Tris(2-hydroxyethyl)ammonium arylchalcogenylacetates, growth stimulants of alcohol yeast *Saccharomyces cerevisiae*

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A number of tris(2-hydroxyethyl)ammonium arylchalcogenylacetates $ArYCH_2CO_2^- \cdot HN^+(CH_2CH_2OH)_3$ (**1**—**4**) (Ar = aryl; Y = O, S, SO_2) were synthesized. Their structure was established by IR and ^1H, ^{13}C, ^{15}N NMR spectroscopy. The influence of compounds **1**—**4** on the growth and development of yeast fungi *Saccharomyces cerevisiae* was studied. Compounds **1**—**4** were found to be efficient, safe, and available synthetic stimulants of these processes. The use of compounds **1**—**4** in low concentrations (from 10^{-8} to 10^{-4} wt.%, depending on the time of cultivation, concentration, and the anion type) reduced the lag-phases of development and increased the generation rate of yeast cells by 2—5 times. The amount of yeast cells increased by 2—3 times, while the content of protein in the biomass increased by 13—15%. The fermentation of sugar- and starch-containing raw material with yeast *Saccharomyces cerevisiae* increased the yield of ethyl alcohol (biofuel) by 5—9%.

Key words: arylchalcogenylacetic acids, tris(2-hydroxyethyl)ammonium salts, stimulants, cultivation, *Saccharomyces cerevisiae* yeast, growth rate, biomass, fermentation, ethanol production.

Microbiological synthesis is the basis for obtaining food acids, vitamins, alcohols (biofuels). The most large-tonnage product obtained by this method is ethyl alcohol, the biosynthesis of which is most often carried out by cultures of yeast fungi of the *Saccharomyces cerevisiae* species. The fermentation of different carbohydrates by yeast requires from 60 to 70 hours and gives about 60% yield of ethyl alcohol.[1—3] Along with ethanol, a significant amount of sedimentary yeast is formed, which is a valuable protein supplement to the fodder for livestock and raw materials for the production of protein-containing agents.[1,2] The activation of the metabolic processes of the yeast cells directed on the increase of the formation of the target fermentation products will make more efficient the use of carbohydrates of the nutrient medium and increase the productivity of yeast, while reducing the consumption of fermented sugars and the total time of cultivation.

The stimulation of growth and ethanol-directed orientation of yeast metabolism can be achieved by application of biostimulants. Nowadays, expensive and poorly soluble natural stimulants such as biotin, ferulic and gibberellic acids are used in the industrial production.[4]

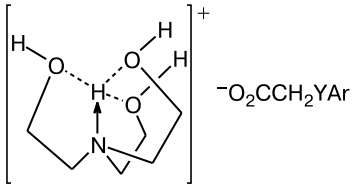
We used biologically active arylchalcogenylacetic acids and biogenic ethanolamines (triethanolamine) to synthesize[5—10] a number of tris(2-hydroxyethyl)ammonium arylchalcogenylacetates (**1**—**4**) by the following Scheme:

$$ArYCH_2CO_2H + N(CH_2CH_2OH)_3 \longrightarrow ArYCH_2CO_2^-HN^+(CH_2CH_2OH)_3$$

1—**4**

where Ar — aryl; Y = O, S, SO_2.

The composition and a tricyclic "atrane" structure of obtained compounds were established by IR and NMR spectroscopy, elemental analysis, and X-ray diffraction.[11—16]



1—**4**

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It is possible that due to the unique structure, compounds **1**—**4** possess a synergistic effect and exhibit biological activity exceeding activity of the parent acids and amines. Thus, among compounds **1**—**4** there were found nontoxic (LD_{50} = 1300—6000 mg kg^{-1}) compounds, which are promising for medicine, clinical microbiology and biotechnology with antioxidant, immunotropic, antiallergenic, antitumor, antimetastatic, protective, growth- and enzyme-stimulating action.[5—10]

The purpose of the present work is the synthesis and study of compounds 4-Cl-$C_6H_4SCH_2CO_2^-$ $HN^+(CH_2CH_2OH)_3$ (**1**), 4-Cl-$C_6H_4SO_2CH_2CO_2^-$ $HN^+(CH_2CH_2OH)_3$ (**2**), 2-Cl-$C_6H_4OCH_2CO_2^-$ $HN^+(CH_2CH_2OH)_3$ (**3**) and 4-Cl-$C_6H_4OCH_2CO_2^-$ $HN^+(CH_2CH_2OH)_3$ (**4**) as stimulants of growth of race XII alcohol yeast *Saccharomyces cerevisiae*.

Results and Discussion

The results of the influence of compounds **1** and **2**, which are the most efficient in stimulation of the yeast cells growth within the entire range of concentrations studied, are shown in Fig. 1.

Compound **3** did not exert significant influence on the biomass growth, while compound **4** possessed a pronounced effect only in the concentrations of $1\cdot10^{-8}$—$1\cdot10^{-7}$ wt.% (Fig. 2). The stimulating effect started to operate after 10—12 h from the beginning of cultivation, and after 36 h the amount of yeast cells increased as compared to the control by 1.8—2.9 times for compounds **1** and **2** and by 2.4—2.8 times for compound **4**.

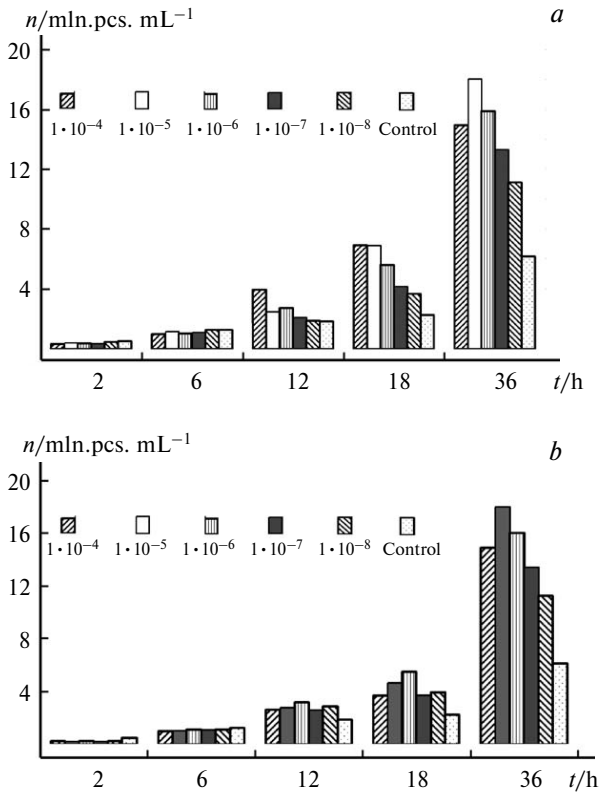

Fig. 1. The yeast biomass growth at different concentrations (wt.%) of compounds **1** (*a*) and **2** (*b*) (*n* is the number of cells, *t* is the cultivation time).

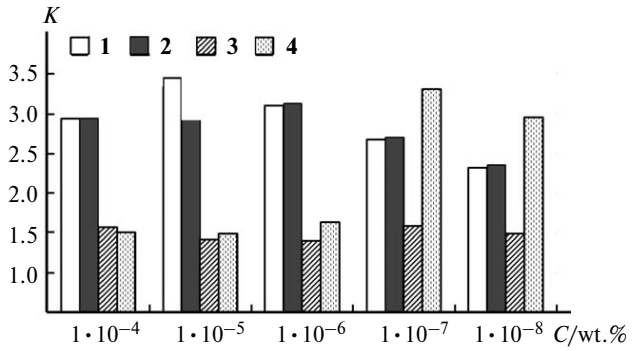

Fig. 2. The number of cells growth factor (*K*) in the media with addition of compounds **1**—**4** as compared to the control.

Microscoping of cultural media did not show significant difference in the viability of yeast cells when compounds **1**—**4** were present in the medium as compared to the control.

Since the methods used gave only estimated results, we obtained curves of microorganisms growth for separate concentrations of compounds **1**—**4** (Fig. 3). As it is seen from Fig. 3, starting from 10—14 hours of cultivation the growth rate of microorganisms in the media containing compounds **1** and **4** increases by 2—5 times as

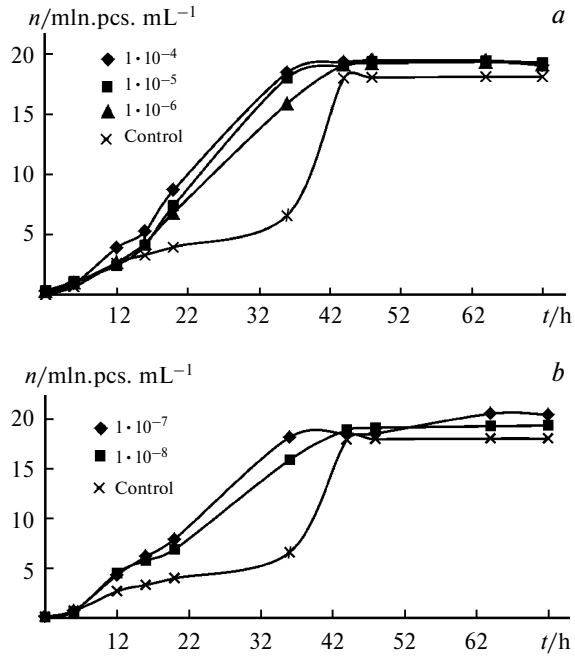

Fig. 3. The accumulation of yeast biomass in the presence of compounds **1** (*a*) and **4** (*b*) at different concentrations (wt.%).

compared to the control. A reduction of the lag-phases is observed for compounds **1**—**4**, *i.e.*, the yeast cells reach the exponential phase of growth faster. In the initial (lag-phase) and in the final (stationary phase) periods, the rates of cell generation in the media containing compounds **1**—**4** and in the control group are similar. It is reasonable to use the indicated specific features of the culture growth in continuous cultivation. The introduction of compounds **1**—**4** in nutrient medium can result in a considerable growth of the biomass of yeast for a shorter period of time. Note that compounds **1** and **2**, which bear a sulfur-containing anion (Y = S, SO_2), stimulate the yeast biomass growth within the whole range of studied concentrations, while compounds **3** and **4** (Y = O) exhibited activity in the narrower range.

The presence of compounds **1**—**4** in the nutrient medium composition stimulates the accumulation of protein compounds in the cell (Fig. 4). The greatest effect was shown by compounds **2** (Y = SO_2) and **3** (Y = O) in the concentration of $1 \cdot 10^{-6}$ and $1 \cdot 10^{-5}$ wt.%, respectively. In their presence, the content of protein in the biomass increased by 13—15% to the dry mass as compared to the control.

It should be noted that compound **3** efficiently stimulates accumulation of protein in the cell, exerting no pronounced influence on the growth of the amount of cells.

The fermentation of synthetic Reeder medium with the content of sucrose increased to 10 wt.% in the presence of compounds **1**—**4** leads to the increase in the rate and the fermentation degree of sugars. Thus, the fermentation degree increases by 8—30%, while the formation of ethanol calculated on assimilated sugar increases in the case of compound **1** by 1.1—1.4 times (Figs 5 and 6). The efficience of fermentation of Reeder medium without addition of compounds **1**—**4** was low. The yield of ethanol was only 37%. The introduction of compounds **1**—**4** in the medium led to the increase in the ethanol production to 58% from theoretical yield (Table 1).

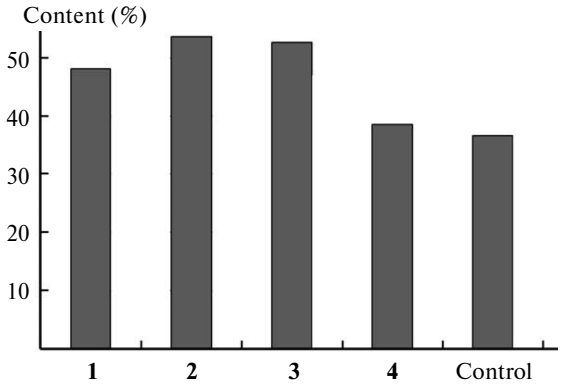

Fig. 4. The content of protein in yeast biomass grown using compounds **1** ($1 \cdot 10^{-4}$ wt.%), **2** ($1 \cdot 10^{-6}$ wt.%), **3** ($1 \cdot 10^{-5}$ wt.%), and **4** ($1 \cdot 10^{-7}$ wt.%).

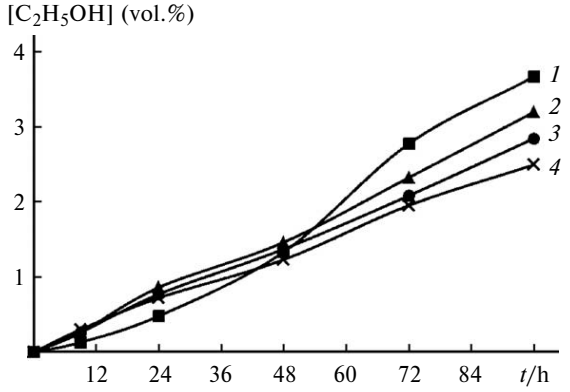

Fig. 5. The dynamics of the formation of ethanol in the presence of compound **1** in different concentrations (wt.%): $1 \cdot 10^{-4}$ (*1*), $1 \cdot 10^{-5}$ (*2*), $1 \cdot 10^{-6}$ (*3*), as well as in the absence of the stimulant (*4* is the control group).

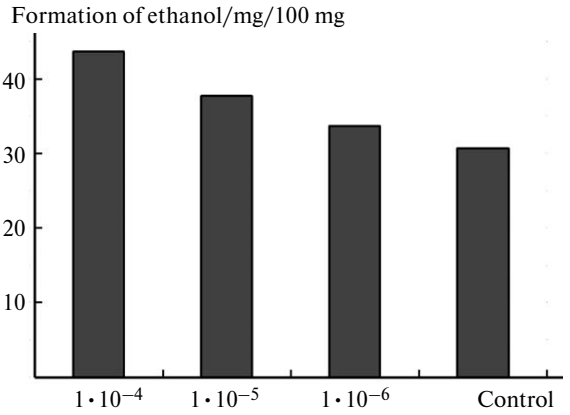

Fig. 6. The formation of ethanol (mg per 100 mg of assimilated sugar) in the medium containing compound **1** in the concentrations $1 \cdot 10^{-4}$, $1 \cdot 10^{-5}$, $1 \cdot 10^{-6}$ wt.% and in the control sample after 96 h of fermentation.

The flour medium, as compared to Reeder medium, is more favorable for cultivation of race XII yeast. The fer-

Table 1. The results of fermentation of Reeder nutrient medium in the presence of compounds **1**—**4**

Compound	Concentration in nutrient medium (wt.%)	Degree of sugar fermentation (%)	Yield of ethanol (% from theoretical)
1	$1 \cdot 10^{-4}$	66.2	54
	$1 \cdot 10^{-5}$	66.8	47
	$1 \cdot 10^{-6}$	66.5	42
2	$1 \cdot 10^{-6}$	49.6	41
3	$1 \cdot 10^{-4}$	73.9	58
	$1 \cdot 10^{-5}$	73.7	58
	$1 \cdot 10^{-7}$	73.3	56
4	$1 \cdot 10^{-7}$	50.2	42
	$1 \cdot 10^{-8}$	58.6	46
Control	—	41.6	37

Table 2. The results of fermentation of flour nutrient medium in the presence of compounds **1**—**4**

Compound	Concentration in nutrient medium (wt.%)	Degree of sugar fermentation (%)	Yield of ethanol (% from theoretical)
1	$1 \cdot 10^{-4}$	96.6	66
	$1 \cdot 10^{-5}$	97.1	70
2	$1 \cdot 10^{-6}$	96.1	66
3	$1 \cdot 10^{-4}$	96.2	70
4	$1 \cdot 10^{-8}$	96.5	65
Control	—	96.5	61

mentation of such medium based on wheat flour results in the higher fermentation degree of sugars for a shorter period of time in both the control group and in the media containing compounds **1**—**4**. The experiment resulted also in the higher yield of ethanol. Thus, the yield in the control was 61% and increased by 5—9% when compounds **1**—**4** were introduced in the flour medium (Table 2).

In conclusion, we found that tris(2-hydroxyethyl)-ammonium arylchalcogenylacetates **1**—**4** are efficient synthetic stimulants of growth and development of race XII yeast *Saccharomyces cerevisiae* and make it possible to intensify the process of their cultivation. This is reflected in the biomass growth and increase in the rate of the culture growth in the logarithmic phase by 2—5 times. The content of protein in the yeast biomass increases by 2—15%. The highest growth of the content of protein compounds is observed when compound **2** is introduced in the nutrient medium in a concentration of $1 \cdot 10^{-6}$ wt.%.

A positive influence of compounds **1**—**4** on the yeast cells allows increasing the depth of sugar fermentation and enhancing ethanol-directed metabolism of yeast cells. The yield of ethanol in the presence of compounds **1**—**4** increases by 5—9%. The use of biostimulants in production of ethanol (biofuel), as well as of fodder and baker´s yeast, will increase the efficiency of the processes and the yields of the target products per mass unit of consumed sugar. The advantage of synthetic biostimulants **1**—**4** is their availability, low cost, good solublility in water, stability on storage, nontoxicity, and efficiency in low concentrations ($1 \cdot 10^{-8}$—$1 \cdot 10^{-4}$ wt.%).

Experimental

Synthesis of compounds 1—4 (general procedure). A solution of tris(2-hydroxyethyl)amine (triethanolamine) and the corresponding acid in ethyl alcohol (molar ratio 1 : 1) was heated for 15—30 min at 65 °C and maintained for 1 h at 20—22 °C. The mixture was poured into diethyl ether (anhydrous) and maintained for 12 h at 5—10 °C. A precipitate was collected by filtration, washed with ether, and dried *in vacuo*. The products were isolated as colorless powders, well soluble in water and ethanol. The composition and the structure of compounds **1**—**4** were confirmed by elemental analysis, IR spectroscopy, ^{1}H, ^{13}C, ^{15}N (relative to $MeNO_2$) NMR spectroscopy, and X-ray diffraction.

Tris(2-hydroxyethyl)ammonium (4-chlorophenylsulfanyl)-acetate (1). The yield was 95%. M.p. 78—79 °C. IR, ν/cm^{-1}: 1362 (ν_s COO$^-$); 1588 (ν_{as} COO$^-$); 2920 (br, N$^+$—H); 3150 (br, OH). ^{1}H NMR, δ: 7.89—7.22 (m, 4 H, C_6H_4); 3.94 (t, 6 H, OCH_2, J = 5.0 Hz); 3.64 (s, 2 H, SCH_2); 3.46 (t, 6 H, NCH_2, J = 5.0 Hz). ^{13}C NMR, δ: 176.71 (C=O); 134.68—128.97 (C_6H_4); 55.19 (OCH_2); 54.93 (NCH_2); 38.15 (SCH_2). ^{15}N NMR, δ: −338.90.

Tris(2-hydroxyethyl)ammonium (4-chlorophenylsulfonyl)-acetate (2). The yield was 96%. M.p. 94 °C. IR, ν/cm^{-1}: 1125 (ν_s SO_2); 1362 (ν_{as} SO_2); 1367 (ν_s COO$^-$); 1611 (ν_{as} COO$^-$); 2925 (br, N$^+$—H); 3210 (br, OH). ^{1}H NMR, δ: 7.42—6.70 (m, 4 H, C_6H_4); 4.55 (s, 2 H, SO_2CH_2); 3.80 (t, 6 H, OCH_2, J = 5.0 Hz); 3.42 (t, 6 H, NCH_2, J = 5.0 Hz). ^{13}C NMR, δ: 178.05 (C=O), 150.09—127.25 (C_6H_4), 64.00 (SO_2CH_2), 59.06 (OCH_2), 57.60 (NCH_2). ^{15}N NMR, δ: −335.15.

Tris(2-hydroxyethyl)ammonium (2-chlorophenyloxy)acetate (3). The yield was 95%. M.p. 80—81 °C. IR, ν/cm^{-1}: 1404 (ν_s COO$^-$); 1590 (ν_{as} COO$^-$); 2925 (br, N$^+$—H); 3182 (br, OH). ^{1}H NMR, δ: 7.25—6.80 (m, 4 H, C_6H_4); 4.48 (s, CH_2CO); 3.92 (t, 6 H, OCH_2, J = 5.0 Hz); 3.42 (t, 6 H, NCH_2, J = 5.0 Hz). ^{13}C NMR, δ: 177.00 (C=O), 156.02—111.39 (C_6H_4), 66.94 ($\underline{C}H_2CO$), 55.14 (OCH_2), 54.89 (NCH_2). ^{15}N NMR, δ: −339.00.

Tris(2-hydroxyethyl)ammonium (4-chlorophenyloxy)acetate (4). The yield was 94%. M.p. 83 °C. IR, ν/cm^{-1}: 1397 (ν_s COO$^-$): 1606 (ν_{as} COO$^-$); 2920 (br, N$^+$—H); 3156 (br, OH). ^{1}H NMR, δ: 7.18—6.70 (m, 4 H, C_6H_4); 4.45 (s, CH_2CO); 3.93 (t, 6 H, OCH_2, J = 5.0 Hz); 3.44 (t, 6 H, NCH_2, J = 5.0 Hz). ^{13}C NMR, δ: 176.51 (C=O), 154.61—112.31 (C_6H_4), 66.98 ($\underline{C}H_2CO$), 55.03 (OCH_2), 54.77 (NCH_2). ^{15}N NMR, δ: −338.60.

Biological tests. The yeast *Saccharomyces cerevisiae* of race XII were cultivated on a synthetic Reeder medium of the following composition (g L^{-1}): sucrose — 20; $(NH_4)_2SO_4$ — 3; $MgSO_4$ — 0.7; NaCl — 0.5; KH_2PO_4 — 1; K_2HPO_4 — 0.1. The dosage of compounds **1**—**4** into the nutrient medium was carried out as follows. A matrix solution with a concentration of 2 mg mL^{-1} (compounds **1**—**4** (200 mg) were dissolved in distilled water (100 mL)) was prepared. Then, a serial dilution of the matrix solution was carried out to a concentration of $1 \cdot 10^{-7}$—$1 \cdot 10^{-2}$ wt.%. The diluted matrix solution was dosed into the nutrient medium in an amount necessary to obtain the desired concentration. The inoculum of the yeast *Saccharomyces cerevisiae* of race XII was prepared by seeding on sloping wort-agar with further rinsing of the grown culture with the Reeder medium. The content of yeast cells in the inoculum was $(7—8) \cdot 10^6$ cell mL^{-1}; the dosage of inoculum is 5% to the volume of the nutrient medium. The cultivation was carried out for 20—36 (72) h at 30 °C. The total amount of yeast cells in the culture medium was determined by optical method on a KFK-3 photoelectrocolorimeter (ZOMS) at 490 nm. The viability of the yeast was assessed by the presence of living and budding cells. The content of living cells was determined by staining with methylene blue, that of the budding cells by microscopy. Control experiments were carried out with the addition of distilled water to the nutrient medium instead of the solution of compounds **1**—**4**. The total biomass of yeast was determined by gravimetric method, the protein content in the biomass was determined by colorimetry with amido-black.[17] The alcohol

fermentation of the Reader medium with a sucrose content of 10% in the presence of compounds **1**—**4** was carried out for 96 h at 30 °C, with the norm of application of the yeast seed material being 5% to the volume of the nutrient medium. The fermentation progress was monitored by determining the content of residual sugar by the Dubois method.[18] The accumulation of ethanol was determined by GC-MS on an Agilent Technologies 7820 A gas chromatograph with an HP 5975 selective mass spectrometer detector. The experiment conditions: the ionization energy 70 eV, the separator temperature 280 °C, the source of ions temperature 230 °C; a 30000×0.25-mm quartz column with a stationary phase (5% diphenyl polysiloxane); the column temperature 50 °C. The components were identified using the NIST11 library of mass spectra. The quantity of the components was calculated from the peak areas using correction sensitivity coefficients. The simulation of the industrial process of alcohol fermentation was carried out on a nutrient medium containing 10% of the 1st grade wheat flour. To obtain fermentable sugars, the kneading flour was saccharified with enzymatic agents Amlosubtilin G3x and Glukavamorin manufactured by Sibbiofarm Ltd. (Berdsk). The dosage of the agents and the conditions of the enzymatic hydrolysis were selected in accordance with the manufacturer's recommendations. The fermentation time was 72 h. The remaining conditions were similar to the fermentation of the Reader medium.

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