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Biotechnological Aspects of Ethanol Biosynthesis from *Miscanthus*

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Abstract—The study demonstrates that *Saccharomyces cerevisiae* VKPM Y-1693 yeast is resistant to the aqueous enzymatic *Miscanthus* hydrolysate. The good morphophysiological condition of the yeast indicates that the media contain no detrimental impurities characteristic of acidic chemical hydrolysates. It is shown that the native active acidity of the hydrolysate (pH 4.5–4.7) is optimal for ethanol biosynthesis from enzymatic *Miscanthus* cellulose hydrolysate. An addition of 1% yeast extract makes the aqueous enzymatic *Miscanthus* cellulose hydrolysate medium complete. Under the conditions of batch fermentation, it is sufficient to introduce 10% yeast inoculum in the log phase of growth. As shown by gas-liquid chromatography, the enzymatic digestion of *Miscanthus* cellulose produces ethanol with low contents of esters and fusel oils. *Miscanthus* bioethanol the contains no methanol.

Keywords: Soranovskii variety of *Miscanthus*, enzymatic digestion, cellulose, *Saccharomyces cerevisiae* Y-1693, strain, bioethanol

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Due to the development of bioenergy, biomass is currently considered one of the key renewable energy resources of the future. According to the preliminary estimates, the world's proven oil reserves and wood reserves are approximately equal; however, the hydrocarbon resources are being rapidly depleted, while the reserves of plant biomass are restored as the result of the natural increase. In the near future, the petrochemical industry will be replaced by the biochemical and chemical processing of wood and other plant materials (Ablaev, 2011).

Currently, due to forest conservation, the most pressing tasks are the search for renewable energy sources alternative to wood, the handling of environmental problems, and the development of energy-saving technologies.

Considerable attention is being paid to the problem of the biomass treatment to produce biofuels. Biorefinery, an integrated processing of lignocellulosic biomass by chemical and/or biotechnological methods in a range of competitive products and energy (Kuznetsova, 2013), is a modern and fundamental area of industrial biotechnology that is developing in industrial countries. In the EU, about 3% (65 million tons of fuel equivalent) of all energy needs are met by the use of biomass; in some countries this parameter reaches the value of 23% (Finland), 18% (Sweden), and 12% (Austria) (Bulatkin et al., 2013). In energy, the biomass can be used directly by incineration or it may be converted to diesel, ethanol, or natural gas by chemical and biotechnological methods. By-products of vegetable origin (straw, sunflower husks, corn stalks, etc.), the annual waste of which is up to 50 million tons in Russia, can be sources of biomass. In agricultural production, *Miscanthus sinensis* Andersson of the grass family Poaceae is one of the sources for the production of cellulose-containing biomass (Shumnyi et al., 2010). A novel form of *Miscanthus*—the Soranovskii variety—was derived at the Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences in 2006. It is regarded as a flammable crude material for the production of biomass, namely, ethanol.

From an economic point of view, the production of second generation bioethanol, i.e., bioethanol, from cellulose-containing material, is costly, since the biomass processing requires physical, chemical, and thermobaric treatments aimed at maximizing the impact on the chemical bonds of the biomass. Cellulose-containing raw material is a solid matrix formed by cellulose and hemicellulose and bonded by lignin. The combination of these components conditions the stability of the matrix to all external actions. For this reason, combinatorial methods are used for its destruction. Pretreatment is required to improve the accessibility of cellulose and hemicellulose to the action of hydrolytic enzymes and reach a high degree of conversion of these polymers into sugars.

Enzymatic conversions of the plant biomass into sugar solution, performed after a chemical pretreatment of raw materials, and subsequent biochemical conversion of sugars into ethanol are actively studied worldwide (Brosse et al., 2009; Somerville et al., 2010;

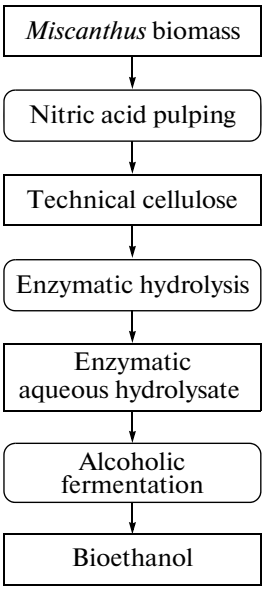

Fig. 1. Process flow diagram for the bioethanol production from the *Miscanthus* biomass.

Jordan et. al., 2012). It is the chemical pretreatment stage that most of the studies are focused on. A sufficiently large series of studies are being carried out to optimize the stage enzymatic hydrolysis (development of novel enzyme complexes and technological conditions for the enzymatic hydrolysis). These processes have disadvantages: the substrate concentrations are low (hence, diluted sugar solutions are obtained—30 g/L), and the use of the acetate buffer is required for enzymatic hydrolysis (therefore, the resulting media is initially unsuitable for yeast metabolism and ethanol biosynthesis). Apparently, this may explain the relatively small number of publications devoted to the production of bioethanol. Moreover, the authors of these publications avoid detailed descriptions of technological production modes. In Russia, the production of bioethanol from *Miscanthus* is not described, despite the fact that such a possibility was considered.

At the Institute for Problems of Chemical and Energetic Technologies, Siberian Branch of the Russian Academy of Sciences (IPCET SB RAS), the *Miscanthus* biomass bioconversion into fuel is performed according to the scheme shown in Fig. 1.

The stages of chemical pretreatment and enzymatic hydrolysis have been worked out in detail and described by us earlier. In this paper, these stages are studied and presented under the optimal conditions that provide a high yield of the target intermediates (technical cellulose (TC) and aqueous enzymatic hydrolysate of the *Miscanthus* TC). The aim of the work was to study some biotechnological aspects of the conversion of reducing sugars from enzymatic *Miscanthus* hydrolysates to bioethanol.

MATERIALS AND METHODS

The complete experiment for obtaining the alcohol (enzymatic hydrolysis and fermentation of the derived hydrolysate) was carried out in triplicate; the average values are presented in the study.

The feedstock for the bioethanol production was the Soranovskii variety of *Miscanthus sinensis* Andersson (the plantation age was 9 years), which was grown at the Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences, Novosibirsk region, and provided to IPCET SB RAS for research on interdisciplinary projects 73. TC was produced from *Miscanthus* at the pilot plant of IPCET SB RAS by the nitric acid method (NA), which comprises pulping of the feedstock in a diluted solution of nitric acid at atmospheric pressure and subsequent treatment with a diluted sodium hydroxide solution (Budaeva et al., 2013; Gismatulina et al., 2013).

Enzymatic hydrolysis of moist TC was performed in a reactor with a total volume of 11 L (Pavlov, 2014) and a multienzyme composition consisting of commercially available enzyme preparations Bryuzaim BGX, Tsellolyuks-A, and Rapidaza TsP, which were taken in an amount of 0.04 g/g of substrate. A special feature of this process is that it is carried out in an aqueous medium instead of an acetate buffer (Skiba et al., 2012).

The enzymatic hydrolysate was a brownish-yellow turbid liquid with a characteristic sour odor of *Miscanthus*. The hydrolysate obtained by the enzymatic hydrolysis was filtered; it was additionally supplied with 10 g/L ammonium sulfate. The hydrolysate was pasteurized at a temperature of 100°C without exposition; it was cooled and directed to fermentation by *Saccharomyces cerevisiae*, strain VKPM Y-1693 (Research Institute for Genetics and Selection of Industrial Microorganisms, Moscow). The strain was isolated from the fermenter of the Kotlas Pulp and Paper Mill and was used for the ethanol production on wood hydrolysates. A special feature of the strain is its resistance to harmful impurities in hydrolysates. Fermentation was carried out anaerobically at a temperature of 28°C batchwise.

The concentration of reducing substances (RSs) was determined spectrophotometrically using a 3,5-dinitrosalicylic acid assay (using a UNICO UV-2804 spectrophotometer, United States) for glucose. Active acidity was measured potentiometrically (using a Checer-1 pH-meter). The volume fraction of alcohol in the brew was determined by an aerometer in a distillate obtained by alcohol distillation from the brew (GOST R 51135-98-2003, 2003). Ethanol from the brew was concentrated by simple distillation; no further purification was performed.

The theoretical concentration of ethanol was calculated according to the stoichiometric equation of fermentation; the ethanol yield was calculated as the ratio of the experimental ethanol concentration to the

theoretical ethanol concentration. The economic coefficient of fermentation $Y_{P/S(PB)}$ is the ratio of the product (ethanol) concentration to the concentration of the RS in the enzymatic hydrolysate.

The ethanol was analyzed by gas-liquid chromatography (GLC) (GOST R 51786-2001, 2001) on a gas chromatograph with a flame ionization detector Crystal 2000M (SKB Khromotek, Yoshkar-Ola, Russia). Experimental conditions: the capillary gas chromatography column ZB-FFAP (USA) was 50 m × 0.32 mm × 0.52 μm; the detector temperature was 220°C; and the evaporator temperature was 190°C. The duration of sample exposition at 77°C was 6 minutes 30 seconds; then, the sample was heated at a heating rate of 10°C per minute to a temperature of 210°C and exposed for 15 minutes. The flow division ratio was 40 : 1; compressed nitrogen was the carrier gas; the pressure of the carrier gas (nitrogen) was 77 kPa; and the ratio of air to hydrogen was 250 : 25. The calibration curve was obtained using calibration mixtures—state standard samples. The gas flow rate (discharge) was 30 mL/min; the gas flow rate (feeding to the FID) was 30 mL/min; the gas flow rate (hydrogen in the FID) was 20 mL/min; the gas flow rate (air in the FID) was 200 mL/min; and the amount of the sample was 1 μL.

RESULTS AND DISCUSSION

This paper discusses some aspects of the alcoholic fermentation on an enzymatic *Miscanthus* hydrolysate medium.

It is known that hydrolysis media for cellulosic materials cannot be used as a complete medium for yeast culturing, because the contents of nitrogen and phosphorus compounds in the media are insufficient, they lack vitamins and growth promoters, and they may contain contaminants reducing the biological goodness of the media. However, the enzymatic hydrolysates are prepared under mild conditions, so some contaminants (furfural, methanol, formaldehyde, terpenes, hydroxymethylfurfural, and volatile acids) are either absent in them or present in lower concentrations.

Resistance of the strain Y-1693 was determined by its long-term cultivation on an enzymatic *Miscanthus* hydrolysate medium for three months. The hydrolysis medium was sterilized at 0.5 atm for 30 min; its sterility was checked. After that, the medium was supplied with 5 wt % yeast inoculum in the exponential phase of growth; the yeast inoculum characteristics were the following: the total amount was 158.5 CFU/mL; the yeast contained 19% budding cells, 70% live cells, and 1% dead cells. After the yeast was added, the media were poured into chemical glass vials under sterile conditions (10 mL of media into each vial) and closed with cotton-gauze plugs. In order to exclude the possibility of infection, a new tube was selected for each of the current analyses.

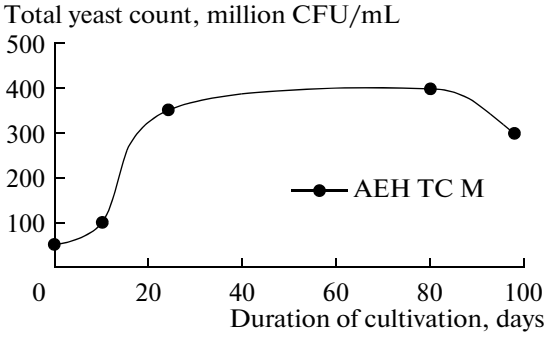

Fig. 2. Dependence of the total yeast count on the duration of cultivation on the aqueous enzymatic hydrolyzate (AEH).

When culturing the strain for 80 days, the total number of yeast cells did not decrease (Fig. 2); after that, it slowly reduced.

It can be concluded that the yeast is sufficiently resistant to hydrolysis media, which indicates the high quality of the aqueous enzymatic *Miscanthus* TC hydrolysate. The good morphophysiological condition of yeast indirectly indicates that the environment contains no harmful contaminants typical for chemical hydrolysates.

The native active acidity of the TC enzymatic hydrolysate is 4.5–4.7 units of pH; the same value is recommended for the production of ethanol from raw grain-potato material (Yarovenko et al., 1999). The pH value of 5.5 is optimal for the yeast action (Yarovenko et al., 1999). The pH value of 3.9 is recommended for obtaining hydrolysis alcohol from nonwood raw materials (Sharkov et at., 1973). These three options have been tested for the aqueous enzymatic *Miscanthus* TC hydrolysate; the concentration of reducing substances was 51 g/L. The yeast inoculum dose in these experiments was 5 wt %.

In all the experiments, the fermentation process ended after three days, as illustrated by a decrease of the RS (glucose) content and an increase of the volume fraction of ethanol (Fig. 3). In the experiment performed at pH 3.9, the yield of ethanol (33%) was minimal, which can be associated with the active acidity initially unfavorable for the yeast. When the pH value of the enzymatic hydrolysate was 4.5–5.5, the population size tripled (from 13 to 36–39 million CFU/mL) and remained constant until the end of the experiment.

The active acidity of 4.5 pH units allows us to obtain the maximum yield of ethanol: 1.7 vol %, that is, 51.5% of the theoretical yield. The low yield of ethanol may be associated with the unbalanced composition of the culture medium or with the unintended use of sugars in the medium for the growth of the yeast population or for endogenous metabolism.

To exclude the version of the unintentional use of sugar, we varied the inoculum dose (5, 10, 15, and

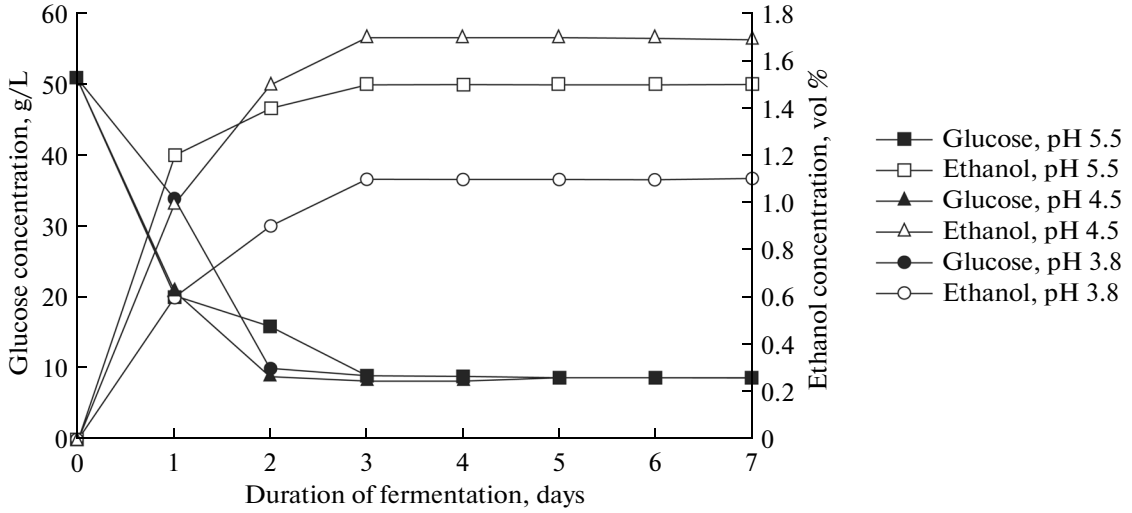

Fig. 3. Dependence of the RS concentration and the ethanol volume fraction on the duration of the fermentation at different active acidity of the medium.

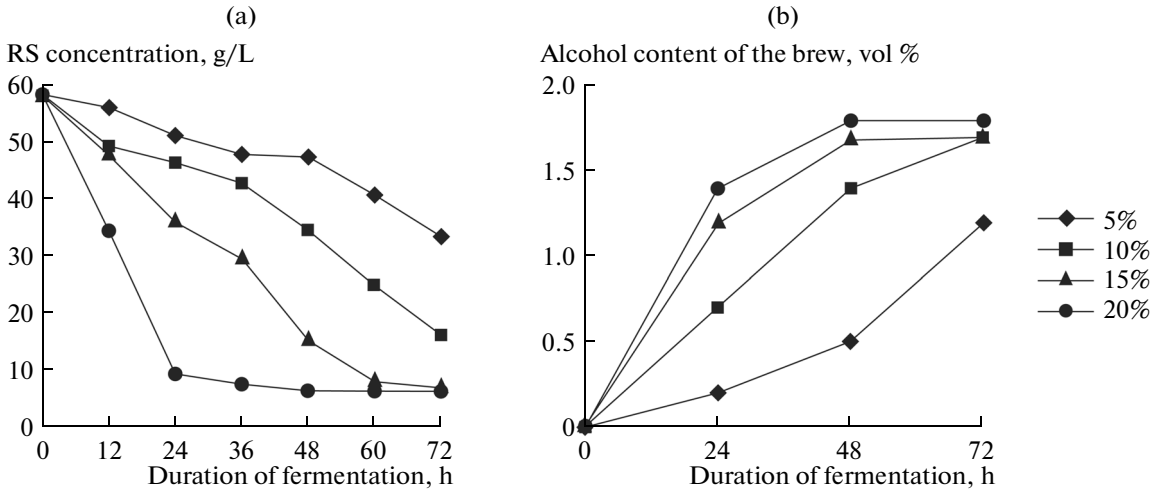

Fig. 4. Dependence of the RS concentration (a) and the ethanol volume fraction (b) on the duration of fermentation at different inoculum doses.

20 wt %) in the next experiment. We used the hydrolysate containing 58 g/L RS; the hydrolysate was additionally supplied with 10 g/L ammonium sulfate. The fermentation is accelerated with the increasing inoculum dose: at a dose of 20 wt %, the duration of fermentation was 24 hours; at 15 wt % and 20 wt %, it took 3 days; and at a 5 wt % dose, half of the RS remained unfermented (Fig. 4). The cell count remained constant during the fermentation and corresponded to the quantity of yeast introduced with the inoculum: 14 million CFU/mL for an inoculum dose of 5 wt %; 28 million CFU/mL for the inoculum dose of 10 wt %; etc. This indicates a lack of vitamins and amino acids in the medium.

Therefore, increased inoculum doses cause an increase in the ethanol yields due to the utilization of the resources of the viable cells. In this case, however, the cells do not proliferate, and the ethanol yield is low: 33, 46, 46, and 49% in four experiments, respectively, which also indicates the imbalance of the medium.

In the next experiment, the medium was supplied with 1% dry yeast extract as a source of amino acids and vitamins. This enabled us to reduce the duration of fermentation to 24 hours at the inoculum doses of 5 wt % and 10 wt % and up to 12 hours at the inoculum doses of 15 wt % and 20 wt %.

The yeast population approximately doubles during the fermentation, and the ethanol concentration in the brew reaches 2.3 vol %, which corresponds to 63% of the theoretical yield. The economic coefficient of fermentation is 0.406. The comparison of fermenta-

The content of impurities in ethanol from nonfood raw materials and in the experimental bioethanol sample from *Miscanthus*

Samples		Parameter			
		mass concentration of aldehydes in the recalculation on anhydrous alcohol, mg/dm^3	mass concentration of fusel oil in the recalculation on anhydrous alcohol, mg/dm^3	mass concentration of esters in recalculation on anhydrous alcohol, mg/dm^3	methanol content in recalculation on anhydrous alcohol, vol %
Raw ethanol from food raw materials	Raw alcohol obtained from all sorts of raw materials (except molasses) or their mixtures	<300	<5000	<500	0.13
	Raw alcohol from molasses	<500	<5000	<700	–
Industrial ethanol	Brand A OKP 91 8213 1100	<200	<500	<80	<0.1
	Brand B OKP 91 8213 1200	<350	<1000	<180	<0.1
Experimental bioethanol sample from *Miscanthus*	Ethanol from NA TC	700–1000	1600–2600	300–1100	0.0007–0.02

tion parameters in the media with no additional growth factors (yeast extract) and in the media with their addition shows that the introduction of growth factors increased the ethanol yield by 14–33%; under these conditions, the yield no longer depended on the inoculum dose.

Thus, it can be concluded that the aqueous enzymatic *Miscanthus* hydrolysate medium contains no impurities that are technologically detrimental for the yeast and for the alcohol fermentation; the medium requires no additional treatments for their removal (distillation under vacuum to remove furfural, filtration with charcoal, etc.). However, the medium is not complete for the yeast metabolism; this can be corrected by adding 1% yeast extract. Under the conditions of batch fermentation, it is sufficient to add 10 wt % yeast inoculum in the exponential phase of development.

The experimental ethanol samples were analyzed by gas-liquid chromatography; the averaged results are presented in the table and compared to the regulations on raw ethanol obtained from food raw materials (GOST R 52193-2003, 2003) and industrial alcohol (GOST 17299-78, 1978).

The volume fraction of methanol in the experimental ethanol samples is very small: it is 0.0007–0.02%, which is less than the threshold of 0.13 vol % stated for the raw alcohol from all kinds of food materials (except molasses). These results can be explained by the chemical composition of the starting raw materials, since the *Miscanthus* TC contained no pentosans that can produce methanol during the yeast metabolism.

Mass concentration of fusel oils in the experimental samples is significantly lower than in the raw alcohol from food raw material (1602 mg/dm^3 against 5000 mg/dm^3), which can be explained by the lack of proteins and peptides in the experimental TC hydrolysates.

The aldehyde fraction concentration in the experimental sample is fairly high; trace amounts of acetone and 2-butanone were also detected in the experimental samples, identifying the produced ethanol as nonfood (GOST R 51786-2001, 2001). The concentration of esters in the experimental sample is sufficiently low: it meets the requirements for raw alcohol from molasses. Since the experimental samples were not purified, the low ether concentration may indirectly indicate the purity of the yeast culture during the fermentation and the favorable conditions for the ethanol biosynthesis.

The results of the impurity analysis of the experimental bioethanol samples from *Miscanthus* (see table) give reason to assume that the alcohol of high quality will be obtained after rectification. Thus, enzymatic hydrolysis of the cellulosic raw material produces ethanol with much better characteristics than does the acid hydrolysis.

According to the results of the presented work, we can draw the following conclusions:

(1) The *Saccharomyces cerevisiae* yeast, strain VKPM Y-1693, is resistant to the aqueous enzymatic *Miscanthus* hydrolysate medium; the good morpho-physiological state of the yeast indicates that detri-

mental impurities characteristic of the acidic chemical hydrolysates are absent in the media.

(2) The native active acidity of the hydrolysate (4.5–4.7 units of pH) is optimal for the ethanol biosynthesis on the enzymatic *Miscanthus* TC hydrolysate medium.

(3) The addition of 1% yeast extract allows us to make the aqueous enzymatic *Miscanthus* TC hydrolysate medium complete; at this, under the conditions of batch fermentation, it is sufficient to add 10 wt% yeast inoculum in the exponential phase of growth.

(4) The enzymatic hydrolysis of the *Miscanthus* TC produces bioethanol with low contents of ethers and fusel oils (as determined by GLC). *Miscanthus* bioethanol contains no methanol.

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