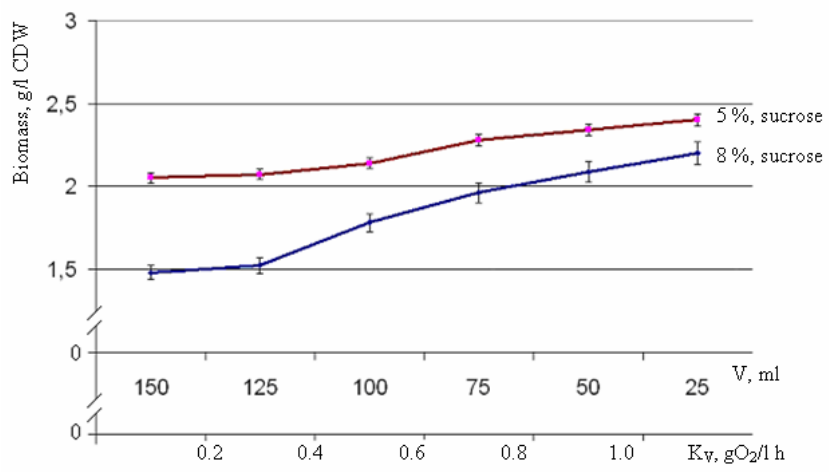

Fig. 3. Biomass yields depending on aeration level and sucrose concentration

Thus, the best cultivating conditions for *K. marxianus* UCM Y-305 were 8% sucrose, 1.25% yeast extract, 0.25% L-phenylalanine, 18 h of cultivation time, 28–30 єC cultivating temperatures, 150 ml medium with aeration 0.48–1.12 g O_2/lh (Fig. 3.), pH value 5.0. Sucrose was completely consumed after 18–26 h of cultivation under different conditions.

After cultivating under the all optimized conditions the levels of the 2-phenylathenol and biomass production were established. It was 0.37 g/l 2-phenylethanol and biomass yield 2.04 $\pm$ 0.22 g/l DWB.

Conclusion

Twenty yeast strains were screened for production of 2-phenylethanol from L-phenylalanine with sucrose as a carbon source. It was found nine producers of 2-phenylethanol among *K. marxianus* strains. The most productive strain was *K. marxianus* UCM Y-305. It can be concluded that 2-phenylethanol production by *K. marxianus* UCM Y-305 can be improved from 0.0268 g/l to 0.37 g/l by selecting the suitable cultivating conditions.

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АНАЛИЗ СИНТЕЗА 2-ФЕНИЛЭТАНОЛА ДРОЖЖАМИ РОДА *KLUYVEROMYCES*

Реферат

Настоящее исследование направлено на определение синтеза 2-фенилэтанола дрожжами рода *Kluyveromyces*. Наиболее продуктивным штаммом является *K. marxianus* УКМ Y-305. Установлено, что подбор оптимальных условий культивирования дал возможность увеличить синтез 2-фенилэтанола дрожжами *K. marxianus* УКМ Y-305 с 0,0268 г/л до 0,37 г/л.

К л ю ч е в ы е с л о в а: *K. marxianus*, продуцент, 2-фенилэтанол, условия.

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АНАЛІЗ СИНТЕЗУ 2-ФЕНІЛЕТАНОЛУ ДРІЖДЖАМИ РОДУ *KLUYVEROMYCES*

Реферат

Дане дослідження спрямоване на визначення продукування 2-фенілетанолу дріжджами роду *Kluyveromyces*. Найбільш продуктивним штамом є *K. marxianus* УКМ Y-305. Встановлено, що підбір оптимальних умов культивування дав можливість збільшити продукування 2-фенілетанолу дріжджами *K. marxianus* УКМ Y-305 з 0,0268 г/л до 0,37 г/л.

К л ю ч о в і с л о в а: K. marxianus, продуцент, 2-фенілетанол, умови.

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NEGATIVE REGULATION OF MOENOMYCIN A BIOSYNTHESIS IN *STREPTOMYCES GHANAENSIS* ATCC14672

Members of the soil-dwelling prokaryotic genus Streptomyces produce around two-thirds of all known antibiotics. Moenomycin A is a potent antibacterial drug against many Gram-positive pathogens, including vancomycin-resistant pathogens. Studying of moenomycin A biosynthesis regulation is of great importance because of need of new tools for combinatorial biosynthesis. In this work we examined the effects of known pleiotropic regulator DasR on moenomycin biosynthesis under heterologous conditions. DasR controls several pivotal cellular processes, including the transport of N-acetylglucosamine (GlcNAc), ubiquitous metabolite and an important source of carbon and nitrogen in soil ecosystems. Gene dasR was inactivated in S. coelicolor and moenomycin biosynthetic gene cluster was expressed in the mutant and parent strains. Moenomycin production increased twofold in dasR-deficient strain, confirming the involvement of DasR in regulation of phosphoglycolipid production.

K e y w o r d s: streptomycetes, moenomycin A, regulator DasR, N-acetylglucosamine.

Streptomycetes are complex multicellular Gram-positive soil bacteria, perhaps best known for their ability to produce over two-thirds naturally derived antibiotics. Moenomycin A (MmA) is produced by *Streptomyces ghanaensis* ATCC14672. Moenomycin A (MmA) is a member of the phosphoglycolipid family of antibiotics, which are the only natural products known to directly target the extracellular peptidoglycan glycosyltransferases involved in bacterial cell wall biosynthesis. The emergence of resistance to existing antibiotics represents a significant threat to public health. New antibiotics with activity against resistant bacterial strains are desperately needed. The structural and biological uniqueness of MmA make it an attractive starting point for the development of new antibacterial drugs.

The entire MmA biosynthetic (*moe*) gene cluster from the producer *Streptomyces ghanaensis* ATCC14672 was cloned and sequenced [1]. The regulation of *moe* cluster gene expression is unclear because it appears to lack dedicated regulatory genes. The aim of this work is to find genes that can regulate MmA biosynthesis. Particularly we focused our attention on gene *dasR*, encoding a protein involved in regulation of transport and metabolism of N-acetylglucosamine (GlcNAc). The latter is a breakdown product of chitin, widely represented polymer in soils. Two molecules of GlcNAc are also present in the MmA, and, therefore, it is logically to suppose that DasR might somehow be implicated in regulation of MmA production. We addressed this question through expression of MmA gene cluster and subsequent analysis of MmA production levels in *dasR*-deficient and parent strains of *S. coelicolor* M145.