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RESEARCH ARTICLE
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Combined Acid-enzymatic Hydrolysis of Plant Waste to Obtain Nutrient Media for the Cultivation of Yeast *Rhodosporidium diobovatum*

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ABSTRACT

This study aims to establish an efficient and eco-friendly approach to beet pulp processing through the integration of acid and enzymatic hydrolysis and to explore the potential for cultivating *Rhodosporidium diobovatum* yeast on the resulting hydrolysate. The combined acid-enzymatic hydrolysis of beet pulp produces hydrolysates containing approximately 80% reducing substances, representing a 40% increase compared to hydrolysates obtained via acid hydrolysis using phosphoric acid alone. The optimal conditions for the initial phosphoric acid hydrolysis stage include a raw material particle size of 0.5cm, a temperature of 170°C, a reaction time of 10min, an acid concentration of 3% (wt.), a liquid-to-solid ratio (hydromodule) of 5.8, a stirring speed of 100rpm, and a pH range of 5.1 to 5.5. The optimal conditions for enzymatic hydrolysis (the second stage) are a temperature of 48°C, a stirring speed of 100min^{-1}, a pH ranging from 5.1 to 5.5 in the presence of buffer solution, a hydromodule of 8, and a duration of 12h. Fermentolysate is a more favorable nutritional source for the yeast *Rhodosporidium diobovatum* compared to acid hydrolysate, resulting in a yeast biomass yield of approximately 75% and a residual raw material content of no more than 25-30%. Thus, integrated processing of sugar beet production waste can yield long-term economic and environmental benefits, facilitating the management of large quantities of waste, mitigating the risk of disruption to natural systems, and producing essential biotechnological products.

Keywords: Plant waste; Beet pulp; Acid-enzymatic hydrolysis; Biotechnology; *Rhodosporidium diobovatum*.

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INTRODUCTION

Beet pulp is the most multi-tonnage by-product of the beet sugar production process (Cieciura-Włoch et al., 2019; Wilkowska et al., 2020; Shurbina et al., 2022; Qian et al., 2023; Cieciura-Włoch et al., 2020). The pulp yield is approximately 70-90% of the mass of the processed beet; if not processed properly, it can lead to an ecological disaster (Cieciura-Włoch et al., 2020; Shurbina et al., 2022). The chemical composition and low cost of beet pulp make this substrate attractive to many chemical industries due to its biotechnological (Modelska et al., 2017; Qian et al., 2023) and chemical (Tomaszewska et al., 2018; Cieciura-Włoch et al., 2020) properties. Various hydrolytic and microbial biomass conversion processes are currently used, including hydrolysis and culturing processes (Lynd et al., 2002; Taherzadeh & Karimi, 2007); however, most of the ongoing studies propose to add value to biomass by producing simple sugars, which are then used for microbial synthesis of various value-added products. Such technologies include beet pulp bioconversion.

Beet pulp is a type of lignocellulosic biomass and consists mainly of carbohydrates (cellulose 22-30%, hemicellulose 24-32%, lignin 1-2%) and polymeric saccharides (pectin 50% of dry matter) (Güell et al., 2015; Modelska et al., 2017; Cieciura-Włoch et al., 2019; Yurtseven et al., 2021; Shurbina et al., 2022; Patelski et al., 2024). Beet pulp also contains 2% protein, 2-3% sugar, and about 2%

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mineral substances. It has a minimal degree of technological processing of raw materials and a short period of preservation of properties. The pulp has a very high carbohydrate content (~80% by weight), predominantly composed of glucose (26% of total carbohydrate weight) in the form of cellulose together with arabinose (23%) and galacturonic acid (15%) in the form of sugar beet pectin (Shurbina et al., 2022; Valeeva et al., 2023). Also, fresh raw pulp contains vitamin C and such essential amino acids in human nutrition as lysine and threonine. The pulp contains more pectin and hemicellulose but less lignin than other types of plant biomass (Qian et al., 2023; Türk et al., 2023).

It is well established that lignocellulosic materials are often subjected to pretreatment processes to enhance the biodegradation of lignin, cellulose, and hemicellulose (Wilkowska et al., 2020; Yurtseven et al., 2021; Shurbina et al., 2022; Singh et al., 2022). Various pretreatment methods reduce the crystallinity of cellulose, which in turn breaks down lignin barriers and increases the available area for enzymatic reactions. These pretreatment methods can be physical, chemical, biological, or enzymatic (Singh et al., 2022). The physical pretreatment method mechanically destroys the lignocellulosic structures using physical force, resulting in a reduction in particle size (Patil et al., 2016; Singh et al., 2022). Destruction of lignocellulosic biomass increases the available surface area for enzymatic or bacterial degradation. Chemical pretreatment involves the use of ionic liquids, acids, alkalis, and other chemicals to degrade lignocellulose (Dehghani, 2015; Singh et al., 2022). Such treatment modifies or removes lignin and hemicelluloses and reduces the crystallinity of cellulose (Singh et al., 2022; Taherdanak, 2016). Thus, enzymatic, chemical, or thermal hydrolysis of these polymeric feedstocks is a crucial step for bioconversion.

Beet pulp is unequivocally recognized as a lignocellulosic material (Cieciura-Włoch et al., 2019; Valeeva et al., 2023), making it a promising substrate for enzymatic hydrolysis aimed at producing fermentolysates, which can subsequently serve as a nutrient source in yeast cultivation processes (Yurtseven et al., 2021). According to the literature, the primary monosaccharides released during beet pulp hydrolysis include glucose, fructose, mannose, galactose, galacturonic acid, rhamnose, raffinose, xylose, and arabinose (Leijdekkers et al., 2013; Berłowska et al., 2016; Cieciura-Włoch et al., 2019). These monosaccharides can be successfully used as sources of sugars for microorganisms (lactic acid bacteria, yeasts, fungi) (Concha et al., 2013; Zheng et al., 2013; Berłowska et al., 2018; Cieciura-Włoch et al., 2019; Mardones et al., 2019).

Although bioconversion can achieve the goal with low energy consumption and pollution, the conversion yield will be low without pretreatment (Barron et al., 2021). Pretreatment aims to maximize the yield of monosaccharides by modifying the chemical composition, deforming physical structures, and unlocking the availability of cellulose to enzymes (Zhao et al., 2022). Considering the carbohydrate composition of beet pulp (high content of hard-to-hydrolyze fiber), it can be assumed that pretreatment before bioconversion is necessary. The most commonly used pretreatment method for lignocellulosic feedstocks is treatment with weak acid solutions. Generally, chemical pretreatment is generally expensive and also poses a threat to the environment if chemicals are not handled or disposed of with care (Singh et al., 2022). The results of chemical pretreatment depend on the reagent added, its concentration, and pretreatment duration (Xu & Sun, 2016). Physicochemical pretreatment methods aim to degrade hemicellulose and lignin by changing the temperature, pH, and moisture parameters of biomass (Abo et al., 2019). Due to limiting factors, weak acid solutions capable of reduction or complete recycling are preferred in pretreatment. For example, phosphoric acid is a tricarboxylic acid, one of the attractive hydrolyzing agents in the processing of waste plant material. The attractiveness of using this acid is that after neutralization of the obtained hydrolysates by adding alkalis NaOH and KOH, salts are formed that can be used by microorganisms as mineral food components; these formed salts are not waste. The phosphorus requirement of microorganisms can be fully met by introducing phosphoric acid salts into the media. Since phosphorus is an energy carrier (part of adenosine triphosphate (ATP) molecules) and plays an important role in the synthesis of nucleic acids, the intensity of assimilation of carbon sources and the growth of microorganisms depend on its presence in the environment. The concentration of phosphorus in the medium affects the lipid and protein content of yeast biomass when grown on both carbohydrate and hydrocarbon substrates, as experimentally demonstrated by numerous researchers (Gamez et al., 2006; Valeeva et al., 2023).

The enzymatic approach for the bioconversion of lignocellulosic materials has gained prominence over hydrothermal and acid hydrolysis processes because of its lower environmental impact and minimal emissions (Wilkowska et al., 2020), lower temperatures, no corrosion of equipment, environmental friendliness, and high specificity (Balat et al., 2008; Silvello et al., 2023). The type and amount of hydrolytic enzymes (cellulases, hemicellulases, pectinases, ligninases, etc.), biological pretreatment methods, type of lignocellulosic feedstock, and amount of substrate can be listed among the factors affecting the reduction of sugar yield from such biomass (Paulová et al., 2015; Sharma et al., 2019; Yurtseven et al., 2021; Singh et al., 2022). Enzyme activity is strongly dependent on temperature, pH and incubation period. However, enzyme costs remain the primary limitation in biochemical hydrolysis (Silvello et al., 2023). Nevertheless, enzymatic pretreatment is generally a promising method to degrade lignocellulosic biomass and utilize it to obtain valuable compounds (Yurtseven et al., 2021). Thus, when considering a method for complete hydrolysis of polysaccharides, both acidic and enzymatic methods have their advantages and disadvantages and are not always advantageous to use separately.

This study aims to develop an effective and environmentally friendly method of beet pulp processing, combining acid and enzymatic hydrolysis, considering the selection of optimal process parameters, as well as assessing the quality of the obtained hydrolysates for the possibility of cultivation of fodder yeast *Rhodosporidium diobovatum* on them under aerobic conditions. The main hypothesis being tested in the study was: enzymatic

hydrolysate will result in higher yeast biomass yield compared to acid hydrolysate.

MATERIALS & METHODS

Preparation and Analysis of Raw Materials

Granulated beet pulp from Buinskiy Sakhar (Russia) was used in the study. The pulp pellets were pre-milled on a VYUGA 3MT laboratory mill (Russia) and sieved through sieves with cells of 0.1 mm (fine fraction), 0.5 mm (medium fraction), and 1.8 mm (big fraction). Beet pulp composition, % wt. of absolute dry matter (ADM): moisture –11.65±0.15, extractive substances – 2.5±0.98, cellulose – 23.25±0.01, easily hydrolyzable polysaccharides – 9.3±0.06%, lignin – 3.9±0.97, total nitrogen – 12.15±1.41, protein according to Barnstein – 4.95±0.28, ash – 5.08±0.61. The moisture content of all fractions was measured on an automatic moisture analyzer (AND MX-50, Japan) in accordance with ASTM E1756-01.

Determination of lignin insoluble in 72% sulfuric acid was done in accordance with ASTM D1106-96 (2007). The quantitative content of extractive substances was determined by the weight method in accordance with ASTM E 1756-08. Easily hydrolysable polysaccharides were determined in accordance with ASTM E1758-01. Determination of ash was done by dry oxidation (at 575±25 °C). The content of total nitrogen – according to the Kjeldahl method (in a wet ashing unit of SELECTA (Spain), with a remote temperature block in a fume cupboard, followed by ammonia distillation), true protein – according to the Barnstein method (ISO-1871-2009). All measurements were performed for two parallel experiments. Statistical data processing was performed using the STATISTICA program, version 13.3 (StatSoft Inc., USA), as well as StatPlus 2009 software.

Acid Hydrolysis

The strategy for determining the "optimal mode" involved successively identifying the optimal values for the following process characteristics: feedstock fraction, acid hydrolysis temperature, acid hydrolysis duration, phosphoric acid concentration, and washing (i.e., presence or absence of washing in the process). The optimum performance was determined by the maximum value of reducing agent yield under the same experimental conditions. The following parameters were not optimized: hydromodule (determined in the previous experiments), temperature of enzymatic hydrolysis (determined based on characteristics of enzyme preparations), stirring speed (determined in the previous experiments), pH=5.1-5.5 units (determined based on characteristics of enzyme preparations), duration of enzymatic hydrolysis (determined in the previous experiments), and enzyme concentration (determined based on feedstock composition and activity of enzyme preparations).

In order to determine the optimal particle sizes for the hydrolysis of beet pulp, acid hydrolysis processes were performed on all raw material fractions obtained prior to the main experiments. The process of acid hydrolysis was carried out in capsule laboratory reactors at the unit of high-temperature hydrolysis with a thermal accumulator (KSTU, Department of Chemical Cybernetics) with a volume of 100 ml at the same technological parameters (hydrolysis temperature of 170±5°C, duration of 20 minutes, hydromodule of 5.8, hydrolyzing agent – 3.0% phosphoric acid solution). At the end of the hydrolysis process, samples were taken for the quantification of reducing substances (RS) in acid hydrolysates. Separation of solid sediment from samples was performed on a Biobase laboratory automatic centrifuge (China) at a rotor speed of 7000 rpm for 15 min. The purified hydrolysates were analyzed for the content of reducing substances according to the methods the Bertrand method (Chidan Kumar et al., 2011; Chandraju et al., 2014). To determine the sugar content using the Bertrand method, 1ml of 2% HCl solution was added to 1ml of the sample in a conical flask. The flasks were then heated on a water bath for 10 minutes at 80°C. Subsequently, 8ml of distilled water and 10ml of Fehling's reagent-1 and Fehling's reagent-2 were added to the flask, and the contents were boiled on an electric stove for a period of two minutes. After cooling the solution, 15mL of 20% sulfuric acid solution was added under the fume hood. Subsequently, 2g of KI was introduced, and titration with sodium thiosulfate was performed until a milk-colored solution was achieved. In titration, starch solution was used as an indicator. The amount of copper was determined by measuring the consumption of sodium thiosulfate. Then, the amount of copper was converted to glucose. Dry weight of reducing agents – drying of samples in ESCO Isotherm laboratory cabinet (China) with subsequent weighing. All measurements were performed in two repetitions.

Dry matter content in hydrolysates was determined using a moisture analyzer AND MX-50 (Japan); acidity of hydrolysates was determined using the Multitest IPL-513 analyzer (Russia). All processes were repeated twice. The following parameters were calculated from the obtained data of the hydrolysis process: conversion, %; R (rate), g RS/L*hour; average dry matter (DM) concentration, %; RS content in DM, %. Considering the obtained data, the optimal fraction of raw materials, the acid hydrolysis of which led to the maximum yield of RS under the same conditions, was used for the main experimental work on acid hydrolysis. The main processes of acid hydrolysis of the beet pulp optimal fraction were executed on the same unit with the following technological parameters: hydrolysis temperature from 150±5 to 190±5°C with a step of 20°C, duration from 5 to 20 minutes with a step of 5 minutes, hydrolyzing agent – 0.5, 1, 2, 3, and 4% solutions of phosphoric acid (Shurbina et al., 2022), and hydromodule of 5.8. Sampling and characterization of the hydrolysates were also performed as in the experiments to evaluate the optimal fraction. According to the results of experiments, the optimal mode of acid hydrolysis (temperature, duration, concentration of hydrolyzing agent) was determined by the maximum yield of RS. After that, the experiment was repeated with a larger sample of beet pulp (about 120g) under optimal conditions. The obtained solid residue after acid hydrolysis under optimal regimes in order to obtain a more accurate dry weight and to eliminate possible substrate microflora was dried to constant weight in an ESCO Isotherm (China) drying cabinet at 120±5°C for 2 hours. After drying, the solid

residue was placed in the desiccator for further enzymatic hydrolysis. It also analyzed the residual quantitative content of cellulose and easily hydrolyzable polysaccharides using the above techniques. The acid hydrolysate obtained under optimal conditions and purified from solid residue was sterilized by boiling and then stored at 4°C for further use as a nutrient medium for the cultivation of *Rhodosporidium diobovatum* microorganisms.

Enzymatic Hydrolysis

Enzymatic preparations Novozymes (Denmark) for the hydrolysis of beet pulp polysaccharides were used for enzymatic hydrolysis of solid residue after acid hydrolysis (optimal mode): NS22074 cellulase complex (activity 1,000 EGU/g), NS50010 β-glucosidase (activity 250 CBU/g), NS22002 β-glucanase and xylanase (activity 45 FBG/g (~470 FXU/g)), and NS22035 glucoamylase (activity 750 AGU/g). Based on the data on the residual quantitative content of cellulose and easily hydrolyzable polysaccharides in the solid residue, the dosages of enzyme preparations were determined and the enzyme composition was prepared. For comparative enzymatic hydrolysis, a part of the solid residue was washed with distilled water for 20 minutes, and another part was used without washing.

Enzymatic hydrolysis with Novozymes preparations was performed as follows. In sterilized 1000-mL rocking flasks, 30g of prepared raw materials (washed solid residue, unwashed solid residue), the calculated amount of enzyme composition, and distilled water (hydromodule 8) were placed. The flasks were then closed with a cotton-gauze plug. Enzymatic hydrolysis was performed in a Kuhner ISF1-X laboratory incubator shaker (Switzerland) at t=48°C, 100 min^{-1}, pH=5.1-5.5 units, maintained with buffer solution (citric acid 0.05 mol/L buffer and NaOH), for 24 hours. Every 2 hours, hydrolysate samples were taken from the flasks, centrifuged on a Biobase centrifuge (China) at 10000min^{-1} for 5min, and the supernatant was used to determine pH (on a Multitest IPL-513 analyzer (Russia)), RS concentration in terms of glucose (Bertrand method), and dry weight of reducing agents (sample drying in an ESCO Isotherm (China) laboratory cabinet followed by weighing). All measurements were performed in two repetitions. Based on the experimental results, the effect of washing on enzymatic hydrolysis was evaluated by the maximum yield of RS. The hydrolysate purified from solid residue with maximum RS content after enzymatic hydrolysis was sterilized by boiling and then stored at 4°C for further use as a nutrient medium for culturing *Rhodosporidium diobovatum* microorganisms (to compare with acid hydrolysate).

Cultivation

Aerobic cultivation was performed on prepared acid and enzymatic hydrolysates. *Rhodosporidium diobovatum* strain VKPM Y-3158, a carotenoid producer with high β-carotene content, was used in the study. The carotenoid yeast *Rhodosporidium diobovatum* contains digestible protein – 380-450g/kg, beta-carotene – 120-170 mg/kg ADM, thiamine (B1) – 45-75μg/g, riboflavin (B2) – 7.5-15μg/g ADM, nicotinic acid (B5) – 660-840μg/g, pyridoxine (B6) – 10.5-21.1μg/g ADM, and biotin (B7) – 0.52-1.32μg/g. The inoculum size – 30mL – suspension of pure yeast culture of *Rhodosporidium diobovatum* stored at -70 °C and thawed at room temperature before use, diluted with sterile water. In a sterile box, 30mL of sterilized water at 30°C was injected into the tubes containing slanted agar medium and culture grown. Then, the culture was carefully removed from the agar medium layer using a wire that had been sterilized over an alcohol burner. The wire was shaken and poured into a flask containing hydrolysate. The flasks were covered with three layers of gauze, placed in a Kuhner ISF1-X shaker incubator (Switzerland), and incubated at 30°C, pH 4.8-5.0 for 24-72 hours, and a rocking speed of 100 min^{-1}. Aeration was natural through a gauze layer. The processes of yeast cultivation with phosphoric acid hydrolysates and fermentolysates were performed simultaneously with the process of cultivation on model Rider medium. The components of the nutrient media are summarized in Table 1.

Table 1: Components of nutrient media for culturing the yeast *Rhodosporidium diobovatum*

Components	Rider medium	Hydrolysate H_3PO_4	Fermentolysate
Hydrolysate	-	+	-
Fermentolysate	-	-	+
Water	+	+	-
Glucose	+	-	-
$(NH_4)_2SO_4$	+	+	+
$MgSO_4 \cdot 7H_2O$	+	+	+
KH_2PO_4	+	-	-
K_2HPO_4	+	-	-
KCl	-	+	+
$Ca(NO_3)_2$	+	+	+
NaCl	+	+	+
Yeast autolysate	+	+	+

To maintain pH at an optimal level of 4.8-5.0 units during yeast biomass growth, each medium was acidified with hydrochloric acid or alkalized with ammonia water. Chemical and microbiological control was performed during the cultivation process. Samples were taken every 2 hours to provide a control. One part was centrifuged, and the pH and concentration of the RS were determined. The second 10-mL part was used to determine the optical density with a KFK-2 photoelectrocalorimeter (Russia) at a wavelength of 590 nm (diluted 1:20 with water). The optical density value reflected the change in yeast biomass concentration. Numerical values of optical density were converted to the concentration of yeast biomass in the medium in g/L. Dry weight of cultivation medium after centrifugation was determined by drying samples in an ESCO Isotherm laboratory oven (China) followed by weighing. The physiological state of yeast cells was assessed by microscopy, and the number of cells was determined in a Goryaev chamber. To ensure the reliability and accuracy of measurements, the reproducibility of experimental data for all processes was assessed by two repeated experiments.

RESULTS & DISCUSSION

Table 2 and Fig. 1 present the results of the acid hydrolysis process of beet pulp. Based on Table 2 and Fig. 1, it can be concluded that the optimum fraction is the fraction with a particle size of 0.5 mm, as it allows obtaining 4.9265% RS under equal conditions and achieving a feedstock conversion of 34.12%.

Table 2: Data on the beet pulp acid hydrolysis processes to determine the optimal fraction of the raw material

Parameters	Results of acid hydrolysis experiments with raw material fraction		
	0.1 mm	0.5mm	1.9mm
RS (mean), % wt.	4.4787	4.9265	3.4044
Conversion, %	31.02	34.12	23.58
R (rate), gRS/L*hour	134.36	295.59	102.13
Average DM (dry matter) concentration, %	10.32	8.28	8.00
RS content in DM, %	43.40	59.50	42.56

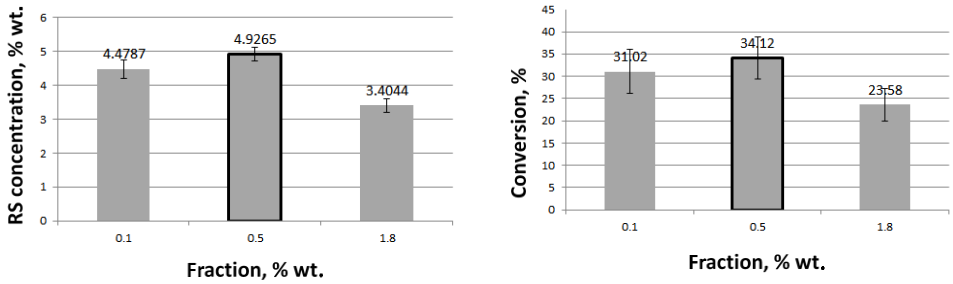

Fig. 1: Comparative data on the beet pulp hydrolysis processes of different fractions.

Apparently, this is due to the specific surface area of the particles and the limited duration of hydrolysis. Thus, smaller particles undergo hydrolysis faster, but with prolonged exposure to acid, partial degradation of monosaccharides occurs, which affects the overall RS yield.

Larger particles require longer hydrolysis time because the hydrolyzing solution needs to penetrate inside the particles, while the yield of dissolved solids also becomes more difficult. This phenomenon has been observed in many processes, both in acid treatment processes (Yurtseven et al., 2021), alkaline treatment processes (Marzo et al., 2021), and microbiological treatment processes (Marzo et al., 2021) of lignocellulosic raw materials acting as substrate.

Further studies were performed with a fraction of 0.5 mm. Fig. 2, a-e, shows the results of the main acid hydrolysis experiment.

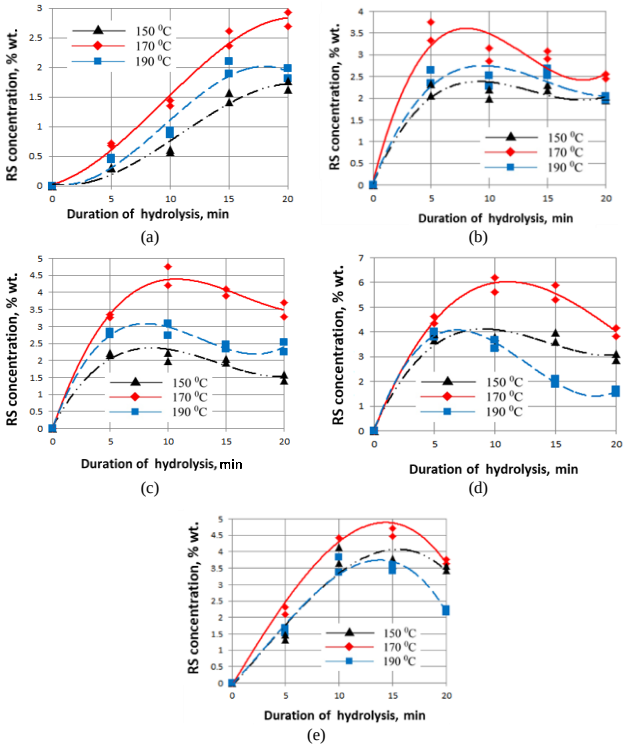

Fig. 2: Variation of RS concentration, % wt., as a function of hydrolysis time, hydrolysis temperature, and phosphoric acid concentration, % wt.: a) 0.5; b) 1; c) 2; d) 3; e) 4.

As can be seen from the presented data, in all studies, the maximum RS yields are observed for a temperature of 170°C. Increasing the concentration of phosphoric acid up to 3% promotes an increase in RS yield; further increases in acid content lead to a decrease in the yield of sugars, especially at elevated temperatures. Gentler hydrolysis conditions (Fig. 2a) allow the maximum to approach 20min of the process, whereas more severe conditions accelerate the carbohydrate degradation process, as evident from the maxima in the rest of the graphs. A lower yield of sugars is observed before the acid concentration of 2% at 150°C. As the strength of the hydrolyzing agent increases, a synergistic effect is observed in conjunction with temperature, with the maximum value becoming higher than in the case of 190°C. The decrease in RS yield at 190°C with increasing acid concentration is explained by the partial decomposition of sugars to simple substances.

At the same time, increasing the acid concentration also decreases the RS yield (in the case of 170°C). It should also be noted that the RS yield maxima shift toward shorter time intervals, indicating an increase in the hydrolysis rate. Under equal conditions (hydromodule), the kinetics of RS yield in the hydrolysis of beet pulp is quite comparable to the data on the hydrolysis of plant-based raw materials. For example, the authors of (Gamez et al., 2006) also showed the effect of temperature and acidity on the RS yield.

The results of the studies for the maximum RS yields are summarized in Table 3 and Fig. 3.

Table 3: Data on beet pulp acid hydrolysis processes

Parameter	Results of acid hydrolysis experiments with acid concentration, % wt.				
	0.5	1	2	3	4
Temperature, °C	170	170	170	170	170
Time, min	20	5	10	10	10
RS max, %	2.82	3.54	4.48	5.91	4.72
Conversion, %	11.53	24.87	31.50	41.60	32.32
R (rate), g RS/L*hour	49.35	424.39	268.72	354.86	94.44
Average DM (dry matter) concentration, %	6.03	4.74	7.90	8.61	13.91
RS content in DM, %	46.71	74.04	56.59	54.91	42.25

The following conclusions can be drawn from the obtained experimental data of the conducted beet pulp hydrolysis processes. The maximum values of reducing agents for the processes were obtained at 5-10 minutes

(except for hydrolysis processes with 0.5% phosphoric acid solution). The maximum values of reducing substances at all concentrations studied increased with increasing temperature from 150 to 170°C and decreased at 190°C. The maximum values of reducing substances at all investigated concentrations increase with increasing phosphoric acid concentration from 0.5% wt. to 3% wt. at all temperature parameters and decrease at 4% wt. phosphoric acid concentration. The maximum value of reducing substances was obtained in the process of high-temperature hydrolysis of beet pulp with 3% phosphoric acid at 170°C for 10min and amounted to 5.9143%. Hydrolysates of beet pulp containing reducing substances in the average amount of 54.9% of the total mass of soluble substances were obtained, which will ensure, when using hydrolysates in microbiological synthesis processes, a sufficiently high conversion of raw materials into target products (Türk et al., 2023).

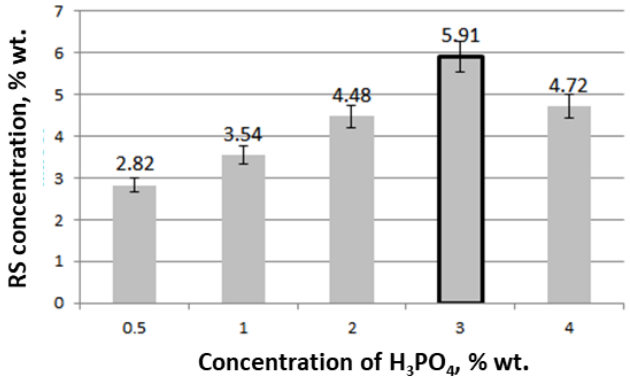

Fig. 3: Comparative data on beet pulp hydrolysis processes at varying concentrations of phosphoric acid.

According to the data presented, the reduction in cellulose content is more than 40% relative to the initial content, as well as in the easily hydrolysable polysaccharides (which include pentosans, glucans, mannans, and soluble oligosaccharides). The conversion of carbohydrates in phosphoric acid is about 40%, and the proportion of reducing agents from soluble substances of hydrolysate is 55% wt. or more. Similar data were obtained by the authors of (Ga´mez et al., 2006), who worked with sugarcane cake, and the proportion of sugars (RS) in % of soluble substances in soluble hydrolysis products was less than 55 wt.%.

Fig. 4 shows the data of RS yield kinetics in the processes of enzymatic hydrolysis of washed and unwashed solid residue after beet pulp hydrolysis.

It could be concluded, that washing of the solid residue after acid treatment significantly affects the RS yield. Thus, the maximum RS for the unwashed residue is 2.14 wt.% and is observed after 12 h, while for the post-washed residue, the RS yield is only 1.36 wt.% and is observed after 24 h of enzymatic hydrolysis. The rates and average RS yields are comparable to the studies of some researchers. Probably, the highest RS yield of the unwashed residue is related to the activity of enzymes, which exhibit maximum activity at lower pH values. Phosphate ions are present in the unwashed solid residue, reducing the pH in the hydrolysate during enzymatolysis into the region of the maximum pH ranges at which enzymes are most active. Maintaining pH during enzymatic hydrolysis is the main objective, so obviously the acid-free solid residue contributes to this support as well as the buffer. The rate of hydrolysis is therefore observed to be higher. In the case of washed material, the pH in the hydrolysate shifts to more neutral ranges, reducing the activity of the enzyme compositions. The main enzyme in the hydrolysis of lignocellulosic mass is cellulase, which is most active at a pH of about 5.0 units. The main mass of RS is formed from cellulose, which affects the overall RS yield. No additional washing is required in our case. However, it should be considered that additional washing of the solid residue yields solutions with RS concentrations of about 1%. Consequently, the solution from washing can be used to dilute concentrated hydrolysates. The total yield of reducing agents at two stages of beet pulp processing is presented in Fig. 5 and is equal to 80% of the raw material weight.

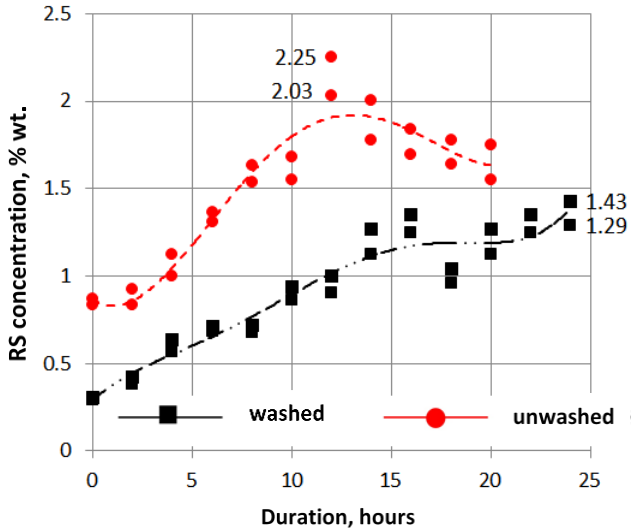

Fig. 4: Data on the enzymatic hydrolysis processes of beet pulp solid residue.

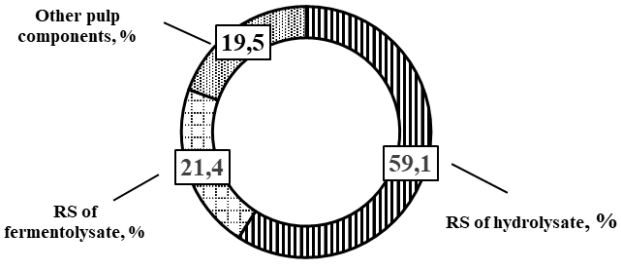

Fig. 5: Total yield of reducing substances in combined acid-enzymatic hydrolysis of beet pulp.

Thus, the optimal modes of combined acid-enzymatic hydrolysis of beet pulp are a raw material fraction of 0.5mm, an acid hydrolysis temperature of 170°C, a duration of acid hydrolysis of 10min, a phosphoric acid concentration of 3%, a hydromodule of 5.8, washing of solid residue after acid hydrolysis is not required, enzymatic hydrolysis at t=48°C, a stirring speed of 100min^{-1}, and pH=5.1-5.5 units in the presence of buffer solution (citric acid 0.05mol/L buffer and NaOH), hydromodule of 8, duration of 12 hours, and enzyme concentration based on the amount of feedstock.

Under such regimes, the total yield of reducing agents in the two-stage process of beet pulp processing is 80% of the raw material weight.

Under such modes, the total yield of reducing agents in the two-stage process of beet pulp processing is 80% of the raw material weight. Table 4 shows the results of culturing *Rhodosporidium diobovatum* yeast after 72 h from the start of culturing, when the maximum concentration of yeast biomass in the culture liquid was reached.

Table 4: Results of benignity assessment of hydrolysates

Hydrolyzate	Increase in optical density, units	Cell growth, cells/ml	Consumption share of reducing sugars, %
Control (Rider medium)	1.096	220	80.79
Acid hydrolysis	0.865	111	66.87
Enzymatic hydrolysis	0.977	154	81.77

Compared to acid hydrolysate, fermentolysate is a more benign nutrient source for the yeast *Rhodosporidium diobovatum*. Similar data were obtained in (Singh et al., 2022; Wilkowska et al., 2020; Yurtseven et al., 2021). The yield of yeast biomass per unit of raw material was 75%, which is due to the consumption of other components of hydrolyzate and mineral nutrition. The reduced biomass growth on acid hydrolyzates is probably due to the presence of large amounts of pentoses and uronic acids formed during acid hydrolysis and being biomass growth inhibitors, along with low molecular weight lignin fractions. The unutilized residue of raw materials in the total technological scheme is about 20%.

The enhanced yield of reducing sugars under optimized conditions specifically with 3% phosphoric acid at 170°C for 10min followed by enzymatic hydrolysis at 48°C for 12h can be attributed to the effective breakdown of cellulose and hemicellulose matrices. Phosphoric acid was chosen due to its dual functionality: acting as a mild acid catalyst and simultaneously enriching the medium with phosphorus, an essential nutrient for yeast metabolism (Gamez et al., 2006). Compared to other mineral acids, phosphoric acid minimizes the formation of fermentation inhibitors such as furfural and hydroxymethylfurfural (Zhao et al., 2022), thereby supporting more favorable growth conditions for *R. diobovatum*. The differential performance between washed and unwashed solid residues during enzymatic hydrolysis indicates the importance of maintaining residual phosphate ions in the matrix. As observed, unwashed solids yielded higher concentrations of reducing sugars, likely due to the buffering capacity and enhanced enzymatic activity at slightly acidic pH values (Silvello et al., 2023). This finding suggests that minimal processing post-acid hydrolysis not only simplifies the workflow but also supports better enzymatic performance. Furthermore, *R. diobovatum* cultivated on fermentolysates exhibited superior biomass yields compared to acid hydrolysates, likely due to the lower concentration of inhibitory compounds and a more balanced nutrient profile in the enzymatic hydrolysate. These results are consistent with prior reports that enzymatic hydrolysates promote higher microbial biomass due to the absence of phenolic inhibitors and better carbohydrate bioavailability (Singh et al., 2022). The successful bioconversion of beet pulp also highlights the potential of integrating such methods into a circular bioeconomy framework. This approach not only enables the sustainable management of agro-industrial waste but also produces high-value microbial biomass enriched in proteins, carotenoids, and vitamins, making it suitable for use in animal feed and bio-based products (Patelski et al., 2024). Moreover, the reduction in residual waste to 25–30% underscores the efficiency of the process and its applicability on an industrial scale.

Conclusion

Considering the low cost of the by-product of beet sugar production (beet pulp) and on the basis of experimental data obtained from the processes of beet pulp hydrolysis with phosphoric acid (average content of reducing substances 56.74% of the total mass of soluble substances), it follows that the use of beet pulp hydrolysates in the processes of microbiological synthesis can provide a high enough conversion of raw materials into target products. The optimal modes of combined acid-enzymatic hydrolysis of beet pulp are a raw material fraction of 0.5mm, an acid hydrolysis temperature of 170°C, a duration of acid hydrolysis of 10 minutes, a phosphoric acid concentration of 3%, a hydromodule of 5.8, washing of solid residue after acid hydrolysis is not required, enzymatic hydrolysis at t=48°C, a stirring speed of 100 min^{-1}, and pH=5.1-5.5 units in the presence of buffer solution (citric acid 0.05mol/L buffer and NaOH), hydromodule of 8, duration of 12h, and enzyme concentration based on the amount of feedstock. Under such modes, the total yield of reducing substances in the two-stage process of beet pulp processing is 80% of the raw material weight. Compared to acid hydrolysate, fermentolysate is a more benign nutrient source for the yeast *Rhodosporidium diobovatum*. The yield of yeast biomass per unit of raw material was 75%. The unused residue of raw materials in the total process flow diagram was about 25-30%. Thus, integrated processing of sugar beet production waste can be economically and environmentally beneficial in the long term and will allow the processing of multi-tonnage wastes of this production, reduce the threat of disturbance of natural systems, and obtain the desired biotechnological products.

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Ethics Statement: This study did not require ethical approval as no animal or human testing was performed.

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