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# Selection of the Optimal Enzyme Composition for Sugar Beet Pulp Conversion

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**Abstract**—It was shown that the presence of cellobiohydrolase, β-glucosidase, endoglucanase, arabinoxylan-arabinofuranhydrolase, pectin lyase, and polygalacturonase is necessary for efficient hydrolysis of sugar beet pulp (SBP). Optimal multienzyme complexes consisting of the same enzymes and additional endoarabinase or exoarabinase, endogalactanase, β-xylosidase, endoxylanase, and/or α-arabinofuranosidase were determined. These components enabled the conversion of SBP based on a yield of total reducing sugars (RSs) of 61–68%, an arabinose yield of 94%, and a glucose yield of 63–79%. The optimal complex from dry multienzyme preparations (EP) of cellulases, hemicellulases, and pectinases, which are produced by the fungal strains *Penicillium canescens*, *P. verruculosum,* and *Aspergillus foetidus*, enabled SBP hydrolysis based on arabinose and glucose yields close to 100% at an initial SBP concentration of 100–250 g/L and an EP concentration of 5–10 mg protein/g SBP after 24–48 h of hydrolysis.

*Keywords:* sugar beet pulp, arabinose, glucose, ethanol, cellulases, arabinases

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## INTRODUCTION

Sugar beet pulp (SBP) is a by-product of sugar beet processing. It is usually used in livestock farming as a valuable and cheap food. After sucrose extraction, 18–23% of dry substances remain in the SBP [1], ~80% of which are polysaccharides, including 22–24% (of the dry matter) cellulose, 24–32% hemicellulose (mainly arabinan), 15–32% pectin [2, 3]. Protein (8–11%), fats (1–2%), and lignin (3–6%) are present in small amounts. Analysis of the SBP composition [4] indicates a high content of arabinose and glucose (each up to 21% of the dry matter), which can be converted into alcohols, amino and organic acids, and other products of microbiological synthesis [5, 6].

SBP polysaccharides were hydrolyzed earlier for the production of pectin [7, 8], arabinan [9], oligosaccharides [3], and monosaccharides [4–6, 10–12]. In the study [10], the process of SBP hydrolysis by commercial enzyme preparations (EPs) of cellulases and pectinases was studied. It was shown that pectinases, which are necessary for the removal of pectin from the surface of cellulose fibers, play an important role in SBP hydrolysis. Commercial EPs of pectinases or a mixture of pectinases with cellulases were used in studies to convert SBP [3, 4, 6]. SBP treatment at a substrate concentration of 6–10% of the dry matter was carried out for several days.

The goal of this study was the selection of the enzyme composition for the implementation of deep SBP conversion with the maximum sugar yield (arabinose, glucose) and the lowest EP cost at the maximum SBP concentration.

## MATERIALS AND METHODS

**Strains and enzymes.** The EPs used in the study were lyophilized culture fluids (CFs) of fungal strains *P. canescens* with cloned homologous pectin lyase (strain PCA-PL), arabinoxylan-arabinofuran hydrolase (PCA-AXH), exoarabinase (PCA-exoA), heterologous endoarabinase (PCA-endoA), fungal strains *P. verruculosum* B1-Cell and F-10 (producers of cellulases and heterologous β-glucosidase, respectively), as well as the fungal strain *A. foetidus* 70a, a producer of pectinases and hemicellulases.

Homogeneous enzymes were isolated from the listed EPs according to the methods described in studies [11, 13–19].

**Reagents.** For the production of buffer mixtures, reagents from Bio-Rad (the United States), Panreac (Germany), Helicon, and Reahim (Russia) were used. Xylose, arabinose, fructose, glucose, galactose, sucrose, and cellobiose (Megazyme, Australia) were

used as standards in a chromatographic study of the hydrolysis products.

Commercial SBP (dry matter content of 96%) was crushed into 0.5- to 1.0-mm particles with a MF10 basic mill (IKA Werke, Germany).

The substrates for the determination of specific activities were linear arabinan and arabinan branched from the sugar beet, arabinoxylan from wheat, and galactan from potatoes (Megazyme, Australia); K-salt from polygalacturonic acid (PGA), Na salt of carboxymethylcellulose (CMC), beech xylan, citrus pectin with an esterification degree of ~70%, *p*-nitrophenyl-α-arabinofuranoside (*p*NPAF), *p*-nitrophenyl-β-glucopyranoside (*p*NPGL), *p*-nitrophenyl-β-xylopyranoside (*p*NPX) (Sigma, United States); and hemoglobin and microcrystalline cellulose (MCC) (Vitek, Russia).

**Determination of enzyme activity.** The unit of the enzyme activity was defined as the amount of the enzyme that catalyzes the formation of 1 μmol of product per 1 min.

The activity with respect to polysaccharide substrates (concentration of 5 g/L in the reaction mixture) was determined based on the initial rates of the formation of reducing sugars (RSs) at a pH of 5.0 and 50°C by the Somogyi–Nelson method [20].

The activity with respect o *p*-nitrophenyl derivatives of sugars (0.9 mM in the reaction mixture) was determined based on the rate of the formation of *p*-nitrophenol at a pH of 5.0 and 40°C [20].

The total proteolytic activity of the EP was determined on hemoglobin (1% solution in the reaction mixture) at a pH of 4.7 and 30°C by the Anson method [21].

The lyase activity was determined by the change in optical density at 232 nm, which corresponds to the accumulation of a 4.5-unsaturated product of pectin transelimination [22].

The glucose concentration in the SBP hydrolysates was determined with a Photoglucose kit (Impact, Russia) according to the instructions.

The protein content in the EP was determined by the Lowry method with BSA as the standard. The concentration of homogeneous enzymes was assessed based on the optical density at 280 nm, with extinction coefficients calculated based on the amino acid sequence.

Electrophoresis in 12% polyacrylamide gel with Na-DDS was performed with MiniProtein equipment (Bio-Rad, the United States) according to the manual. The content of individual proteins in EP was estimated by densitometry.

**Enzymatic SBP hydrolysis.** SBP hydrolysis was performed under the action of homogeneous enzymes or their mixtures. A total protein dosage of 2 mg/g of substrate or 0.2 mg/mL of the reaction mixture was maintained (the weight proportions of individual enzymes in their mixtures were equal unless otherwise is specified) in tubes of 2 mL (reaction volume was 1.5 mL) in a temperature-controlled shaker. SBP EP hydrolysis was performed at an EP concentration of 10 mg of protein/g of substrate or 1 mg/mL of the reaction mixture in plastic cells with a volume of 60 mL (the reaction mixture volume was 20 mL) in a thermostatically controlled shaker. In all cases, the hydrolysis process was carried out in the presence of 0.1 g/L of the antibiotic Ampioxum and 0.001 M sodium azide at a pH of 5.0 and 40°C.

During the process, aliquots were taken and the concentrations of RS (by Somogyi–Nelson method), glucose (using the "Photoglucose" kit), and protein (by the Lowry method) were determined. The composition of low molecular weight products was determined via ion-exchange chromatography on an Agilent 1100 Series HPLC system (Agilent, United States) on a Diaspher-110-Amin column 5 μm 4.0 × 250 mm (the eluent was acetonitrile–water at 77 : 23, the flow rate was 1 mL/min, and the sample volume was 10–100 μL). Acetonitrile (0.8 mL) was added to 0.2 mL of hydrolysate for sample preparation, followed by centrifugation for 5 min at 9000 *g*.

The efficiency of SBP hydrolysis was calculated based on the contents of polysaccharides, arabinose, and glucose [2–4].

## RESULTS AND DISCUSSION

**SBP hydrolysis by homogeneous enzymes.** Twelve purified enzymes (homogeneous according to electrophoresis in PAGE with Na-DDS) were isolated from an EP of fungal strains *P. verruculosum, P. canescens,* and *A. foetidus,* and their specific activities were determined (Table 1).

The enzymes of the cellulase complex, cellobiohydrolase 1 (CBH1), endoglucanase 2 (EG2), and β-glucosidase (βG), had high activities with respect to MCC, CMC, and *p*NPGL, respectively. Pectinases—pectin lyase (PL) and polygalacturonase (PG)—were active against pectin and PGA, respectively. Endoarabinase (endoA) and exoarabinase (exoA) were highly active on linear arabinan, and arabinoxylan-arabinofuranhydrolase (AXH) was highly active on branched arabinan (a substrate that is not specific for this enzyme) and arabinoxylan. Endoxylanase (EX) and endogalactanase (EGal) were highly active on xylan and galactan, respectively. β-xylosidase (βX) and α-arabinofuranosidase (AF) were highly active on *p*NPX and *p*NPAF, respectively.

SBP hydrolysis with individual homogeneous enzymes (the exception was CBH1, which was mixed with an excess of βG in a ratio of 9 : 1), as expected, led to negligible sugar formation (Table 2). The highest RS concentration (11–14 g/L) after 48 h of hydrolysis with an initial SBP concentration of 100 g/L was observed with the introduction of PL, AXH, and AF; the action of a mixture of CBH1 with βG and individ-

**Table 1.** Homogeneous enzymes and their specific activities on specific substrates

| Enzyme | Molecular weight, kDa | Source (strain) | Activity (substrate), U/mg protein |
|---|---|---|---|
| CBH1 | 66 | *P. verruculosum*, B1-Cell | 0.19 ± 0.02 (MCC) |
| EG2 | 39 | *P. verruculosum*, B1-Cell | 19.81 ± 0.97 (CMC) |
| βG | 116 | *A. niger*, F-10 | 94.54 ± 4.73 (*p*NPGL) |
| PL | 38 | *P. canescens*, PCA-PL | 19.01 ± 0.95 (pectin) |
| PG | 38 | *A. foetidus*, Af-70a | 360.30 ± 18.02 (PGA) |
| EndoA | 40 | *A. foetidus*, PCA-endoA | 52.40 ± 2.62 (AL*) |
| ExoA | 47 | *A. niger*, PCA-exoA | 113.03 ± 5.65 (AL*) |
| AXH | 70 | *P. canescens*, PCA-AXH | 81.19 ± 4.06/27.98 ± 1.40 (AX**/AB***) |
| EX | 31 | *P. canescens*, PCA-exoA | 119.26 ± 5.96 (xylan) |
| EGal | 42–44 | *A. foetidus*, Af-70a | 497.19 ± 9.86 (galactan) |
| βX | 110 | *A. foetidus*, Af-70a | 34.85 ± 1.74 (*p*NPX) |
| AF | 65 | *A. foetidus*, Af-70a | 18.06 ± 0.90 (*p*NPAF) |

* Linear arabinan; ** arabinoxylan; *** branched arabinan.

**Table 2.** Content of RSs and monosaccharides (g/L) in the reaction mixture after 48 h of SBP hydrolysis (100 g/L) with homogeneous enzymes (2 mg/g SBP)

| Enzyme | RS | Arabinose | Fructose | Glucose | Xylobiose | Oligosaccharide | Cellobiose |
|---|---|---|---|---|---|---|---|
| CBH1 + βG | 7.52 ± 0.77 | 0 | 0.73 ± 0.07 | 2.42 ± 0.21 | 0 | 0 | 0 |
| EG2 | 2.39 ± 0.25 | 0 | 0.32 ± 0.03 | 0.44 ± 0.04 | 0 | 0 | 0.13 ± 0.01 |
| PL | 14.09 ± 1.14 | 4.20 ± 0.41 | 0.39 ± 0.03 | 0.26 ± 0.02 | 0 | 0 | 0 |
| AXH | 11.70 ± 0.72 | 1.99 ± 0.19 | 0.99 ± 0.09 | 1.44 ± 0.14 | 0 | 0 | 0 |
| EX | 6.94 ± 0.50 | 2.07 ± 0.24 | 0.45 ± 0.04 | 0.39 ± 0.03 | 0.23 ± 0.02 | 0 | 0 |
| ExoA | 8.94 ± 0.62 | 2.93 ± 0.35 | 0.75 ± 0.08 | 0.74 ± 0.07 | 0 | 0 | 0 |
| EndoA | 7.99 ± 0.37 | 1.30 ± 0.12 | 0.68 ± 0.06 | 1.04 ± 0.09 | 0 | 0 | 0 |
| EGal | 9.75 ± 0.94 | 0.59 ± 0.05 | 0.57 ± 0.05 | 0.60 ± 0.06 | 0 | 6.27 ± 0.61 | 0 |
| βX | 1.73 ± 0.16 | 0.24 ± 0.02 | 0.20 ± 0.02 | 0.19 ± 0.02 | 0 | 0 | 0 |
| PG | 6.14 ± 0.30 | 0 | 0.80 ± 0.07 | 1.09 ± 0.10 | 0 | 0 | 0 |
| AF | 10.48 ± 0.75 | 1.90 ± 0.18 | 1.00 ± 0.09 | 1.59 ± 0.14 | 0 | 0 | 0 |

ual EX, endoA, exoA, EGal and PG resulted in the formation 6–10 g/L RS; individual EG2 and βX produced ~2 g/L RS.

With SBP incubation under the conditions of the hydrolysis reaction without enzymatic treatment, a small amount of fructose and glucose (0.2 g/L) was detected. The effect of EG on SBP led to the accumulation of a small amount of cellobiose (0.13 g/L). The action of the mixture of CBH1 with βG resulted in 2.42 g/L glucose; EX produced 2.07 g/L arabinose and 0.23 g/L xylobiose; βX, AXH, AF, endoA, and exoA produced arabinose as the main product (0.24–2.93 g/L); EGal resulted in an oligomeric product with a degree of polymerization (DP) of 2 (6.27 g/L); and PL resulted in 4.20 g/L arabinose.

SBP was later hydrolyzed with mixtures of different compositions (Table 3). A mixture of all enzymes (no. 1) provided an RS concentration of 33.6 g/L, which corresponded to a 42% SBP polysaccharide conversion. The arabinose yield was 12.6 g/L (60% of the maximum possible), and the glucose yield 9.1 g/L (43% of the maximum possible).

To identify key enzymes for SBP conversion and optimization of the enzyme-mixture composition, enzymes were excluded one at a time from mixture no. 1, which in some cases led to results comparable to those for mixture no. 1 based on the RS yield (data not shown). The exceptions were enzyme mixtures without CBH1 and βG, mixtures without AXH, and those without PL, the exclusion of which led to RS yields of 27.4, 30.5, and 28.8 g/L, respectively. Thus, the CBH1 enzymes with βG, AXH, and PL were necessary for effective SBP hydrolysis. However, when a mixture of only these enzymes (no. 2) was used, 31.3 g/L of RS

**Table 3.** Content of RSs and monosaccharides in the reaction mixture after 48 h of hydrolysis of SBP (100 g/L) with mixtures of homogeneous enzymes (2 mg/g SBP)

| No. of the mixture: composition | RS, g/L | Arabinose, g/L | Galactose, g/L | Glucose, g/L |
|---|---|---|---|---|
| 1: All enzymes | 33.6 ± 1.7 | 12.6 ± 0.6 | 0.8 ± 0.1 | 9.1 ± 0.4 |
| 2: CBH1 with βG, AXH, PL | 31.3 ± 1.1 | 11.5 ± 0.6 | 0.7 ± 0.1 | 7.8 ± 0.3 |
| 3: CBH1 with βG, AXH, PL, EG2, PG | 35.7 ± 1.9 | 14.5 ± 0.7 | 0.9 ± 0.1 | 10.9 ± 0.6 |
| 4: CBH1 with βG, AXH, PL, EG2, PG, PF, exoA | 43.1 ± 2.5 (49.1 ± 2.9)* | 19.6 ± 1.0* | 1.3 ± 0.2* | 15.8 ± 0.8* |
| 5: CBH1 with βG, AXH, PL, EG2, PG, AF, exoA, EGal | 41.2 ± 2.8 (53.5 ± 3.5)* | 19.3 ± 0.9* | 2.1 ± 0.3* | 16.7 ± 0.7* |
| 6: CBH1 with βG, AXH, PL, EG2, PG, EX, βX, endoA, EGal | 42.7 ± 2.3 (54.1 ± 3.3)* | 17.8 ± 0.8* | 1.4 ± 0.2* | 13.2 ± 0.6* |
| 7: CBH1 with βG, AXH, PL, EG2, PG, βX, AF, exoA, EGal | 44.9 ± 2.6 (54.5 ± 3.7)* | 17.6 ± 0.9* | 1.5 ± 0.2* | 14.3 ± 0.7* |

* Data per 72 h.

was obtained (11.5 g/L of arabinose, 7.8 g/L of glucose, Table 3)—less than the yield with mixture no. 1, which indicates the need to add other (auxiliary) enzymes.

At the next stage, two enzymes not on the above list of key enzymes were excluded from mixture no. 1. It was noted that no more than 39 g/L of RS formed with the use of mixtures without EG2 and/or PG, while mixtures leading to RS concentrations > 40 g/L were detected in other cases (when EG2 and PG were present but EX, βX, AF, endoA, exoA, and EGal were excluded). Thus, EG2 and PG were also considered key for SBP hydrolysis. Enzyme mixture no. 3, which contained CBH1, βG, AXH, PL, EG2, and PG, resulted in the formation of 35.7 g/L RS (14.5 g/L arabinose, 10.9 g/L glucose, Table 3).

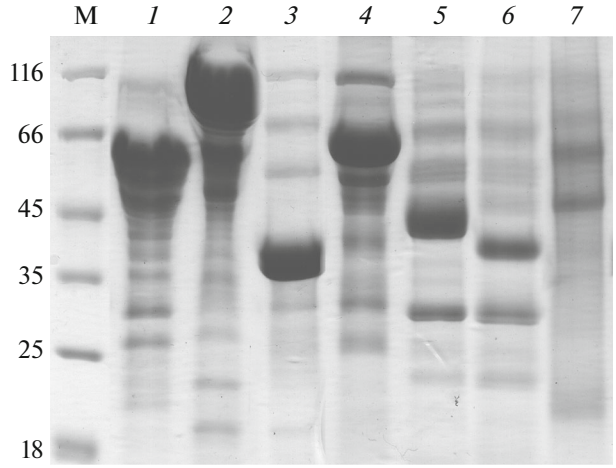


**Fig. 1.** Electrophoresis of dry EP in polyacrylamide gel with Na-DDS: *1*—B1-Cell, *2*—F-10, *3*—PCA-PL, *4*—PCA-AXH, *5*—PCA-exoA; *6*—PCA-endoA, *7*—Af-70a. M-markers, the molecular weights of standard proteins are indicated.

Based on mixture no. 3, four mixtures (no. 4–7) were then selected by the addition of EX, βX, AF, endoA, exoA, and/or EGal in different combinations (with the addition of one to five of these enzymes), the use of which made it possible to obtain the highest concentrations of RS and monosaccharides. With these enzyme mixtures, SBP was hydrolyzed for 72 h: the RS concentration was 49.1–54.5 g/L, which corresponded to a SBP polysaccharide conversion of 61–68%, the arabinose concentration was 17.6–19.6 g/L (94% of the maximum possible), and the glucose concentration was 13.2–16.7 g/L (63–79% of the maximum possible).

**Hydrolysis of SBP by EPs.** For SBP conversion, laboratory EPs (EP electrophoregrams are shown in Fig. 1) with a predominant content of one cloned enzyme were used: F-10 (the βG content was 70% of the total protein), PCA-PL (47% PL), PCA-AXH (29% AXH), PCA-exoA (20% exoA, 12% EX), and PCA-endoA (22% endoA, 11% EX). Multicomponent preparations were also used: cellulase (B1-Cell) (a total content of CBH1 and CBH2 of 50%, EG1, EG2, and EG3 17%), A. foetidus (Af-70a) (an AF content of 24%, PG content of 12%, βX and EGal contents of less than 1%). Table 4 shows the EP activities in relation to several substrates. It should be noted that high activities were revealed toward arabinan from EP PCA-AXH, PCA-endoA, and PCA-exoA (23–42 U/mg), and the presence of activity toward CMC, like the activity of cellulases B1-Cell and F-10 (7.8 U/mg) and the rest of the EP (1.2–1.4 U/mg), indicated the presence of endoglucanase activity in these EPs.

During SBP hydrolysis (100 g/L) by individual EPs (with the exception of the B1-Cell mixture with F-10 at a ratio of 9 : 1, Table 5), arabinose accumulation (6–

**Table 4.** Specific activities of EP on a number of substrates (U/mg protein)

| EP | MCC | CMC | PGA | AL* | Galactan | Xylan | AX** | *p*NPG | Pectin | Hemoglobin |
|---|---|---|---|---|---|---|---|---|---|---|
| B1-Cell | 0.4 ± 0.1 | 7.8 ± 0.7 | 0.5 ± 0.1 | 0.5 ± 0.1 | 0.3 ± 0.1 | 27.7 ± 2.1 | 12.7 ± 0.8 | 1.0 ± 0.1 | 0 | 0 |
| F-10 | 0.1 ± 0.1 | 7.8 ± 0.6 | 0.6 ± 0.1 | 0.4 ± 0.1 | 0.1 ± 0.1 | 4.4 ± 0.3 | 2.9 ± 0.3 | 53.4 ± 2.6 | 0 | 0 |
| PCA-AXH | 0 | 1.2 ± 0.1 | 0.8 ± 0.1 | 41.8 ± 2.9 | 1.0 ± 0.1 | 3.4 ± 0.2 | 32.5 ± 2.6 | 0.4 ± 0.1 | 0 | 0.1 ± 0.1 |
| PCA-exoA | 0 | 1.3 ± 0.1 | 0.7 ± 0.1 | 36.3 ± 2.4 | 0.6 ± 0.1 | 17.6 ± 1.4 | 20.2 ± 1.9 | 0.1 ± 0.1 | 0 | 0 |
| PCA-endoA | 0 | 1.4 ± 0.1 | 0.6 ± 0.1 | 23.2 ± 0.8 | 0.4 ± 0.1 | 34.2 ± 2.1 | 32.0 ± 2.3 | 0.1 ± 0.1 | 0 | 0.1 ± 0.1 |
| PCA-PL | 0 | 0.6 ± 0.1 | 0.7 ± 0.1 | 1.3 ± 0.1 | 0.5 ± 0.1 | 1.9 ± 0.2 | 4.3 ± 0.4 | 0 | 8.3 ± 0.9 | 0.1 ± 0.1 |
| Af-70a | 0 | 1.4 ± 0.1 | 17.6 ± 1.3 | 0.7 ± 0.1 | 0.5 ± 0.1 | 1.6 ± 0.2 | 1.2 ± 0.2 | 1.5 ± 0.1 | 0 | 0.6 ± 0.1 |

* Linear arabinan; ** arabinoxylan.

**Table 5.** Content of RSs, monosaccharides, and protein in the reaction mixture after 48 h of hydrolysis of SBP (100 g/L) EP and their mixtures (10 mg of protein/g SBP)

| EP | RS, g/L | Arabinose, g/L | Fructose, g/L | Galactose, g/L | Glucose, g/L | Protein, g/L |
|---|---|---|---|---|---|---|
| B1-Cell+F-10 | 34.2 ± 1.7 | 0.2 ± 0.1 | 1.5 ± 0.2 | 1.1 ± 0.2 | 11.2 ± 0.8 | 6.2 ± 0.1 |
| PCA-AXH | 43.6 ± 2.4 | 7.2 ± 0.7 | 3.1 ± 0.5 | 1.8 ± 0.2 | 8.4 ± 0.4 | 6.5 ± 0.3 |
| PCA-exoA | 31.7 ± 1.6 | 5.8 ± 0.5 | 2.8 ± 0.3 | 0.9 ± 0.1 | 5.5 ± 0.3 | 4.7 ± 0.1 |
| PCA-endoA | 35.0 ± 1.7 | 6.4 ± 0.6 | 3.0 ± 0.3 | 0.6 ± 0.1 | 4.9 ± 0.3 | 5.3 ± 0.2 |
| PCA-PL | 37.0 ± 1.8 | 7.8 ± 0.4 | 3.4 ± 0.4 | 0.5 ± 0.1 | 3.3 ± 0.2 | 6.7 ± 0.2 |
| Af-70a | 36.6 ± 1.7 | 6.0 ± 0.3 | 3.2 ± 0.1 | 0.8 ± 0.1 | 7.3 ± 0.6 | 6.5 ± 0.2 |
| Mixture no. 8: B1-Cell+F-10, PCA-AXH, PCA-PL, Af-70a, PCA-exoA | 56.5 ± 1.2 | 18.5 ± 1.7 | | 4.6 ± 0.7 | 24.7 ± 1.2 | 9.5 ± 0.9 |
| Mixture no. 9: B1-Cell+F-10, PCA-AXH, PCA-PL, Af-70a, PCA-endoA | 58.6 ± 1.9 | 19.9 ± 2.1 | | 3.4 ± 0.6 | 25.5 ± 1.3 | 9.7 ± 0.7 |

8 g/L in 48 h) was observed for all EPs. The glucose concentration was 11.2 g/L for the B1-Cell and F-10 mixture and 3–8 g/L for the other EPs. The galactose concentration for all EPs was 0.5–1.8 g/L. It should be noted that fructose accumulation up to 1.5–3.4 g/L was observed for all used EPs. The used EPs had relatively low proteolytic activity (Table 4); however, as the result of SBP conversion, a relatively high yield of soluble protein was observed: 4.7–6.7 g/L (which accounted for 50–65% of the total protein content in SBP). This was probably due to the destruction of the pulp polysaccharide net and the release of bound protein.

SBP hydrolysis was then simulated with mixtures of various EPs. We established earlier [15] that homogeneous exoA and endoA did not show synergism during joint arabinan hydrolysis. The effect of these enzymes on branched arabinan was significantly enhanced in the presence of enzymes that are active against lateral arabinose substrate residues, AXH and AF. Preliminary experiments on SBP (data not shown) confirmed the absence of a synergistic effect of EP PCA-exoA and PCA-endoA; thus, either PCA-exoA or PCA-endoA was included in the optimal EP mixture. In addition, it was established (data not shown) that the proportion of cellulase EP should be at least half of the total protein. In this case, the glucose accumulation rate becomes comparable with the arabinose accumulation rate.

Two mixtures of EPs nos. 8 and 9 were created. They were characterized by the presence of PCA-exoA (EP mixture no. 8) or PCA-endoA (EP mixture no. 9) of the following composition: B1-Cell + F-10 (considered as one EP): PCA-AXH : PCA-PL : Af-70a : PCA-exoA/or PCA-endoA = 10 : 2 : 2 : 5 : 1. The mixture was composed such that the total protein dosage in the reaction mixture was 10 mg/g SBP (the SBP concentration was 100 g/L). The removal of any component from the formulated mixtures led to a decrease in the product yield (data not shown). Thus, after 48 h of SBP hydrolysis with EP mixtures nos. 8 and 9, RS concentrations of 56.5 and 58.6 g/L were achieved. These concentrations corresponded to a polysaccharide conversion of 71–73%. the arabinose concentrations were 18.5 and 19.9 g/L (97% of the maximum possible), the glucose concentrations were 24.7 and 25.5 g/L (up to 100% of the maximum possible), the galactose concentrations were 4.6 and 3.4 g/L (70–90% of the maximum possible [4]), and the protein concentrations were 9.5 and 9.7 g/L (95–97%) (Table 5).

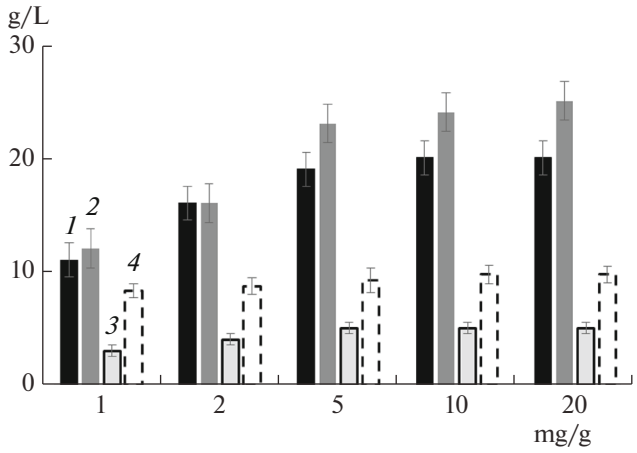


**Fig. 2.** Content of arabinose (*1*), glucose (*2*), galactose (*3*) and protein (*4*) after 48 h of SBP hydrolysis (100 g/L) with respect to the concentration of mixture no. 9.

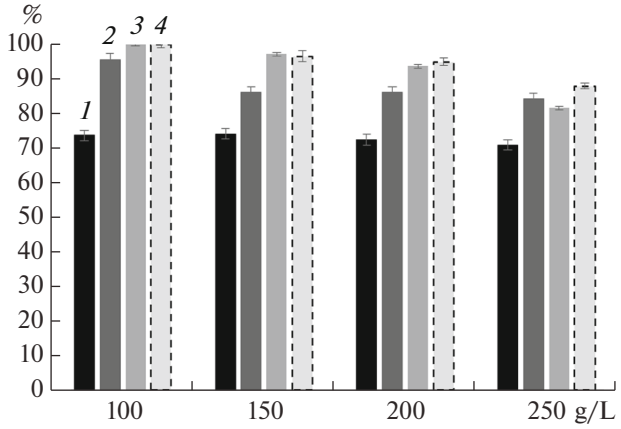


**Fig. 3.** Degree of SBP hydrolysis (250, 200, 150 and 100 g/L) after 48 h of hydrolysis with mixture no. 9 (10 mg/g): *1*—polysaccharide conversion, %; *2*—arabinose; *3*—glucose; *4*—protein.

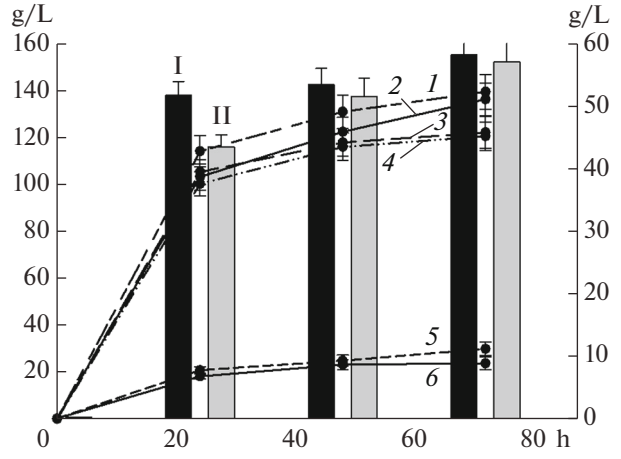


**Fig. 4.** Changes in the concentrations of RSs (I-II) and monosaccharides (*1–6*) (g/L) during SBP hydrolysis (250 g/L) for 72 h with mixture no. 9 at a concentration of 10 (I, 1*, 3, 5*) and 5 mg/g (II, *2, 4, 6*): *1, 2*—glucose concentration; *3, 4*—arabinose; *5, 6*—galactose.

SBP was then hydrolyzed (100 g/L) with EP mixtures no. 8 and no. 9 with different concentrations of EP per protein: 1, 2, 5, 10, and 20 mg of protein/g SBP (Fig. 2 shows the results only for EP mixture no. 9, since the data for mixture no. 8 were close to this data). After 48 h of hydrolysis with EP mixture no. 9 with 5–20 mg of protein/g SBP, the yield of the main products was the same and did not depend on the EP concentration; its further decrease led to a significant decrease in the sugar yield.

SBP was hydrolyzed at initial substrate concentrations of 100, 150, 200, and 250 g/L with EP mixtures nos. 8 and 9 (Fig. 3 shows the data for EP mixture no. 9). An increase in the SBP concentration to 250 g/L resulted in a slight decrease in the concentration of products after 48 h of hydrolysis. The conversion of substrate polysaccharides by RSs decreased from 74 (for 100 g/L) to 71% (for 250 g/L), the arabinose yield of the maximum possible ranged from 84 to 96%, the glucose yield ranged from 100 to 90%, and the protein yield ranged from 100 to 88%. The final product yield at an initial SBP concentration of 250 g/L was 142.4 g/L RS, 44.2 g/L arabinose, 49.1 g/L glucose, 9.3 g/L galactose, and 22.1 g/L protein.

Figure 4 shows the dynamics of the accumulation of the main products of SBP hydrolysis (250 g/L) under the influence of EP mixture no. 9 at concentrations of 10 and 5 mg/g for 72 h. After 24 h, the yield of products of SBP hydrolysis was 75% of the maximum reached in 72 h, even at an EP concentration of 5 mg/g. At the same time, the rates of accumulation of arabinose and glucose were comparable. The total product yield per 72 h was 155.2 g/L for RSs (polysaccharide conversion of 78%), 45.8 g/L for arabinose (89% of the maximum possible), 52.3 g/L for glucose (99%), 11.2 g/L for galactose (88%), and 22.9 g/L for protein (92%).

## CONCLUSIONS

Thus, in the course of the research, the enzymes necessary for deep SBP conversion to monosaccharides were found: CBH1, βG, EG2, AXH, PL, and PG. The addition of hemicellulase endoA, exoA, EGal, βX, EX, and/or AF to the main enzymes made it possible to obtain the most effective composition of individual enzymes, which result in a 1.2–1.4 times higher yield of RSs and monosaccharides.

The optimal EP mixture was selected. It consisted of cellulases (24% CBH1, 8% EG2, 4% βG), pectinases (5% PL, 3% PG), and hemicellulases (4% AXH, 1% endoA or exoA, 6% AF, 3% EX, less than 1% βX and EGal). This EP mixture at a concentration of 5–10 mg of protein/g substrate made it possible to reach a 100% degree of SBP conversion on arabinose, glucose, and protein after 24–48 h of hydrolysis at a substrate concentration of up to 250 g/L. At the same time, the accumulation rates of arabinose and glucose

were comparable, which indicated an optimal ratio of cellulases (responsible for glucose accumulation), hemicellulases (arabinose), and pectinases (which allow access to cellulose fibers due to the destruction of surface pectin).

No studies on the successful conversion of SBP at such a high initial substrate concentration (250 g/L) were found in the literature. Usually, an SBP concentration of 50–100 g/L was used, and the degree of conversion to monosaccharides was 70–100% [3, 4, 6].

The role of pectinase in SBP conversion was also noted in the study [4]. It was also shown that PL in a small amount (about 0.24 mg/g SBP) effectively diluted SBP for 12–15 h up to a flowing state, even at a concentration of 250 g/L, at which SBP represented a swollen mass without a liquid fraction. The involvement of PG in this process was negligible.

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## COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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