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BIOCATALYSIS

Preparing Bioethanol from Oat Hulls Pretreated with a Dilute Nitric Acid: Scaling of the Production Process on a Pilot Plant

O. V. Baibakova*, E. A. Skiba, V. V. Budaeva***, and G. V. Sakovich******

Institute of Problems of Chemical and Energy Technologies, Siberian Branch, Russian Academy of Sciences, Biysk, 659322 Russia

**e-mail: olka_baibakova@mail.ru*

***e-mail: ipcet@mail.ru*

****e-mail: budaeva@ipcet.ru*

*****e-mail: admin@ipcet.ru*

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Abstract—The full cycle of bioethanol production from pretreated oat hulls is scaled for a pilot plant. The one-stage pretreatment of oat hulls with a dilute nitric acid at atmospheric pressure is scaled for a 250-L reactor. The total amount of hydrolysable polysaccharides in the resulting substrate is 87.2%. Using the commercially available enzyme preparations CelloLux-A and BrewZyme BGX and the industrial strain BKPM Y-1693 of *Saccharomyces cerevisiae* yeast, the process of enzymatic hydrolysis and alcoholic fermentation is successfully scaled for a 63-L reactor. The scaling factor is 1 : 400. Bioethanol is obtained with a high yield of 17.9 daL/t. After rectification, the test sample of bioethanol meets the standards for high-purity alcohol from food raw materials according to the mass concentration of aldehydes, esters, and by the content of methanol.

Keywords: scaling, oat hulls, pretreatment, nitric acid, enzymatic hydrolysis, alcoholic fermentation, bioethanol

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INTRODUCTION

The use of biotechnological approaches for the production of second-generation bioethanol from cellulose-containing raw materials with zero nutritional value is a most promising line of study in modern science [1–5]. The diversity of such nontimber raw materials as agricultural wastes [6, 7] and energy crops [8, 9] allows us to consider many potential sources of high-energy biomass with different chemical compositions and properties.

Abroad, cellulose-containing raw materials have now displaced traditional food sources (e.g., sugar cane, manioc). Large transnational corporations produce bioethanol from sugar cane bagasse, cellulose wastes from palm oil production, cellulosic components of fruits [10], straw [11], and hulls of cereals [12]. In the Russian Federation, there are no plants for the production of bioethanol from cellulose-containing raw materials. Studies on the technology of bioethanol production and the scaling of this process are therefore extremely relevant.

Key factors that determine the industrial efficiency of bioethanol production are the chemical composition of cellulose-containing raw materials and the means of their pretreatment [13–15]. Preliminary chemical treatment of biomass greatly alters the physicochemical properties of raw materials by reducing the crystallinity of the lignocellulosic matrix and disrupting the structure of lignin [16]. This in turn raises the reactivity of the biomass in enzymatic hydrolysis. Pretreatment is considered effective if it meets the following criteria: it increases the yield of fermentable sugars at the stage of enzymatic hydrolysis, suppresses the formation of by-products that inhibit the growth of microorganisms, and reduces capital and operating costs [17].

The implementation of enzymatic hydrolysis under industrial conditions requires effective and commercially available cellulolytic enzyme preparations of carbohydrases that convert different plant polysaccharides to simple sugars. The main industrial producers of such preparations are microscopic fungi of the genus *Trichoderma*; the great potential of *Penicillium*, *Chrysosporium*, and *Acremonium* fungi has also been demonstrated [18]. The tasks of finding the best composition of an enzyme complex for the conversion of cellulose-containing raw materials to glucose are currently being solved using the ways and means of genetic engineering [19, 20].

When scaling the process according to volume, it is important to optimize the stage of alcoholic fermentation. As an alternative to successive implementation of the stages of enzymatic hydrolysis and alcoholic fermentation, advanced studies [21, 22] now consider a

simultaneous process that leads to cost savings by reducing the duration of technological stages [23].

In this work, oat hulls were used as raw materials. Oat hulls are agricultural wastes, so their prime cost can be considered zero. Oat hulls comprise 28% of the grain's mass and they are accumulated on an industrial scale at grain-processing plants. Oat hulls are a morphologically uniform type of raw material that is naturally calibrated according its size and thickness and requires no grinding during processing, which additionally increases its manufacturability. According to the Russian Federal State Statistics Service, the gross harvest of oats in Russia was on average 4674570 metric tons in 2009–2015, which corresponds to 1308900 metric tons of oat hulls. In addition, this type of raw material is ubiquitous.

Oat hulls were pretreated with dilute nitric acid in one stage. We first proposed the use of nitric acid for the pretreatment of raw materials in [24]; it is now extensively used by our colleagues [25]. Unique results are achieved by combining the chemical effect of three functions: hydrolysis, nitration, and oxidation. The possibility of using nitric acid for the pretreatment of cellulose-containing raw materials in order to biosynthesize ethanol is not obvious, since the products of the oxidative nitration of lignin can act as inhibitors at the stage of enzymatic hydrolysis (for a complex of cellulolytic enzymes) or at the stage of alcoholic fermentation (for a complex of yeast enzymes. The products can inhibit biocatalysis via the Embden–Meyerhof–Parnas pathway or the subsequent decarboxylation of pyruvate with the formation of acetaldehyde and the reduction of acetaldehyde to ethanol) [26]. We have shown that nitric acid not only hydrolyses polysaccharides but also nitrates; it also oxidizes lignin, yielding two types of products: water-soluble lignin, which inhibits enzymatic hydrolysis even when the residual concentration of the inhibitor in the substrate is only 0.019 ± 0.001 wt %, and water-insoluble and acid-insoluble lignin, the presence of which in the solid phase of the substrate in amounts of 12.5 ± 0.1% does not reduce the yield of reducing substances (RSes), recalculated to hydrolysable components [27].

Studies on the enzymatic hydrolysis of pretreated oat hulls in an acetate buffer [26] and an aqueous medium and studies on the microbiological synthesis of bioethanol under laboratory conditions with successive [28] or simultaneous [29] steps of enzymatic hydrolysis and alcohol fermentation give grounds for optimism in the industrialization of the developed technology.

The aim of this work was to scale the cycle of the preparation of bioethanol from pretreated oat hulls in reactors at a pilot plant using industrially available biocatalysts.

EXPERIMENTAL

Our experiment on scaling the process of bioethanol preparation from pretreated oat hulls was conducted in duplicate. The averaged data are provided in this study.

Oat hulls for our study were obtained under industrial conditions at the Biysk Elevator Plant. The substrate for enzymatic hydrolysis was obtained in a 250-L reactor at the pilot plant of the Institute of Problems of Chemical and Energy Technologies (Siberian Branch, Russian Academy of Science) by treating oat hulls with 4% nitric acid for 4 hours at a water : acid ratio of 1 : 20 and temperatures of 94–96°C at atmospheric pressure.

The treated oat hulls were drained from the acid solution on a filter press to separate the acid liquor from the solid residue. The drained solid residue (pretreated oat hulls) was washed with water to neutral reaction. To accumulate the substrate for the subsequent synthesis of bioethanol, two series of boiling wereperformed, after which the obtained substrates were combined. The main characteristics of the substrates (the mass fractions of cellulose (by Kushner), pentosans, acid-insoluble lignin, and ash) were determined according to standard methods [30].

Technological stages of enzymatic hydrolysis and alcoholic fermentation were carried out simultaneously. Enzymatic hydrolysis of the obtained substrate was performed in a 63-L reactor at the pilot plant of the Institute of Problems of Chemical and Energy Technologies (Siberian Branch, Russian Academy of Science). The substrate concentration was 60 g/L (on an absolutely dry weight basis). Commercially available enzyme preparations (EP) were used in the following doses: 0.04 kg of EP per kg substrate (EP CelloLux-A) and 0.02 L of EP per kg substrate (EP BrewZyme BGX). EPs CelloLux-A (manufactured by OOO Sibbiofarm, Berdsk) and BrewZyme BGX (supplied by Rusferment, Moscow) were standardized according to the cellulase and xylanase activities in their analytical passports (Table 1). The ratios and doses of the preparations were substantiated in [31]. Enzymatic hydrolysis was conducted at 46 ± 2°C for 24 hours. The medium was then cooled to 28°C and supplied with seed yeasts. Alcoholic fermentation and enzymatic hydrolysis were then performed simultaneously for 48 hours.

Alcoholic fermentation was conducted using the *Saccharomyces cerevisiae* Y-1693 strain (Russian National Collection of Industrial Microorganisms, Moscow). The inoculum dose was of 12%. Fermentation occurred under anaerobic conditions at 28°C. The total amount of yeasts was determined using a Goryaev chamber. The inoculum was prepared as follows: a pure culture was transferred to a medium of unhopped malt wort in amounts of 5% of the total volume of the medium. Cultivation lasted for 24 h at a temperature of 28°C. Yeasts in amounts of 5% were transferred to the medium, which was comprosed of

Table 1. Activities of the individual enzyme preparations

Preparation	Enzymatic activity
CelloLux-A (cellulase-standardized preparation), the preparation of fungal origin	Cellulase: 2000 ± 10% carboxymethylcellulase activity units per cm^3; Xylanase: 8000 ± 10% xylanase activity units per cm^3; β-Glucanase: 1500 ± 10% β-glucanase activity units per cm^3
BrewZyme BGX (hemicellulase-standardized preparation), the producer of *Trichoderma longibrachiatum*	Cellulase: 2100 ± 5% carboxymethylcellulase activity units per cm^3; Xylanase: 6500 ± 5% xylanase activity units per cm^3; β-Glucanase: 1700 ± 5% β-glucanase activity units per cm^3

Table 2. Chemical characteristics of the raw materials and the substrate

Raw materials	Wt %, on oven-dry basis	
	oat hulls	pretreated oat hulls
Cellulose (by Kushner)	44.7 ± 0.2	79.8 ± 0.3
Pentosans	30.8 ± 0.2	7.4 ± 0.1
Acid-insoluble lignin	18.1 ± 0.1	7.7 ± 0.1
Ashes	4.6 ± 0.1	4.8 ± 0.1

enzyme hydrolysate and unhopped malt wort (ratio, 1 : 1). Cultivation lasted 24 h at a temperature of 28°C. The obtained yeasts in a doses of 12% were added as inoculum to the enzyme hydrolysate during pilot-plant production. The total number of cells was 114.5 ± 10 million CFU/mL, 20.9% of which were budding.

At the stages of enzymatic hydrolysis and alcoholic fermentation, the level of active acidity was maintained manually in a range of 4.9 ± 0.1 pH units by adding solutions of orthophosphoric acid and ammonium hydroxide. Deviation of the acidity from the given range negatively affected the functioning of the enzymatic complex and the zymase enzymes in yeasts.

The concentration of RSes, recalculated to glucose in the hydrolysate, was determined spectrophotometrically on a UNICO UV-2804 (United States) using a reagent based on 3,5-dinitrosalicylic acid (Panreac, Spain). The relative error of the method was 3.45%. The yield of RSes was calculated using a coefficient of 0.9. This coefficient was associated with the addition of water molecules to anhydroglucose residues of the respective monomeric units as a result of enzymatic hydrolysis.

The volume fraction of alcohol in the mashes was determined using a hydrometer in a distillate obtained by distilling alcohol from the mash according to GOST R (Russian State Standard) 51135–2003 [32]. At the end of fermentation, ethanol was isolated from the mash via simple distillation and then subjected to fractional separation on a GS-2 mash column (produced in Shchelkovo). Ethanol was analysed via gas–liquid chromatography (GLC) on a gas chromatograph equipped with a Crystall-2000M flame-ionization detector (Chromatech, Yoshkar-Ola, Russia) according to GOST R 51786-2001 [33]. The experimental conditions were: a ZB-FFAP gas-chromatographic capillary column (United States) 50 m × 0.32 mm × 0.52 μm; a detector temperature of 220°C; and an evaporator temperature of 190°C. Each sample was incubated at 77°C for 6 min 30 s, then heated to a temperature of 77°C at a rate of 10°C/min and incubated for 15 min. The flow division ratio was 40 : 1. The carrier gas was compressed nitrogen. The pressure of the carrier gas (nitrogen) was 77 kPa. The air-to-hydrogen ratio was 250 : 25. The calibration curve was plotted using calibration mixtures (State Standard samples). The gas flow rate (discharge) was 30 mL/min, the gas flow rate (FID blowing) was 30 mL/min, the gas flow rate (FID oxygen) was 20 mL/min, and the gas flow rate (FID air) was 200 mL/min. The sample volume was 1 μL.

RESULTS AND DISCUSSION

Table 2 presents the characteristics of oat hulls before and after pretreatment.

Preliminary chemical treatment of oat hulls with a nitric acid solution reduced the content of pentosans by a factor of 4.2 and the content of acid-insoluble lignin by a factor of 2.4; the mass fraction of cellulose grew 1.8 times, while the ash content remained the same. For the pretreated oat hulls, the total fraction of hydrolysable polysaccharides was 87.2%. This value was comparable to the results obtained in [24, 28] and allows us to assume high reactivity of the substrate in enzymatic hydrolysis and the successful microbiological synthesis of bioethanol.

Figure 1 shows micrographs of oat hulls before and after treatment with a dilute nitric acid solution. Before treatment, the surfaces of oat hulls were

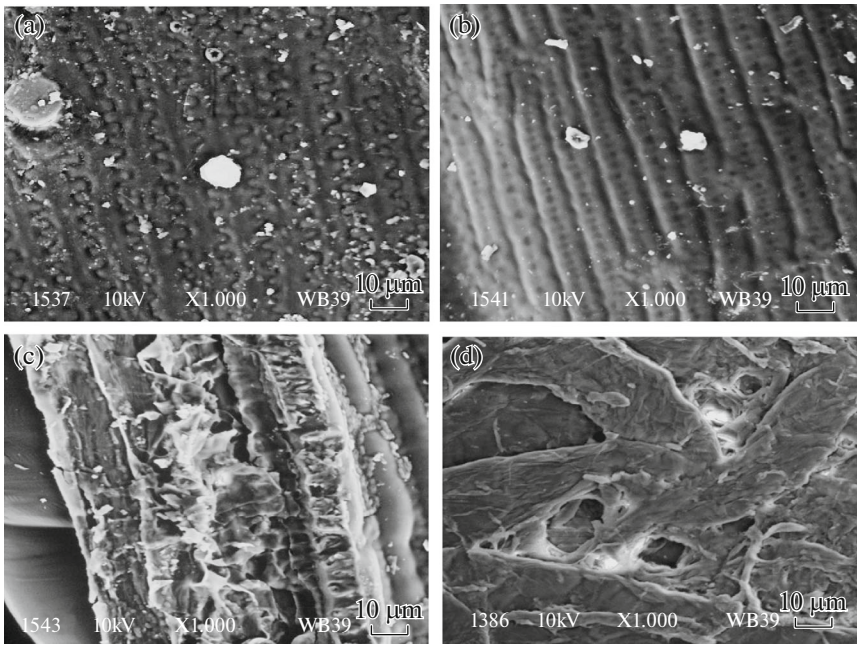

Fig. 1. Micrographs of oat hull fibers before pretreatment of (a) their outer surfaces, (b) their inner surfaces, and (c) fractures; and (d) after pretreatment. SEM, 1000× magnification.

smooth and their fibers were arranged in rows. The rows on the outer side of oat hulls visually resemble zippers (see Fig. 1a), while those on the inner side resemble stitches of a knitted fabric (see Fig. 1b). Layers of hulls, which perform protective functions in the plant, have visible fractures (see Figure 1c). After treatment, the surface became more developed and nonhomogeneous, and the inner layers break down into ribbon fibers (see Fig. 1d).

Figure 2 shows the dependences of the concentration of RSes and the strength of the mash on the duration of enzymatic hydrolysis and alcohol fermentation in the reactor. For the first 24 hours, enzymatic hydrolysis proceeded at 46 ± 2°C, and the concentration of RSes grew exponentially: after 24 hours, it reached 44 ± 0.2 g/L. This value corresponds to a yield of 66.0 ± 0.3% of the substrate's concentration. Compared to the process conducted in Erlenmeyer flasks in an aqueous medium, the yield of RSes fell by a factor of 1.1 (66.0 ± 0.3 versus 73.3 ± 0.3%) [28]. Since the scaling factor was 1 : 400, this drop was negligible.

Figure 3 shows micrographs of the substrate at the initial moment and 24, 48, and 64 hours after the start of the process.

At the initial moment, the fibers of the substrate are smooth-edged ribbons that are close to one another (see Figure 3a). At 24 hours after the start of the process, the fibers have decomposed into individual bands and partially destruct with the formation of smaller particles with notched edges (see Figure 3b). After 48 hours, further decomposition of individual fibers into separate fragments with different sizes and shapes (mainly into particles with notched edges) is observed (Figure 3c). At 64 h after the start of the process, the particles of the substrate are almost completely destroyed under the action of the enzyme complex, so only small particles with notched edges remain (see Fig. 3d).

The yeast inoculum was introduced 24 hours after the start of enzymatic hydrolysis (the reaction mass was cooled to 28°C for 3.5 hours). Analyzing the morphophysiological state of yeast cells during fermentation showed that the enzymatic hydrolysate of the pretreated oat hulls was not an adequate nutrient medium for their development. Oval medium-sized yeast cells were visible during a microbiological analysis. The yeasts mostly formed clusters of 10–30 cells, indicating that autolysis had begun (see Figs. 3c,d). In addition, some cells were adsorbed on the surface of the substrate (see Figure 3c).

At 48 hours after the start of the reaction, the percentage of budding cells was 4.9 ± 0.1%; after 64 h, it was 6.5 ± 0.1%. This does not meet the standards accepted in industry (for mature industrial yeasts, the percentage of budding cells in a cereal–potato medium must reach 10–15% [34]). The total number of cells fell from 114.5 ± 10 to 10.7 ± 1 and 10.9 ± 1 million CFU/mL after 48 and 64 hours, respectively, which also does not meet industrial standards (the number of yeast cells must be no lower than 80–100 million CFU/mL [34]). Hydrolysate is a poor medium, and correcting its composition with nitrogen and phosphorus will improve the functional state of yeast and increase the yield of bioethanol. Foreign

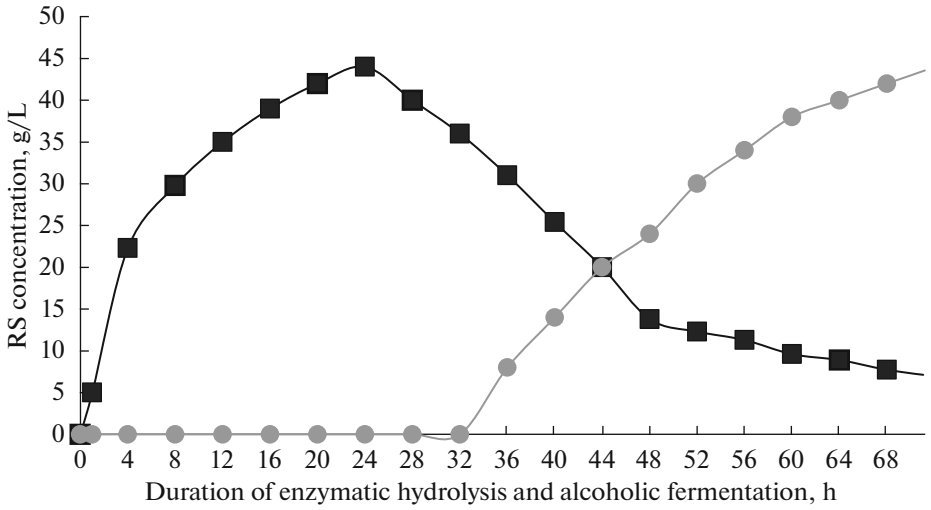

Fig. 2. Dependences of (■) the RS concentrations and (●) strength of the mash on the duration of the process.

microorganisms (micrococci) were found in the medium under plant conditions, so Cefotaxime antibiotic was added.

The accumulation of bioethanol is associated with the substrate consumption: 36 hours after the start of the process, the strength of the mash was 0.4–0.1 vol %; it then grew exponentially and reached 2.3 ± 0.1 vol % by the end of fermentation (see Fig. 2). The resulting strength of the mash differed from that of mashes obtained via alcoholic fermentation of chemical hydrolysates of wood (2.3 ± 0.1 versus 1.0–1.5 vol %) [35]. When enzymatic hydrolysis and alcoholic fermentation proceed simultaneously, it is impossible to calculate the yield of RSes from the mass of the substrate, or to calculate the yield of bioethanol from the mass of RSes, since they begin to leave the system before their concentration reaches its maximum. At 100% conversion of 60 g/L of pretreated oat hulls to RSes, it is theoretically possible to obtain 66.7 g/L of RSes, which (according to the stoichiometric fermentation equation) would yield 4.32 vol % ethanol. Under production conditions, the bioethanol yield reached 53.5 ± 0.3% of the concentration of the substrate (or 17.9 dal/ton of the mass of the raw material), which is slightly lower than the results obtained under laboratory conditions in [29]. The yield of bioethanol under production conditions is comparable to the yield of technical alcohol from chemical hydrolysates of wood, which is 17.0–18.0 daL/ton [35]. Given the large mass fraction of cellulose in wood (which ranges from 42 to 52%), we may state the obvious advantages of producing bioethanol from oat hulls by the enzymatic method.

Table 3 shows the content of impurities in the test samples of bioethanol before and after rectification.

It is obvious that before rectification, the content of methanol in the test sample of bioethanol was very low, which is unusual for technical alcohol [36]. This was because there were no pectic substances (precursors for the synthesis of methanol) in the raw material, and because of the mild conditions of obtaining the enzymatic hydrolyzate.

Mass concentrations of aldehydes and esters in bioethanol prior to rectification are determined by the nature of the raw materials. Rectification allows us to remove these impurities. After rectification, the concentration of aldehydes is 50 times lower and the concentration of ether is 80 times lower than the respective values (200 mg/dm^3 and 80 mg/dm^3) established

Table 3. Content of impurities in test samples of bioethanol

Indicator	Before rectification	After rectification
Mass concentration of aldehydes, recalculated to anhydrous alcohol, mg/dm^3	3800 ± 100	4.0 ± 0.3
Mass concentration of esters, recalculated to anhydrous alcohol, mg/dm^3	90 ± 3	1.0 ± 0.5
Mass concentration of fusel oil, recalculated to anhydrous alcohol, mg/dm^3	4300 ± 100	3300 ± 100
Methanol content, recalculated to anhydrous alcohol, vol %	0.005 ± 0.001	0.011 ± 0.001
Volume fraction of bioethanol, vol %	n/a	91.6 ± 5

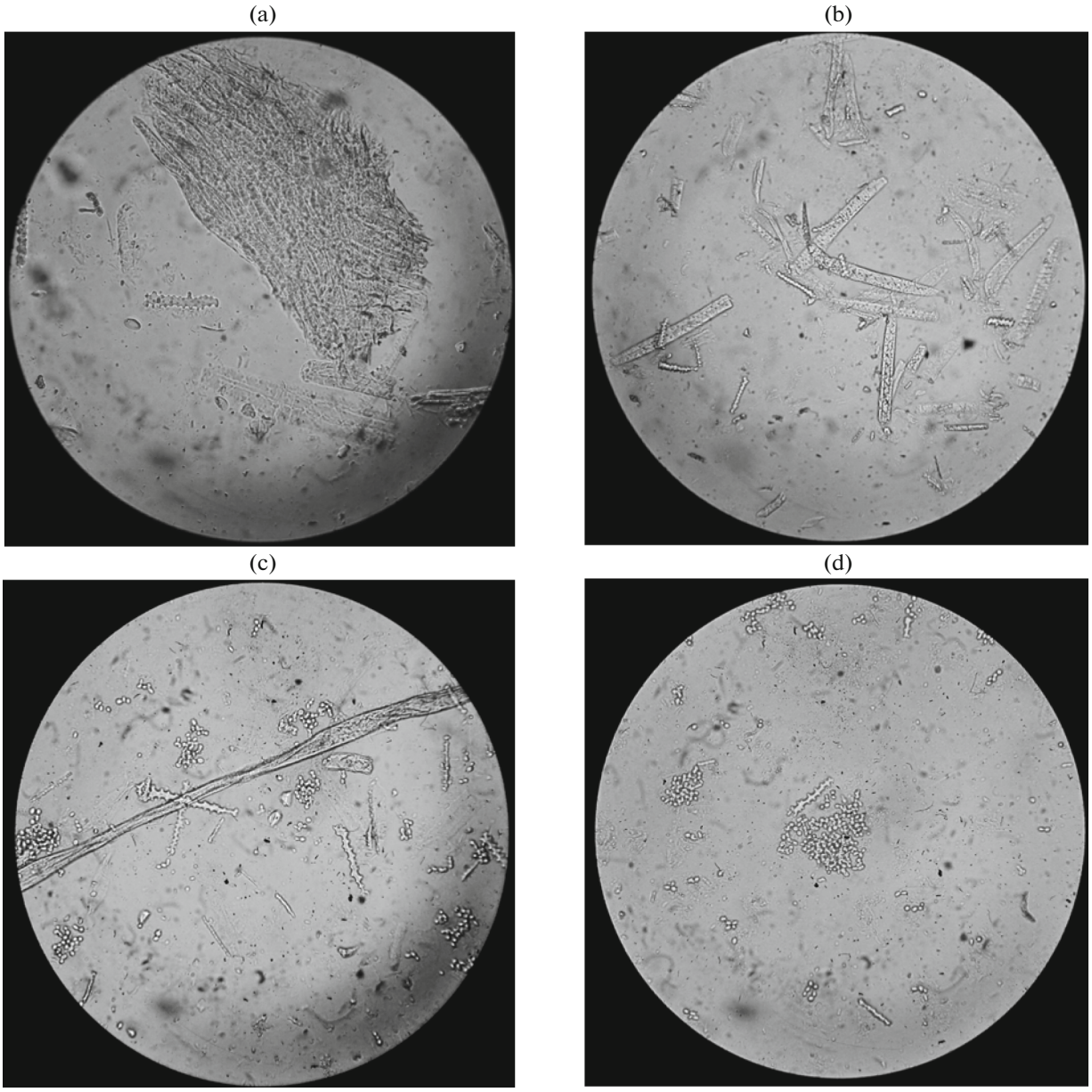

Fig. 3. Micrographs of pretreated oat hull during enzymatic hydrolysis and alcoholic fermentation (a) at the initial moment, (b) after 24 hours, (c) after 48 h, and (d) after 64 h of the process. Optical microscopy, 400× magnification.

for alcohol of technical grade A. This testifies to the high quality of the produced technical alcohol. Purification of the test samples of bioethanol on a distillation column helps to remove fusel oils, the content of which exceeds the respective parameter for alcohol of technical grade A by a factor of 6.6 (500 mg/dm^3) [36]. By comparing the quality indicators obtained for bioethanol after its rectification with the indicators established for rectified ethanol from food raw materials [37], we may state that in terms of the mass concentration of aldehydes and ethers and the content of methanol, the test samples met the standards for high-purity alcohol, for which the respective indicators are 4 mg/dm^3, 13 mg/dm^3, and 0.03 vol %.

CONCLUSIONS

The preparation of substrates from oat hulls via one-stage treatment with dilute nitric acid at atmospheric pressure was scaled for a 250-L reactor. The total fraction of hydrolysable polysaccharides in the obtained substrate was 87.2 ± 0.3%.

Enzymatic hydrolysis of pretreated oat hulls performed using the commercially available enzyme preparations CelloLux-A and BrewZyme BGX in an aqueous medium was scaled for a 63-L reactor. A 110% drop in the yield of reducing substances was noted for a scaling factor of 1 : 400.

The bioethanol production process was scaled in a 63-L reactor. Ethanol was obtained with a high yield of 17.9 daL/ton.

It was established that after rectification, the test sample of bioethanol met the standards for high-purity alcohol from food raw materials according to the mass concentration of aldehydes and esters, and the content of methanol.

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