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Results of Miscanthus Cellulose Fermentation in the Acetate Buffer and in Water Medium

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Abstract

Enzymatic hydrolysis of technical cellulose of miscanthus in the acetate buffer with enzymatic preparation BrewZyme BGX was investigated. A linear dependence of the final concentration of reducing substances on the initial substrate concentration was established. The enzymatic hydrolysis of cellulose in aqueous medium with sequential addition of enzymatic preparations BrewZyme BGX, Celluxil, CelloLux-A was studied. The results of fermentation of the hydrolysates of Miscanthus technical cellulose obtained in the aqueous medium provide evidence of their sound quality, so these hydrolysates can be used for conversion into ethanol, gel film of bacterial cellulose and other products of microbiological transformation.

Key words: enzymatic hydrolysis, reducing substances, Miscanthus technical cellulose, BrewZyme BGX, fermentation into ethanol, bioconversion into bacterial cellulose

INTRODUCTION

Processing of non-wood lignocellulose raw material into fuel ethanol and other useful products became important direction in modern industrial biotechnology and the subject of numerous studies. For example, a description of enzymatic hydrolysis as an alternative to acid hydrolysis in processing non-wood raw material was presented in [1, 2]; results of the investigation of mechanical activation of cellulose enzymatic hydrolysis were reported in [3]. The authors of [4] studied the preparation of lignocellulose material for enzymatic hydrolysis, while the examples of biotechnological processes of agricultural wastes are reported in [5]. The published data point to the urgency of this problem.

This direction has received thorough development abroad, which is evidenced by the reviews of Russian researchers [6, 7] and the works of foreign scientists dealing with different kinds of non-wood raw material [8–16]. The major part of the works carried out abroad deals with miscanthus processing.

Miscanthus is a typical representative of energy cultures. After planting once, crops may be collected during 30 years in the amount of 30 t/ha, while expenses for growing this culture are less than the cost of energy carriers from traditional sources. Investigations of the enzymatic hydrolysis of the products of preliminary treatment of miscanthus, carried out abroad, showed that it is promising to use this kind of non-wood cellulose-containing raw material for biotechnological and chemical purposes for the sake of sustainable development [17–20].

Miscanthus cropped in 2008 from the plantations of the Institute of Cytology and Genetics, SB RAS (Novosibirsk Region) was used to prepare the substrate. Works on growing, preliminary treatment and fermentation are carried out during the recent five years [21–24].

Investigations of the enzymatic hydrolysis of pure cellulose or the products with cellulose

content more than 90 % have the fundamental nature and are becoming urgent again during the recent years [2, 25].

The goal of the present work is investigation of fermentation of miscanthus cellulose in the acetate buffer and in water.

EXPERIMENTAL

Technical-grade cellulose of *Miscanthus sinensis* obtained at the experimental industrial works through the nitric method was used as the substrate. Nitric pulping of miscanthus was carried out in a reactor 250 L in volume, equipped with a turbine mixer. A 4 % solution of nitric acid was prepared in the reactor, and then the raw material ground preliminarily with a chaff-cutter was added. The mixture was heated to a temperature of 90–95 °C and mixed at this temperature for 20 h. After cooling, the suspension was filtered under vacuum and washed with water two times. This prepared lignocellulose material was again loaded into the reactor containing a 2 % solution of sodium hydroxide, heated to 60 °C and mixed at this temperature for 2 h. Then the suspension of cellulose was filtered under vacuum, washed with a 1 % solution of sodium hydroxide and twice with water. After drying, technical-grade cellulose was obtained with the yield of 38.4 %.

The chemical composition of the substrate (calculated for absolutely dry substance) is represented mainly by the hydrolysable part – α-cellulose (90.3 %) and pentosans (1.5 %), the rest is non-hydrolysable admixtures: residual lignin (3.6 %) and ash (4.2 %). The humidity of the substrate was 2.7 %, polymerization degree 660 units.

The BrewZyme BGX (Poland) enzymatic preparation (EP) was used as the source of cellulase in our work. This is a liquid preparation of cellulase, hemicellulase and xylanase; it catalyses the decomposition of cellulose into glucose, cellobiose and higher molecular reducing substances. BrewZyme BGX preparation is characterized by the following activities: xylanase (6500 + 5 % units XA/cm^3); β-glucanase (1700 + 5 % units β-GlS/cm^3) and cellulase (1500 + 5 % units CMC/cm^3).

Determination of the basic characteristics of substrates (mass concentrations) of α-cellulose, residual (acid-insoluble) lignin, ash and pentosans was carried out according to standard procedures described in [26]. Humidity was determined using the Ohaus MB 23/MB 25 specific humidity meter (USA). Cellulose polymerization degree was measured on the basis of viscosity of solutions in cadoxene using a VPZh-3 viscometer with the capillary 0.92 mm in diameter according to the procedure described in [27].

At the fist stage, the procedure of the investigation of enzymatic hydrolysis involved the following: a weighted portion of the substrate, 150 mL of acetate buffer (pH 4.7) and EP BrewZyme BGX were placed in Erlenmeyer flask 500 mL in volume and kept at a temperature of (50±2) °C for 72 h under permanent mixing [24]. The reaction mixture was stirred on a horizontal platform PE-6410M (Russia) with the oscillation frequency of 150 min^{-1}. The suspension was sampled (2 mL) after each 8 h; the concentrations of reducing substances (RS) calculated for glucose [28] were determined according to the spectrophotometric method with the help of UNICO UV-2804 (USA) using eth reagent based on 3,5-dinitrosalicylic acid (Panreac, Spain). This method is distinguished by the simplicity of analysis and low reagent consumption. Its relative error is 3.45 % [29].

Preliminarily, calibration graphs showing the dependence of optical density on the concentrations of the solutions of glucose, xylose and a mixture of glucose and xylose at the ratio of 1 : 1 were plotted (Fig. 1). One can see that the calibration plots are almost identical

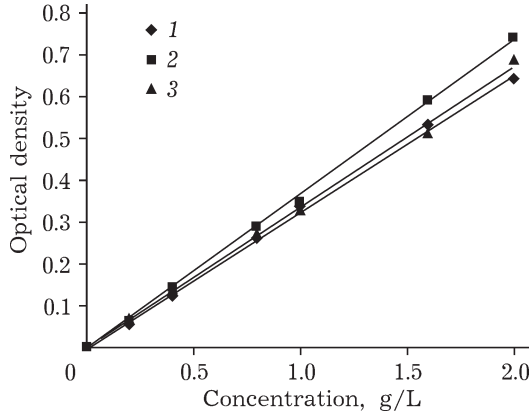

Fig. 1. Dependence of optical density on concentration in solution: *1* – glucose, *2* – xylose, *3* – glucose + xylose.

to each other. Therefore, deviation from the true concentration of RS calculated for glucose will be insignificant in a solution containing also pentoses in addition to hexoses.

At the second stage of the investigation of fermentation, a weighted portion of the substrate was placed in a round-bottom flask 4 L in volume, then distilled water acidified with diluted orthophosphoric acid to pH 4.7 (laboratory ion meter I-160 MI, Russia) and EP BrewZyme BGX were added. Hydrolysis was carried out according to the above-described procedure using a vertical mixing device Velp (Italy) in the continuous mode (round-the-clock) in order to exclude the effect of temperature difference on the action of the EP. The reaction mixture in the flask was heated with the help of a LTHS 4000 flask heater (Czechia). Measurements of pH were performed during the entire process [30, 31].

After the process, the suspension was filtered under vacuum, weighted, then humidity was determined and mass loss was calculated. The degree of polymerization in the precipitate was determined [27]. The precipitate was fermented once more in the humid state by adding sequentially EP: BrewZyme BGX, then Celluxil (Germany) and CelloLux-A (Sibbiofarm Co., Berdsk city, Novosibirsk Region).

The aqueous hydrolysate was fermented with yeast culture *Saccharomyces cerevisiae* (Y-1693 strain) (GosNIIgenetika, Moscow).

RESULTS AND DISCUSSION

Fermentation of pre-treated raw material with substrate concentration up to 30 g/L was described previously in the works dealing with enzymatic hydrolysis of cellulose-containing raw material [21]. It is evident that after fermentation the concentration of RS in hydrolysates, even of pure cellulose, cannot exceed 30 g/L in these cases. In this connection, we studied hydrolysis of technical-grade cellulose with different initial substrate concentrations (g/L): 15, 30, 60, 90, and 120.

Results of the studies of enzymatic hydrolysis are presented in Fig. 2.

One can see that with an increase in the initial concentration of substrate the final concentration of RS in hydrolysates increases. This is also the evidence of the fact that the high concentration of RS – the products of hydrolysis – does not suppress the hydrolytic action of EP BrewZyme BGX.

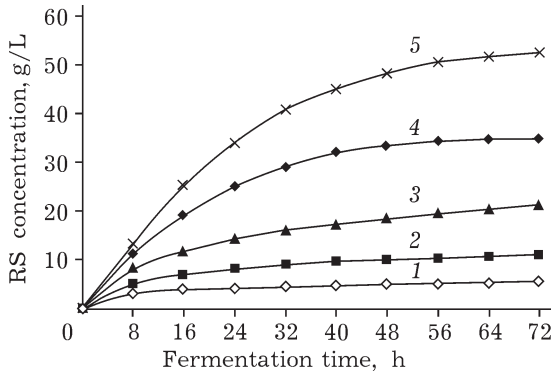

Fig. 2. Dependence of reducing substances (RS) concentration calculated for glucose on fermentation time for experiments with different initial substrate concentrations (g/L): 15 (*1*), 30 (*2*), 60 (*3*), 90 (*4*), 120 (*5*).

In the experiments with low substrate concentrations (15 and 30 g/L) the final concentration of RS after 72 h does not exceed 10 g/L, while for substrate concentration of 60 g/L it reaches 21 g/L. However, for the case of substrate concentration 90 and 120 g/L, the concentrations of RS were 20 g/L as early as after 16 h.

According to the results obtained, it may be assumed that fermentation of technical-grade celluloses with substrate concentrations 90 and 120 g/L provide the formation of hydrolysates with high RS concentrations (35–52 g/L) in aqueous medium. In addition, it is

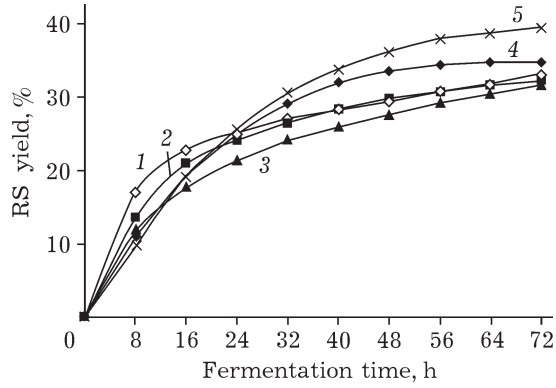

Fig. 3. Dependence of the yield of reducing substances (RS) calculated for glucose on fermentation time for experiments with different initial substrate concentrations (g/L): 15 (*1*), 30 (*2*), 60 (*3*), 90 (*4*), 120 (*5*).

possible to obtain hydrolysates with required RS concentration by choosing the initial substrate concentration.

The dependence of RS yield (with respect to the initial substrate mass) on fermentation time for experiments with different initial substrate concentrations is shown in Fig. 3. The yield of RS (the ratio of RS mass to substrate mass) was calculated taking into account the coefficient connected with the addition of water molecule to anhydrocellulose residues of the corresponding monomer links as a result of enzymatic hydrolysis.

The dependence of the yield of RS on fermentation time for samples with different initial substrate concentrations has different appearances: the first two dependences (curves *1*, *2*) are characterized by high initial hydrolysis rates (2.4 and 1.9 %/h, respectively). In experiments with substrate concentrations 90 and 120 g/L (curves *4*, *5*, respectively) with low initial rates of hydrolysis (1.5 and 1.4 %/h) the maximal yield is achieved after 72 h: 35 and 39 %, respectively. In this situation, only curve *4* reaches a plateau, which is the evidence, that hydrolysis of the available part of substrate is complete. It should be noted that even for the lowest initial concentrations the degree of substrate conversion does not exceed 32–33 %. This phenomenon may be explained either by the low reactivity of the substrate or by the insufficient activity of EP.

It should be noted that the character of the obtained dependence of RS concentration on fermentation time (high initial hydrolysis rate and its substantial decrease with time) correspond to the general notions of fermentation in a reactor of periodic action with mixing [32].

The dependence of RS yield (related to the initial substrate mass) on initial substrate concentration is presented in Fig. 4. One can see that for the concentration range under study (from 15 to 120 g/L) the yield of RS after hydrolysis for 72 h is 32–39 %.

The presence of acetates in hydrolysates after fermentation in acetate buffer does not allow using them in bioconversion processes (ethanol and so on), so we studied enzymatic hydrolysis of cellulose in distilled water. Substrate concentration of 90 g/L was chosen as optimal because it provides high yield of RS

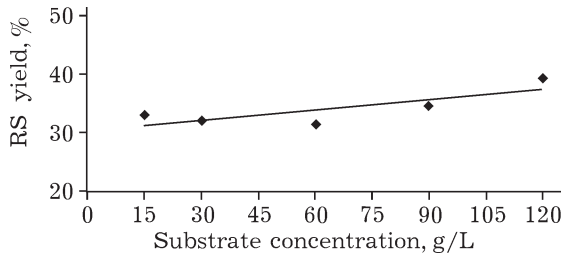

Fig. 4. Dependence of the yield of reducing substances (RS) calculated for glucose after hydrolysis for 72 h on initial glucose concentration.

even after fermentation for a short time and does not require further treatment of hydrolysates for concentrating the RS solution.

Hydrolysis was carried out according to the above-described procedure using a vertical mixing device in the continuous mode (round-the-clock) in order to exclude the effect of temperature difference on the action of the EP.

Results of the investigation of enzymatic hydrolysis in distilled water are presented in Fig. 5.

A comparison of the data shown in Figs. 2 and 5 shows that the dependences of RS concentration on fermentation time in acetate buffer and in aqueous medium for the experiment with substrate concentration 90 g/L are similar exhibiting close initial hydrolysis rates and reaching plateau after fermentation for 56 h.

Mass loss was 31 %; final RS concentration was 30 g/L, which corresponds to the yield of 30 %. The degree of substrate polymerization decreased from 660 to 560 units as a result of enzymatic hydrolysis.

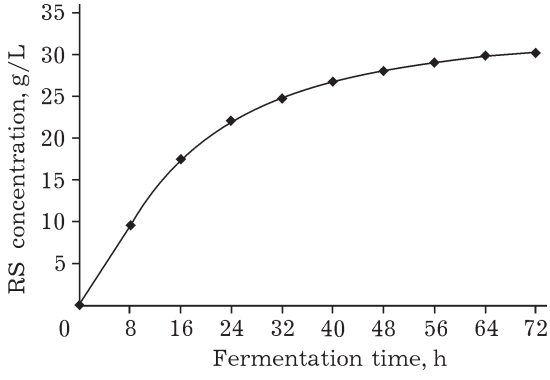

Fig. 5. Dependence of the concentration of reducing substances (RS) calculated for glucose on the time of fermentation of technical-grade cellulose of miscanthus with initial substrate concentration 90 g/L in aqueous medium.

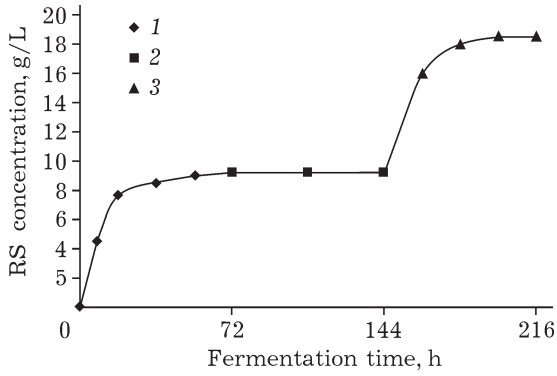

Fig. 6. Dependence of the concentration of reducing substances (RS) calculated for glucose on the time of fermentation for successive addition of EP (initial concentration 44 g/L, aqueous medium): *1* – BrewZyme BGX, *2* – Celluxil, *3* – CelloLux-A.

For all experiments carried out both in the periodic mode and in the continuous mode, fermentation is evidently complete after 72 h. This may be connected with the absence of a part of substrate available for hydrolysis by EP BrewZyme BGX.

To test this assumption, the residue of the substrate obtained in the previous experiment was used in the humid state for repeated fermentation similarly to above-described (EP BrewZyme BGX); initial substrate concentration was equal to 44 g/L. The dependence of RS concentration on fermentation time during 72 h is shown in Fig. 6 (region *1*). The observed increase in RS concentration refutes our assumption that there is no part of substrate accessible for hydrolysis in solution. Hydrolysate with RS concentration 9.25 g/L was obtained, which corresponds to the RS yield of 18 % of the initial substrate mass calculated for absolutely dry raw material.

Then, without hydrolysates separation, cellulolytic EP Celluxil was added; its action did not lead to an increase in RS (fermentation time 72–144 h), which is evidenced by the plateau (see Fig. 6, region *2*). This fact can be explained by the absence of enzymes in EP Celluxil that would be able to hydrolyze the part of substrate after the double action of EP BrewZyme BGX.

Then EP CelloLux-A was added into the reaction mixture. This caused an increase in RS concentration to 18.5 g/L (see Fig. 6, region *3*). The yield of RS increased by 19 %, that is, it reached 38 % as a total after 216 h.

So, after the first 72 h of cellulose fermentation, some part of the substrate available for hydrolysis remains in the system. Only the removal of RS and consequent addition of EP BrewZyme BGX and CelloLux-A allowed us to increase in yield to 38 %, which made 68 % as a total during the whole time of hydrolysis.

It should be stressed that any description of the enzymatic hydrolysis of miscanthus cellulose with sequential addition of EP is absent from the literature. Our results may be compared with the data on fermentation of delignified miscanthus obtained by alkaline delignification with peroxide bleaching of the raw material which was preliminarily ground in a ball mill and separated into fractions [19]. The authors of [19] succeeded in achieving a 90 % conversion of glucose from the hexose component, xylose from pentose component by adding two EP – Celluclast 1.5 L and Novozyme 188 (Sigma, USA) to the substrate. It is necessary to stress that the chemical composition of the substrate included 51 % hexose component, 38 % pentose component, 9 % lignin and 2 % other components. In addition, supplementary grinding of the substrate in a ball mill was accompanied by a decrease in crystallinity degree from 56 to 46 % depending on fraction. The yield of RS in that case [19] was 80 %, calculated for the weighted portion of the substrate, which exceeds the results obtained by us. This fact can be easily explained by the differences in the chemical compositions of the substrates: unlike for [19], the mass fraction of easily hydrolysable component (pentose) in technical-grade cellulose is only 1.5 %. One cannot also neglect the initial concentration of the substrate for fermentation: it was 20 g/L in the case of [19], while it was 15–120 g/L in our work.

We were the first to carry out enzymatic hydrolysis of cellulose obtained by nitric method from miscanthus, through subsequent use of EP BrewZyme BGX, Celluxil and CelloLux-A.

The resulting hydrolysates with RS concentration 30 g/L, calculated for glucose, was fermented by *Saccharomyces cerevisiae* yeast culture (Y-1693 strain). The concentration of alcohol in fermented hydrolysate was 1.3 vol. %, which corresponds to alcohol yield of 75 % of the theoretical value. The used yeast culture is intended for fermenting only the glucose part

of RS, which is 95 % in the hydrolysates obtained [33]. This fact provides the evidence of the high quality of aqueous hydrolysates, and therefore it is promising for the conversion of bacterial cellulose into gel film for medical properties and for obtaining other products of microbiological transformation [34].

CONCLUSIONS

1. Enzymatic hydrolysis of technical-grade cellulose of miscanthus in acetate buffer was investigated; it was established that the final concentration of glucose in hydrolysates increases linearly with an increase in the initial concentration of the substrate. It was revealed that for the initial cellulose concentration of 90–120 g/L, the maximal accumulation of RS in hydrolysates is observed.

2. Fermentation of technical-grade cellulose in distilled water was investigated; hydrolysate with RS concentration 30 g/L was obtained. The results of RS fermentation by *Saccharomyces cerevisiae* yeast (Y-1693 strain) point to the good quality of the hydrolysates.

3. It was established that continuation of miscanthus cellulose after 72 h of hydrolysis is possible in the case if RS are removed from the hydrolysates, and the enzyme is added once more. The joint successive use of BrewZyme BGX and Cellolyux-A preparations allows one to increase the final yield of RS from 30 to 68 %.

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