Stimulation of Glucose Bioconversion to Ethanol by Humic Substances of Oxidized Brown Coal

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Abstract

The effect of humic substances on bioconversion of glucose into ethanol is investigated. It is established that their presence in the reaction mixture with the optimal concentration of 0.001 % accelerates fermentation process. The reaction of glucose fermentation can be recommended for use as a new rapid and economic test for the biological activity of various humic preparations and optimization of their concentrations in aqueous solutions, which can be used, for stimulation of the growth of microorganisms, plants, and animals.

INTRODUCTION

Humic substances (HS) are a group of natural organic acids; increased interest to them is connected with their physiological activity (PA), which is a valuable practical specific property [1].

The positive effect of HS on the life activities of microorganisms was noted more than 60 years ago for yeast cultures as example (*Saccharomyces cerevisiae*). Addition of peat-extracted sodium humate to culture medium with the concentration of 0.001 % accelerated their reproduction. Having a positive effect on the life activities of yeast, this compound accelerated the processes catalyzed by yeast, for example alcoholic fermentation [2].

The goal of the present work was to study the effect of HS obtained from oxidized brown coal on the yeast culture *Saccharomyces cerevisiae*. Brown coal differs from peat substantially not only by the increased concentration of HS but also by the element composition, molecular mass distribution, structural fragment features (higher content of aromatic fragments and low content of aliphatic ones, absence of carbohydrate fragments). Several series of experiments on glucose fermentation were carried out to investigate relative PA of HS of oxidized coal with different technologies of their preliminary treatment, concentration in reaction mixture, and fermentation time. The activity of separate groups of HS from one coal kind was compared as well as that of humates from oxidized coals of different deposits and of the commercial preparation "Humate+" which showed itself practically useful as a highly efficient stimulator of plant growth.

EXPERIMENTAL

Mainly HS from coal of Kholboldzhinsky pit of Gusinoozerskoye deposit (Buryatiya) were used in experiments. To obtain these substances, coal samples were subjected to preliminary treatment: either sieved through a sieve with the mesh size of 1 mm, or additionally

mechanically treated in a multipurpose disintegrator UDA of flow type or in a ball mill Pulverisette-6. The granulometric analysis of resulting products was carried out with Analisette-20 sedimentograph.

According to the procedures proposed by Stevenson [3] and Lowe [4], the following groups of HS were isolated from the initial oxidized coal not subjected to mechanical treatment: fulvic acids (FA), humic acids (HA), sum of FA and HA (HFA), hymatomelanic acids (HMA), HA without HMA, brown HA, gray HA (Fig. 1). The procedures of isolation of the indicated groups of HS, characterization of their fragment composition on the basis of ^{13}C NMR spectra, element composition and molecular mass distribution were presented by us earlier [5].

The following reagent ratios were used in the experiments on fermentation: *D*-glucose (All-Union State Standard GOST 6038–79) – 10 g, yeast (*Saccharomyces cerevisiae*) – 1.5 g, distilled water – 35 ml, sodium humate – 0.0005–0.01 % of the mass of water. The process was carried out in a thermostatically controlled room (20±1) °C.

The ethanol concentration which characterizes the efficiency of yeast, was determined directly in the reaction mixture by means of ^1H NMR spectroscopy (Varian VXR-500S spectrometer, 500 MHz) by recording quantitative spectra on protons and calculating relative content of ethanol in this mixture on the basis of the integral intensity of the signal of its methyl group. The experimental data on ethanol yield presented below and normalized to the reference sample are based on mean values for two parallel experiments with a convergence of 5 % and better.

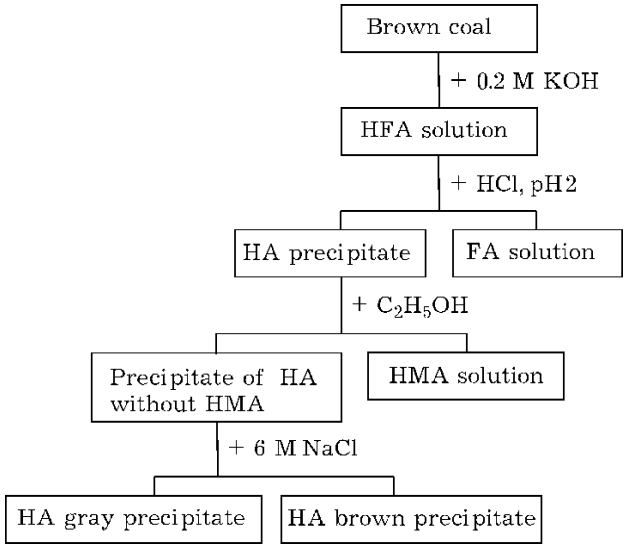

Fig. 1. Scheme of the isolation of HS from coal of the Kholboldzhinsky pit of Gusinoozerskoye deposit.

On the basis of results of the measurements of ethanol yield for the samples of initial humate (H_{in}) obtained with the interval of 24 h, three exposures were chosen for process monitoring: 72, 120, and 168 h. The choice was due to both the experimental possibilities and the fact that for exposure shorter than 72 h the low intensity of ethanol signal resulted in an increased error of the determination of its content, while the results for exposures 168 h and longer demonstrated actual completeness of the process. Conversion degree was 80 %.

RESULTS AND DISCUSSION

At first, in order to establish the effect of HS on bioconversion of glucose and to choose the most efficient concentration to accelerate the process, we used humates of the oxidized coal from the Kholboldzhinsky pit of Gusinoozerskoye deposit, treated with the help of different technologies: humate without preliminary treatment (H_{in}) and humate treated mechanically in Pulverisette-6 for 2 h ($H_{m/t}$) and in UDA (H_{dis}). The reaction medium was distilled water (reference) or aqueous solutions of sodium humate with the mass concentration of 0.0005, 0.001, 0.005, and 0.01 %. Reaction mixtures were left for fermentation for 72, 120 and 168 h, then the concentration of ethanol in them was measured with respect to that of the reference sample (without humate) (Table 1). Errors of the measurement of relative bioconversion here and below did not exceed 3 %.

The data on the relative content of ethanol in the reaction mixture indicate that the maximal acceleration of bioconversion of glucose into ethanol by sodium humate is observed for its concentration 0.001 %: ethanol concentration after reaction for 120 h is more than two times as high as that in the reference. It is significant that HS concentrations 0.0005 and 0.005 % (that is, only two times smaller or five times higher

TABLE 1

Glucose bioconversion into ethanol for different concentrations of sodium humates and process duration with respect to the reference reaction mixture containing no humates

Concentration of sodium humate, mass %	Time, h								
	72			120			168		
	H_{in}	H_{dis}	$H_{m/t}$	H_{in}	H_{dis}	$H_{m/t}$	H_{in}	H_{dis}	$H_{m/t}$
0.0005	1.04	1.15	1.01	1.04	1.01	0.98	0.91	0.79	0.85
0.001	1.46	1.48	1.71	2.22	2.29	2.03	0.96	1.06	1.04
0.005	1.05	1.16	1.00	1.11	1.03	0.99	0.91	0.88	0.89
0.01	1.18	1.16	1.06	1.10	1.14	1.04	0.97	0.99	0.98

TABLE 2

Granulometric composition of humic compounds

Sample	Particle size, μm									
	10	20	30	40	50	60	70	80	90	100
	Content, %									
H_{in}	17.5	28.0	42.0	57.0	72.0	97.0	122.5	185.0	230.0	270.0
H_{dis}	9.0	13.0	14.5	15.9	17.5	21.0	24.0	28.0	36.0	48.0
$H_{m/t}$	–	–	–	–	–	6.0	10.0	15.0	19.5	25.0

than the optimal one) turned out to be practically ineffective for all the three samples (Fig. 2).

It also follows from the data shown in Fig. 2 that the stimulating action of HS increases but little as a result of preliminary treatment in the case of H_{dis} and even decreases for $H_{m/t}$. The high efficiency of preliminary treatment on the particle size of initial oxidized coal can be estimated from the granulometric composition: as a mean, particles decrease for H_{dis} and $H_{m/t}$ by a factor of 5 and 10, respectively (Table 2). In the latter case, almost the whole preparation is represented by particles smaller than 20 μm. It is necessary to note that the insignificant effect of preliminary treatment on the PA of humates in the process under consideration is contrast with their growth-stimulating activity with respect to the seeds of some plants as we have investigated previously [7].

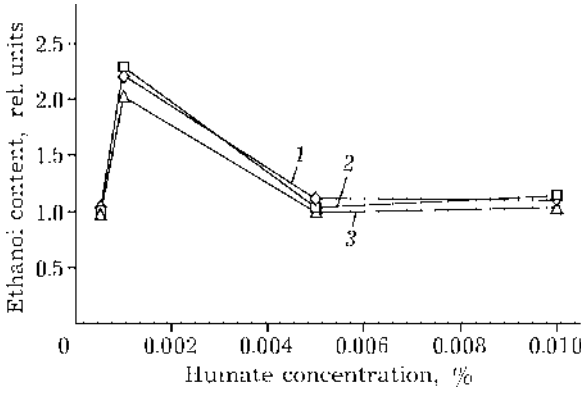

Fig. 2. Dependence of the relative content of ethanol in the reaction mixture on the concentration of humate: initial (*1*), disintegrated (*2*) and treated mechanically (*3*) for the reaction time of 120 h.

For thus determined efficient humate concentration (0.001 %), we compared the glucose the activating action of HS isolated from different oxidized coal samples on the process of glucose fermentation. For this purpose, we used sodium humates from oxidized coal from a number of deposits of Eastern Siberia: Shchetkinskoye (without or with mechanical treatment in Pulverisette-6), Glinkinskoye, commercial preparation "Humate+" (manufactured in Irkutsk, Russia), and H_{in} of Kholboldzhinsky pit. Similarly to previous experiments, samples were taken after 72, 120, and 168 h (Fig. 3).

One can see that the highest stimulating effect determined on the basis of ethanol concentration in the reaction medium is also exhibited for all the preparations after 5 days (120 h). The high efficiency of commercial preparation "Humate+" observed as early as after 72 h is likely to be due to the presence of macro- and microelements which serve as the cultrure medium for the yeast culture catalyzing

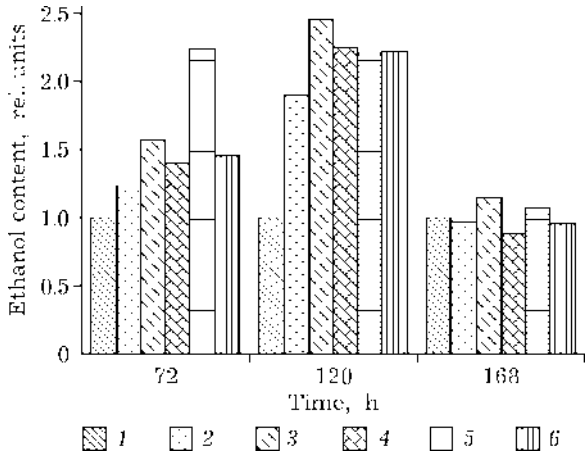

Fig. 3. Effect of humates of different origin on the dynamics of alcoholic fermentation: *1* – reference, *2, 3* – humates of the Glinkinskoye and Shchetkinskoye deposits, *4* – commercial preparation "Humate+", *5* – mechanically treated humate from the Shchetkinskoye deposit, *6* – initial humate.

bioconverison process. It is characteristic that after 168 h the degree of glucose conversion into ethanol becomes equal for all the humates and close to that in the reference. This is natural since total yield of ethanol upon completion of the optimized process is always determined by the amount of glucose taken for experiments.

The main result of the series of experiments lies in the fact that the presence of HS used, independently of their nature, in the concentration of 0.001 % increases the relative yield of ethanol at 20 °C and 120 h by a factor of 2 as a mean. These results may be of interest for the use of potassium and sodium humates for the acceleration of the process in the production of edible ethanol and in wine industry because these compounds have no toxic effects in the amounts used (10 mg/l).

For separate groups of HS isolated from the same oxidized coal (see Fig. 1) and fully characterized by the element and fragment composition, molecular mass distribution (Tables 3, 4), their effect on glucose bioconversion was investigated in order to reveal the interconnection between structure and properties. The data on the PA against glucose fermentation for the investigated HS groups and for three exposures are shown in Table 3 along with the data on auxin-like activity of these HS groups for the concentration of 0.001 % in water toward wheat coleoptiles which had been measured by us previously with respect to the reference (water without HS additives) [5].

Comparison of the PA of separate groups of HS toward the yeast culture (PA_1) for the exposition of 120 h (PA_1^{120}) and the growth of wheat coleoptiles (PA_2) showed that a linear connection is observed between them; it can be represented as follows:

$$PA_2 = 0.48 + 0.56PA_1^{120}, r = 0.83, s = 0.17, n = 7 \quad (1)$$

Since the point for HA without HMA deviates from the regression line most essentially, its exclusion provides a reliable formal connection of the PA of HS groups in essentially different processes:

$$PA_2 = 0.34 + 0.66PA_1^{120}, r = 0.95, s = 0.10, n = 6 \quad (2)$$

Previously [5] we revealed a connection between an increase in coleoptiles in comparison with the reference (DPA_2 and the

TABLE 3

Relative physiological activity of HS groups towards alcoholic fermentation (PA_1), auxin-like activity (PA_2) and the content of carbon atoms in different structural fragments, %

HS group	PA_1 for fermentation* for, h			PA_2**	Carbon content, %					
					C=O	COOH	$C_{ar}O$	$C_{ar}C,H$	$C_{alk}O$	C_{alk}
	72	120	168							
HFA	1.59	1.29	0.92	1.20	4.5	11.2	10.2	46.0	8.0	20.1
HA	1.93	1.58	0.93	1.43	4.1	14.0	10.3	53.0	3.6	15.0
HMA	1.68	1.69	0.95	1.34	5.1	15.7	9.5	46.1	6.9	16.7
HA without HMA	1.74	1.76	1.00	1.26	5.5	13.8	9.1	45.1	5.0	21.5
HA brown	1.53	1.58	0.91	1.46	5.4	14.8	10.2	49.3	6.6	13.6
HA gray	1.60	1.84	0.98	1.60	5.6	10.1	11.9	42.5	12.4	17.5

*An increase in the yield of ethanol in comparison with reference is indicated (PA_1 = 1.00).
**An increase in the length of coleoptiles in comparison with the reference is indicated (PA_2 = 1.00).

TABLE 4

Element composition and molecular mass distribution of HS groups

HS group	Concentration, mass %				Molecular mass distribution*, kD			
	C	H	N	O	M_z	M_w	M_n	M_w/M_n
HFA	72.52	4.54	1.93	21.01	98.8	38.3	1.6	23.9
FA	62.49	4.62	2.07	30.81	53.3	31.1	2.2	14.1
HA	69.45	4.19	3.58	22.79	38.1	20.4	1.2	17.0
HMA	55.21	3.77	4.31	36.72	43.8	20.5	1.8	11.4
HA without HMA	65.80	4.17	2.97	27.06	44.3	22.4	1.1	20.4
HA brown	72.23	4.20	3.29	20.28	35.0	19.8	1.7	11.6
HA gray	66.59	4.13	2.49	26.79	58.8	29.3	1.3	22.5

*The characteristics of the molecular mass distribution: M_z – average, M_w – weight-average, M_n – number-average, M_w/M_n – the index of polydispersity degree.

fragment composition from ^{13}C NMR spectra of HS groups; the connection may be represented as follows:

$$\Delta PA_2 = 3.6(C{=}O) + 4.1(COOH) + 32.2(C_{ar}O) - 5.6(C_{ar}C,H) - 8.5(C_{alk}O) - 2.3(C_{alk}) \quad (3)$$

Such a formal description of the connection between ΔPA_1^{120} and the fragment composition of HS gives the following equation:

$$\Delta PA_1^{120} = 3.0(C{=}O) + 15.6(COOH) + 69.7(C_{ar}O) - 15.6(C_{ar}C,H) - 21.8(C_{alk}O) + 1.1(C_{alk}) \quad (4)$$

A strict linear connection was detected between the corresponding coefficients at the six arguments of equations (3) and (4) (a_i and b_i, respectively):

$$a_i = 0.09 + 0.45b_i,\ r = 0.99,\ s = 2.39,\ n = 6 \quad (5)$$

On the basis of equations (3)–(5), it may be assumed that, in spite of the difference in objects, methods and conditions of the chosen version of biotesting of HS groups, in both cases the PA is mainly determined by the relative content of the same structural fragments in HS (carboxylic, aroxy, and alkoxy groups). The content of ketone and aliphatic carbon atoms is less essential. One can also see from the coefficients at the arguments of equations (2)–(5) that for the chosen experimental conditions the yeast test is about two times more sensitive to the features of the fragment composition of HS groups.

The established fact of a reliable connection between PA of HS groups from the same oxidized coal towards quite different processes for which a common feature is only the medium (water with 0.001 % of different HS groups added) does not allow us to exclude the hypothesis that the PA of this medium may be connected, as we have mentioned previously [7], with structuring of the water matrix by insignificant additives of HS (1 molecule of a HS with the mean mass of 40 kD per 200 mln water molecules). This question is the subject of our further investigation of water structure by means of relaxometry of ^1H and ^{17}O NMR.

No connection between the PA of HS groups toward glucose bioconversion and the results of measurements of their element composition and characteristics of the molecular mass distribution was detected. This agrees with the results of similar attempts in our investigations of PA of these preparations for wheat coleoptiles as example [5].

CONCLUSIONS

Thus, all the humates investigated have unambiguously activating action on glucose bioconversion into ethanol. The most efficient concentration of humates is ~0.001 %, the recommended duration of experiment at 20 °C is 120 h (5 days). Under these conditions, the relative increase in bioconversion under the action of HS reaches the maximum value. It should be noted that the found effective HS concentration in water is in good agreement both with the results of our previous laboratory and field tests [7] of a number of HS and with the known literature data [2, 8, 9] of testing these HS for auxin-like activity of HS [6].

According to our estimations, the developed experimental approach is one of the most sensitive and rapid potential test methods for the determination of the PA of various HS. The exposure of the reaction under consideration can be substantially decreased either with temperature rise to 30 °C or with a more careful choice of the component ratio glucose – yeast – water. To monitor the degree of glucose conversion into ethanol, instead of ^{1}H NMR spectroscopy, it is reasonable to measure the volume of evolving CO_2, which is proportional to the yield of ethanol. This will allow us not only to increase the economy and flexibility of the proposed method of biotesting of the growth-stimulating activity of HS but also to carry out continuous monitoring of the kinetics of bioconversion processes.

The discovered interconnections between structure and properties stimulate a more detailed investigation of the nature of HS activity using the methods of biotesting and microbiology. In these investigations, it is reasonable to broaden the range of objects due to HS from other kinds of raw material and individual model compounds with the fragment composition close to HS.

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