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OPTIMIZATION OF THE NUTRIENT MEDIUM COMPOSITION FOR BIOETHANOL PRODUCTION BASED ON GRAPE POMACE.

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Abstract

This article describes the yeast strains, enzyme preparations, and mineral components used to optimize the nutrient medium composition in bioethanol production through the processing of grape waste. Methods for obtaining second-generation biofuels are among the most relevant topics of modern research. The production of biofuel from food waste helps address two major issues simultaneously. First, it reduces the negative environmental impact by utilizing food waste. Second, considering the growing demand for energy resources driven by economic and demographic growth, it serves as a renewable energy source. In this study, the nutrient medium composition was optimized, and experimental results demonstrate an increase in bioethanol yield during the alcoholic fermentation process.

Keywords*: bioethanol, enzyme, yeast, hydrolysis, alcoholic fermentation, grape, substrate, sugars.*

INTRODUCTION

It is well known that many countries around the world, including the United States, Brazil, and numerous European nations, use bioethanol as a fuel. According to data provided by the Prognozlashtirish va makroiqtisodiy tadqiqotlar instituti, global bioethanol production reached 112.29 billion liters in 2024 and is projected to increase to 144.34 billion liters by 2029.

In the biofuel industry, producing bioethanol from food waste is considered one of the most pressing challenges of today. In this context, grape pomace (grape marc) is one of the most common biological wastes generated by the grape-processing industry. It consists of grape skins, seeds, and stems remaining after juice extraction.

In 2023, grape production in Uzbekistan amounted to 1,800 thousand tons, which consequently led to the generation of large quantities of secondary waste products. During the processing season, one of the major environmental concerns associated with grape processing plants is the accumulation of substantial amounts of grape stems (281×10^3 t), grape pomace (787×10^3 t), wine lees (337×10^3 t), and wastewater (24×10^6 m³).

Some polluting characteristics of these residues, such as low pH (high acidity) and the phytotoxic and antibacterial properties of phenolic compounds, hinder their natural decomposition. Globally, approximately 60 million tons of grapes are produced annually for processing. According to data from FAO and World Health Organization, 5–10 million tons of waste are generated worldwide each year, including 300–400 thousand tons of solid waste in Uzbekistan alone.

Failure to implement effective recycling measures for such waste leads to both environmental and economic problems. Currently, only 20–25% of grape residues are utilized industrially, while the majority are not processed and are often accumulated in open areas near production facilities. This practice contributes significantly to environmental pollution.The main types of waste generated

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during grape processing include grape stems, grape pomace, and wine lees. In this regard, due to their chemical composition, grape residues represent an important raw material base for the production of second-generation biofuels (advanced biofuels), particularly bioethanol.

Globally, the role of bioethanol as an alternative energy source in the fuel and energy complex is steadily increasing. One of the key indicators in bioethanol production is the energy balance coefficient (EBC), which represents the ratio of the energy contained in the produced biofuel to the energy consumed during its production.

A number of studies indicate that first-generation bioethanol (FG biofuel) produced from corn in the United States does not demonstrate highly efficient energy performance compared to advanced biofuels. A significant amount of energy is consumed in raw material cultivation, processing, planting, harvesting, and subsequent conversion stages. According to an official report by the United States Department of Agriculture, the EBC of corn-based bioethanol is 1.24, meaning that the net energy gain is about 24%.

In contrast, cellulosic bioethanol demonstrates a significantly higher EBC of 5–6. Compared to gasoline, second-generation biofuel (SG biofuel) production and utilization can reduce greenhouse gas emissions by up to 85%. Bioethanol has a high octane rating (99 by motor method and 105 by research method), a lower combustion temperature, and produces cleaner exhaust gases due to the absence of sulfur compounds. Furthermore, as a second-generation fuel, bioethanol burns without forming ash; therefore, when alcohol-blended gasoline is used, no carbon deposits accumulate on engine spark plugs, and engine overheating during operation is minimized.For these reasons, producing second-generation bioethanol from lignocellulosic raw materials is considered highly relevant. This approach aligns with modern economic principles and fully corresponds to contemporary sustainable development concepts.

In recent years, particular attention has been paid in Uzbekistan to expanding the use of biofuel products. Although research on biofuel production has been conducted by scientists such as A. Azizov, N. Mo‘minov, Sh. Imomov, and B. Raxmatov, insufficient attention has been given to developing technologies for producing second-generation environmentally friendly biofuels through the processing of fruit, vegetable, and grape waste from canning and wine factories.According to the Presidential Resolution of August 22, 2019, Uzbekistan aims to increase the share of renewable energy sources in electricity generation to 25% by 2030. Currently, this indicator stands at approximately 10–12%. The Development Strategy of New Uzbekistan for 2022–2026 also emphasizes ensuring uninterrupted electricity supply to the economy and actively introducing green economy technologies across all sectors. In order to fulfill these strategic objectives, conducting scientific research aimed at improving technologies for producing second-generation bioethanol fuel from grape pomace is considered one of the most urgent tasks.

Research Methodology

The experimental study was carried out in a 2 L conical flask equipped with a Unimax 1010 shaker using the Incubator 1000 device. The temperature of the conical flask was controlled by a TC 1/80 thermostat to maintain optimal process conditions.To optimize the dosage of enzyme preparations used in developing the multi-enzyme complex for the enzymatic hydrolysis process, the software STATISTICA (StatSoft, version 7.0) was applied. In this study, the rate of action of enzyme preparations (x, y, z) on cellulose over time and the concentration of accumulated sugars formed at the end of the reaction were determined.

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Unlike conventional experiments, the total proportion of enzyme preparations participating in the reaction was kept constant (e.g., 100%). A total of 12 experiments were conducted for the enzymatic hydrolysis process. The experimental results are presented in references [1–5], and the experimental design developed using the proposed software is also described in the same sources.During enzymatic hydrolysis, a three-factor triangular diagram was constructed using the enzyme preparations SelloLux-A, Ultraflo Core, and Brewzyme BGX. The dependence of accumulated sugar concentration on the proportion of enzyme preparations added to the reaction mass is expressed by the following equation (x, y, z):

$$RS=23,2631x + 22,5956y + 14,1481z + 7,5317xy + 30,3815xz + 29,1999yz;$$

For the ideal enzymatic hydrolysis process, the effect of the multi-enzyme complex developed in references [1–5] was incorporated into the study. For sugar analysis, hydrolysate and yeast samples obtained during the fermentation process were analyzed using a **MiniSpin** centrifuge (Eppendorf, Germany). The samples were centrifuged for 5 minutes at a rotational speed of 10,000 rpm. For the alcoholic fermentation process, the effects of yeast strains Y-1693, PM-16, and Fm17 belonging to the genus *Saccharomyces* were investigated. During fermentation, the yeast biomass growth and the concentration of bioethanol formed in the reaction medium were determined.

The alcoholic fermentation process was carried out under the following conditions: temperature range of 28–32 °C, pH 4.6, and a process duration of 72 hours. To determine the optimal integration time for the combined operation of enzymatic hydrolysis and alcoholic fermentation processes, six experiments were conducted. The results of these experiments are presented in references [1–5].

Research Results.

To obtain the maximum yield of bioethanol, it is necessary to develop a nutrient medium with an optimal composition for alcoholic fermentation. During the experiment, enzymatic hydrolysis was first carried out, where POA (nitric acid–treated substrate) served as the raw material. Initially, a substrate with a dry matter content of 60 g/L was loaded into the fermenter. Hydrolysis was performed in an 11 L fermenter (designed by Pavlova).During the experiment, the acidity of the medium was monitored using an **InPro 42xx** pH meter. The fermenter was equipped with heat exchange elements and a mixing system to ensure proper reaction mass distribution. A transmitter **M200** was also used.During hydrolysis, enzyme preparations were added to the fermenter together with the substrate in the following amounts (mg/g):

- **SelloLux-A** – 40
- **Ultraflo Core** – 25
- **Brewzyme BGX** – 50

The hydrolysis process was conducted for 42 hours at 46 ± 2 °C and pH 4.6 ± 0.2. As a result of enzymatic hydrolysis, the total amount of reducing sugars (RS) reached 48.5 g/L, including 42.6 g/L of fermentable sugars and 5.9 g/L of pentoses.

After completion of enzymatic hydrolysis, the liquid fraction of the hydrolysate was separated from solid residues by vacuum filtration. The effect of potassium phosphate, ammonium sulfate, and yeast extract concentrations on bioethanol yield was investigated (Table 1).A three-factor experimental statistical model was developed for 10 different nutrient medium compositions. In addition to the main components, each variant contained 0.2 g/L potassium chloride and 1 g/L magnesium sulfate. After sufficient accumulation of carbohydrate compounds during hydrolysis, the nutrient medium

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was sterilized in an autoclave at 5 atm for 20 minutes. Yeast cells were then inoculated into the fermenter to initiate alcoholic fermentation.

According to the study results, 10 different nutrient medium variants were developed. For each experiment, yeast cells were added at a concentration of 8–12% of the reaction mass. The initial yeast concentration for all variants was 12 million CFU/mL. During alcoholic fermentation, yeast cell counts increased to 100–130 million CFU/mL, indicating active fermentation. The budding index of yeast cells ranged between 12–20%.Under laboratory conditions, the hydrolysate enriched with additional active components was sterilized and fermented in a 2 L flask using the TS-1/80 thermostat at 28 °C. The analysis of experiments enriched with additional nutrient components is presented in Table 1.

Based on the analysis of 10 experimental variants, bioethanol yield ranged from 48.84% (lowest-performing variant) to a maximum of 76.87%. The statistical model was constructed using experimental statistical modeling methodology based on ternary graphical dependencies presented in reference [5]. The results were illustrated in a three-dimensional dispersion coordinate diagram (Figure 1).Processing of the experimental data confirmed that the maximum bioethanol yield during alcoholic fermentation strongly depends on the composition of the nutrient medium. The results of the three-factor experiment were described by the following equation (1):

$$K = 64{,}8x + 56{,}8y + 49{,}9z + 24{,}4xy + 47{,}9xz + 70{,}2yz \quad \textbf{(1)}$$

Here, **K** represents the bioethanol concentration (%), while **x, y, z** denote the dimensionless concentrations of $(NH_4)_2SO_4$, KH_2PO_4, and yeast extract, respectively.

During the experiment, the lower limit of the dimensionless coefficient was –1 and the upper limit was +1. The minimum value for each component in the three-component mixture was 0 g/L. The maximum concentrations added to the nutrient medium were as follows: ammonium sulfate – 4 g/L, potassium monophosphate – 4 g/L, and yeast extract – 20 g/L.

Based on the developed statistical model, these supplements were introduced into the nutrient medium in 10 different ratios. The reliability of the equation was confirmed using Fisher's criterion. The three-factor response surface diagram of function **K** is shown in Figure 1. The diagram illustrates the dependence of bioethanol concentration on the composition of the nutrient medium. The optimal bioethanol yield (77%) is indicated by a dark red point in the triangular diagram, representing the maximum yield region, whereas the dark green region corresponds to the minimum bioethanol yield.

Analysis of Results

Analysis of the scheme and equation revealed that the most significant factors influencing bioethanol formation during alcoholic fermentation were yeast extract and ammonium sulfate solution.

The concentration of potassium monophosphate in the range of 0–2 g/L did not significantly affect bioethanol production. However, increasing its concentration to 2–4 g/L led to a decrease in bioethanol concentration during alcoholic fermentation.

Thus, during enzymatic hydrolysis, the active acidity of the nutrient medium is regulated by orthophosphoric acid. It can therefore be assumed that sufficient phosphorus accumulates in the native hydrolysate and serves as an adequate nutrient source for yeast cells. Fermentation of the native hydrolysate (Variant No. 1) resulted in a bioethanol yield of 66.92%, indicating the absence of toxic components or inhibitors in the medium.

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By adjusting the mineral and vitamin composition, a high-quality nutrient medium was obtained. For example, in Variant 10, a bioethanol yield of 76.87% was achieved.

Optimization of the nutrient medium composition for alcoholic fermentation allowed the maximum bioethanol yield to be obtained by adding the following components to the reaction mass:

- 1.90 g/L ammonium sulfate
- 0.78 g/L potassium monophosphate
- 6.77 g/L yeast extract

Under these conditions, the bioethanol yield reached 79.88%. The volumetric concentration of bioethanol during fermentation was 2.65 vol.%.

Conclusion

When these nutrients were added to the reaction mixture, bioethanol production increased by 12.96% compared to Experiment 1 (control).The concentration of monophosphate did not significantly affect the mass of bioethanol produced. If monophosphate was excluded from the nutrient composition, bioethanol was obtained at a yield of 79.28%, with a volumetric concentration of 2.63 vol.%.Table 1 presents the calculated efficiency of the alcoholic fermentation process and the rate of carbohydrate consumption by yeast cells. Analysis of Table 1 shows that in all 10 experiments, the biochemical activity constant of yeast cells ranged from 49.72 to 50.60 × 10^{-3}, indicating similar biochemical activity across all variants. It should also be noted that the lower the residual concentration of accumulated sugars, the higher the substrate consumption rate constant.

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