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# Combined Effect of Ultrasound Treatment and a Mix of Krebs Cycle Acids on the Metabolic Processes in *Saccharomyces cerevisiae*

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**Abstract:** This article describes the effect of organic acids and ultrasound on the physiological and biochemical properties of yeast, which was used to obtain biologically active peptides. The research featured brewer's yeast *S. cerevisiae* W-34/70 cultivated in 11% beer wort. A mix of Krebs cycle acids served as an activator. It included succinic, malic, fumaric, citric, and oxaloacetic acids (1:1:1:1:1). The concentration of the Krebs cycle acids was $1 \times 10^{-10}$ M/L at 1% to the suspension volume. The ultrasound treatment had an intensity of 10 W/m$^2$ and lasted 3–10 min. The combined effect increased the fermentation activity of the yeast by 98%. The activity of individual biocatalysts of constructive and energy metabolism rose by 108–330%, while that of proteolysis enzymes increased by 15% in comparison with the samples exposed to individual factors. The stimulation increased the rate of amine nitrogen consumption by the yeast. The amount of accumulated amino acids was larger by 80% than in the control, and that of protein larger by 7%. The maximal content of the synthesized protein was reached 1–2 h earlier. The combination of chemical and physical factors intensified the biosynthesis of protein and its intermediates during yeast processing, thus facilitating the subsequent extraction of biologically valuable components.

**Keywords:** yeast; metabolism; ultrasound; Krebs cycle acids; enzyme activity; amino acid composition; protein synthesis



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## 1. Introduction

Functional ingredients of microbial origin are an actively developing and promising area of food biotechnology [1–11]. They include microbial proteins [1–7], amino acids, and short peptides [6,8–11]. The high growth rate of microorganisms and targeted biosynthesis make this biological resource more efficient than high-yielding crop production or animal husbandry. *Saccharomyces cerevisiae* (*S. cerevisiae*) is an effective source of valuable biologically active components, e.g., nitrogen (proteins, oligopeptides, amino acids), carbohydrates (β-glucan, mannan), lipids, vitamins (group B), minerals (chromium, zinc, selenium, copper, phosphorus, magnesium, calcium, iron, manganese), etc. [4,6,7,9–11].

Bioactive peptides have become the subject of numerous investigations [1–21]. Peptides of various molecular weights have antihypertensive, antioxidant, antimicrobial, immunomodulatory, antiobesity, antidiabetic, and mitogenic properties [2,3,11–13,15,17,20].

The range of their application is wide and covers such industries as medicine, pharmacy, production of functional food and feed products, etc. [1–21]. Bioactive peptides can be obtained in various ways, the two most popular methods being the biocatalysis of high-molecular nitrogenous matters in microbial mass cells and physically- or chemically-induced synthesis (Figure 1) [11,16,18,21–23].

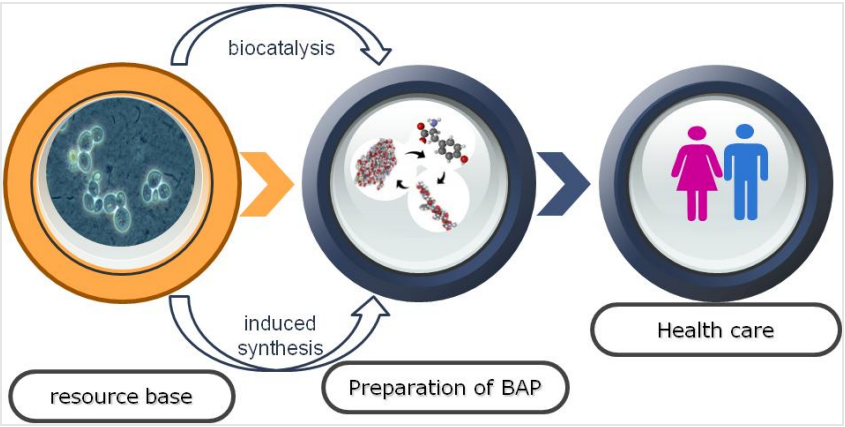



**Figure 1.** *S. cerevisiae* as a source of biologically active peptides (BAP).

Cell metabolism can be used to synthesize specific compounds, e.g., nitrogenous ones. It is regulated by different methods [24–27]. Most of these methods are associated with the activation and synthesis of enzymes involved in substrate preparation, intermembrane transport, energy production, macromolecular biosynthesis, etc. Biological membranes transfer substances into the cell and to organelles. They are also important for cellular metabolism. Organic acids, as well as their salts and esters, are chemical agents that stimulate the vital activity of alcohol, beer, wine, and baker's yeast [27–29]. If introduced exogenously, organic acids easily penetrate into the cell and enter the mitochondria, where they are as effective as their endogenous forms. In this regard, malic and succinic acids are quite popular, usually at concentrations of 0.01–1.0 g/L (or g/kg). However, not only individual intermediates have a positive effect on biological objects: various mixes of Krebs cycle acids have been reported effective at much lower doses than individual acids [29–35]. Homeopathic doses (from $10^{-23}$–$10^{-30}$ M to $10^{-23}$–$10^{-30}$ M) of biologically active substances activate biocatalysts, facilitate biomass growth and reproduction, intensify the accumulation of the target component, and increase stress resistance [28–30,34,35]. The mechanism may be related to the structural transformation of water as a solvent, the allosteric regulation of enzyme catalytic sites, or the parametric resonance [30,32,36].

Physical activation includes thermal, light, radiation, electrical, electromagnetic, or acoustic treatment of biological objects. The use of these methods is currently expanding, because they are available and environmentally friendly: they have a much lower load on the product and the environment [37–39]. Ultrasound remains the best-known of these factors [37,40–42]. Its biological effect depends on the properties of the object, as well as on the volume and time of treatment. A low-level and short-term ultrasound treatment has a positive effect on the biological object; otherwise, it might have an irreversible damage on the tissue, cellular, and molecular levels [37,40,43–50]. In particular, the sterilizing effect of ultrasound manifests itself at a frequency of $\geq$20 kHz and intensity of $\geq$0.5 W/cm$^2$. The main action of ultrasound is associated with cavitation. Cavitation zones appear in a liquid medium when a high-intensity acoustic wave passes through an area of low pressure. As a result, cavitation bubbles collapse, triggering shock waves with a large pressure amplitude that can reach several hundreds of atmospheres. Cavitation bubbles damage microbial cells. The cavitation process is facilitated by the areas in the liquid phase that contain the smallest bubbles of gases, vapors, suspensions, or microbial cells.

In addition to cavitation, ultrasound treatment owes its effect to changes in temperature, the permeability of cell membranes, and the rate of physiological and biochemical processes. When the temperature becomes critical, it activates biochemical and, subsequently, physiological processes in biological objects. If the ultrasound is too intensive, it breaks down polysaccharides, nucleic acids, globular and fibrillar proteins, etc. The object becomes too hot inside, and the high temperature destroys biological structures [37,40,42,47].

Brewer's yeast can be cultivated to obtain biomass. Residual yeast that remains after wort or beer fermentation is a sustainable source of valuable biologically active substances [51,52]. The physiological, biochemical, and technological functions of a microbial culture change as a result of chemical or physical factors, which can be combined [53–56]. For instance, brewer's yeast suspended in whey and treated with low-frequency acoustic vibrations were reported to have a better enzymatic activity, growth, and reproduction than after each of these factors used separately [53]. The present research objective was to study the combined effect of ultrasound and an ultra-low concentration of organic acids on the physiological and biochemical profile of brewer's yeast in the production of biologically active peptides.

## 2. Materials and Methods

### *2.1. Research Materials*

The research featured bottom-fermenting industrial brewer's yeast *S. cerevisiae W-34/70*. Hopped beer wort with 11% solids served as the medium for yeast suspension and cultivation. The yeast and wort were provided by the Kellers Brewery (Kemerovo, Russia). The wort included light malt produced by Grainrus-Kursk Malt (Kursk, Russia) with a moisture content of 4.7%, an extractivity of 82% on a dry matter (DM) basis, and the infusion method of mashing. The hops were of the Select variety (Simon H. Steiner, Hopfen, GmbH, Mainburg, Germany) with an α-acid content of 4.8% and a moisture content of 8.4%. The resulting wort had 11% of solids, 8.84 g/100 mL of fermentable sugars, 27.40 mg/100 mL of amine nitrogen, and a pH of 5.4. These indicators are correspondent to the standard brewing requirements [25].

A mix of Krebs cycle acids was served as a biostimulant. It included succinic, malic, fumaric, citric, and oxaloacetic acids in a ratio of 1:1:1:1:1 in the form of an aqueous solution with a concentration of $1 \times 10^{-10}$ M/L. Pure analytical acids were obtained from LenReaktiv (St. Petersburg, Russia).

### *2.2. Experiment Settings*

#### 2.2.1. Effect of Ultrasonic Treatment on Yeast Fermentation Activity and Non-Viable Cell Count

The bottom-fermenting brewer's yeast strain *S. cerevisiae W-34/70* was mixed with hopped beer wort with an extractivity of 11% in a ratio of 1:1. After that, the mix was subjected to ultrasonic treatment: the sound intensity was 5–15 W/m$^2$, and the treatment time was 1–20 min. The processing modes came from the literature [40–46] and our preliminary studies. The untreated yeast sample served as the control. The concentration of yeast cells was $(38.0 \pm 1.6) \times 10^6$ in 1 mL of suspension in the test and control samples. The ultrasonic treatment involved a UTA-1000 (Volna-T) unit developed by the Center for Ultrasonic Technologies of the Altai State Technical University (Biysk, Russia). It had a power of 1000 W and an oscillation frequency of 22 ± 1.65 kHz.

#### 2.2.2. Effect of Combined Treatment of Yeast with Ultrasound and a Mix of Krebs Cycle Acids on the Glycolysis Enzymes and the Tricarboxylic Acid Cycle

Table 1 represents the yeast treatment parameters. The concentration and dose of the Krebs cycle acids, as well as the treatment time, were based on previous studies [35] and the research results described in Paragraph 3.1. The content of yeast cells in the samples was $(38.0 \pm 1.6) \times 10^6$ in 1 mL of suspension.

**Table 1.** Combined treatment of yeast with ultrasound and a mix of Krebs cycle acids: variants and parameters.

| Variant | Treatment | Concentration of Krebs Cycle Acids, mol/L | Dose of Krebs Cycle Acids, % to Suspension Volume | Ultrasound Treatment Volume, W/m$^2$ | Treatment Time, min |
|---|---|---|---|---|---|
| Control 1 (C1) | No treatment | - | - | - | - |
| Control 2 (C2) | Krebs cycle acids | $1 \times 10^{-10}$ | 1 | - | 60 |
| Control 3 (C3) | Ultrasound | - | - | 10 | 10 |
| Test sample 1 (TS1) | Krebs cycle acids + ultrasound | $1 \times 10^{-10}$ | 1 | 10 | 10 |
| Test sample 2 (TS2) | Krebs cycle acids + ultrasound | $1 \times 10^{-10}$ | 1 | 10 | 5 |
| Test sample 3 (TS3) | Krebs cycle acids + ultrasound | $1 \times 10^{-10}$ | 1 | 10 | 3 |

#### 2.2.3. Combined Treatment with Ultrasound and a Mix of Krebs Cycle Acids: Effect on the Assimilation and Synthesis of Nitrogenous Substances

The test samples underwent an ultrasonic treatment for 5 min at 10 W/m$^2$. Beer wort (11%) with a mix of Krebs cycle acids (1% to mix volume) served as incubation medium. The cell concentration in the samples was (38.0 ± 1.6) × 10$^6$ in 1 mL of suspension. After that, the cultivation proceeded on beer wort with an extractivity of 11% at 25 °C. An untreated inoculum served as control. The pretreated and non-pretreated yeast was introduced into the culture medium at the rate of (20.0 ± 0.8) × 10$^6$ cells per 1 mL of wort.

### *2.3. Research Methods*

The enzymatic activity of the culture was determined in the initial yeast before the treatments, during the treatments, and during the cultivation. The fermentation activity indicates the ability of yeast to ferment the sugars of the medium. Its value was determined by the amount of released $CO_2$ using the Warburg method modified by S. N. Davydenko [57]. The modified method took into account the amount of carbon dioxide released by yeast in 1 h. We placed 1 mL of test yeast in a 50 mL glass beaker and added 0.5 mL of 40% glucose or maltose, 0.25 mL 8× YP medium (8 × 2% peptone, 8 × 2% yeast extract), and 0.25 mL of water. After everything was thoroughly mixed, we took a 2 mL sample in a 10 mL disposable medical syringe, trying to avoid air bubbles. The remaining air was squeezed out of the syringe, and the syringe was hermetically sealed by heating in the flame of an alcohol lamp and squeezing the softened plastic with metal tweezers. The hermetically sealed syringes remained in a thermostat at of 30 °C for 1 h. After that, we measured the height of the piston in the syringe. The amount of $CO_2$ was determined as the difference between the final and the initial value of the piston position.

α-glucosidase (EC 3.2.1.20), β-fructofuranosidase (EC3.2.1.26), and the zymase complex were determined by the polarimetric method [55], while 6-phosphofructokinase (EC2.7.1.11) was measured by the aldolase method [58]. The activity of NAD-dependent dehydrogenases, i.e., isocitrate, succinate, malate dehydrogenase, and pyruvate dehydrogenase, was assessed by the spectrophotometric method. The experiment involved the NAD reduction rate at a wavelength of 340 nm [58] and a PE-5400UV spectrophotometer (Shanghai Mapada Instruments Co., Ltd., Shanghai, China). The enzyme activity was expressed in μmol NADH/min·g of yeast dry matter. The activity of various dehydrogenases was expressed in cell-free yeast homogenates, which were prepared as follows: The yeast was triturated with quartz sand and double phosphate buffer at pH 7.5 in a porcelain mortar placed in an ice bath. After that, it was centrifuged for 20 min at 1000 min$^{-1}$. The resulting supernatant was used to determine the enzymatic activity.

The Anson method determined the activity of intracellular and extracellular proteases [59]. A 1% casein solution in a phosphate buffer served as the substrate at pH 7.31. The yeast mass was calculated by isolating the yeast from the suspension medium. The

suspension was centrifuged for 20 min at 3600 $min^{-1}$, followed by double washing with water and drying to constant weight at 105 °C. The Kjeldahl method made it possible to define the protein content in yeast and wort [58]. The concentration of amine nitrogen in the yeast mass and in the culture medium (beer wort) was determined by iodometry [58]. The amino acid composition of yeast was determined using an AZURA chromatograph (KNAUER, Berlin, Germany). The components were registered on a Smartline UV Detector 2500 spectrophotometer at a wavelength of 570 nm. The microscopy of the yeast suspension involved a Micromed 3 microscope (St. Petersburg, Russia) with a total magnification of ×600. The device was equipped with a 5.1 MP 1/2.5″ ColorUSB2.0 video eyepiece (Hangzhou, Zhejiang, China).

The physiological analysis of the yeast samples during cultivation was based on the total concentration of cells and the non-viable cell count. The experiment involved an automatic yeast cell counter LUNA-II YF™ (Logos Biosystems, Dongan-gu Anyang-si, Gyeonggi-do, South Korea). The yeast suspension was stirred until homogeneous. After that, 18 µL of the yeast suspension was mixed with 2 µL of the acridine orange dye and kept for 10 min at room temperature. Then, 10–12 µL of the obtained yeast sample was loaded onto a slide and placed into the port of the cell counter. The number of non-viable cells was expressed as a percentage of the total yeast cell count. The results were processed statistically and expressed as mean values with a standard deviation (SD). The differences were considered significant at a probability level of $p \leq 0.05$.

## 3. Results and Discussion

### *3.1. Effect of Ultrasonic Treatment of Brewer's Yeast on Fermentation and Non-Viable Cell Count*

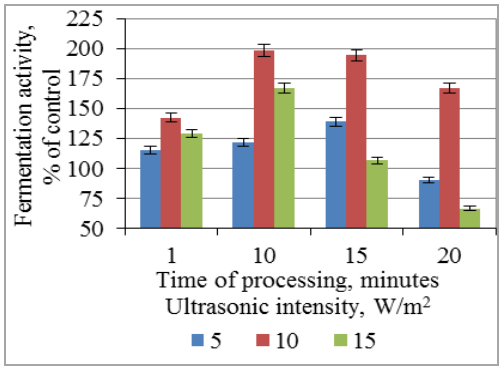
The results of ultrasound treatment on the fermentation activity and the non-viable cell count of the yeast samples are illustrated in Figure 2. The fermentation activity grew during the first 15 min of ultrasonic treatment, regardless of its intensity. After that, the value of this indicator started to decrease (Figure 2a). A more powerful ultrasound treatment also increased the fermentation. However, the fermentation was 16% less active at 15 $W/m^2$ than at 10 $W/m^2$, although the contact time remained the same (10 min). The fermentation activity reached its peak after 10–15 min of ultrasonic treatment at 10–15 $W/m^2$. It exceeded the initial value by 94–98%. The non-viable cell count (Figure 2b) increased by 1.5–4.2 times compared with the control, following the increase in the time and volume of ultrasonic exposure. A significant increase in the population of nonviable cells occurred at 15 $W/m^2$ and a contact time of ≥10 min (Figure 2c). This phenomenon resulted from the cell destruction caused by cavitation centers that appeared during the ultrasonic treatment (Figure 3). The obtained results were consistent with those published in [40,45,49,60,61]. Kaluzhina et al. [49] treated Safale T-58 brewer's yeast with ultrasound at an oscillation frequency of 44 kHz for 5 min. The treatment increased the fermentation activity by 36% and contributed to the maximal increase in biomass, budding cells, and glycogen. However, a longer ultrasound exposure led to excessive cell death. Del Fresno et al. [45] observed the effects of lysis of wine yeast cells after 20 min of ultrasound treatment at 24 kHz and 52 $W/cm^2$. They observed cell death at a sound frequency of 20 kHz after 30 min [61].

The obtained data made it possible to define the optimal ultrasonic treatment intensity (10 $W/m^2$) and time (≤10 min).

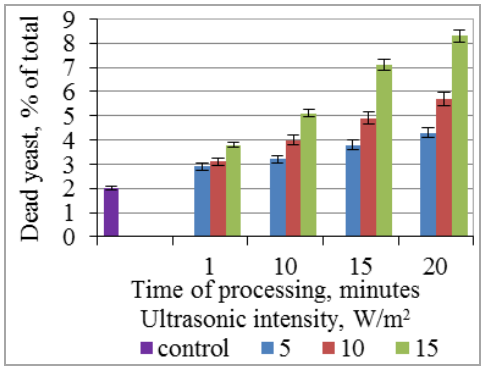
### *3.2. Effect of Combined Treatment of Yeast with Ultrasound and a Mix of Krebs Cycle Acids on the Glycolysis Enzymes and the Tricarboxylic Acid Cycle*

The biosynthesis of enzymes and other vital substances, e.g., proteins and their intermediate fragments, depends not only on internal factors, but also on external cultivation conditions, i.e., temperature, pH, redox potential, the required amount of digestible carbohydrates in the medium, nitrogenous substances, minerals, lipids, vitamins, etc. [24,25]. Pentoses, hexoses, α-keto acids, malate, acetate, malonate, etc. are intermediate compounds and serve as building blocks. Their development depends entirely on the composition of the nutrient medium and the cultivation conditions [24]. A combination of several factors,

both uni- and polydirectional, can have a significant impact on the metabolic processes of living organisms, e.g., yeast.




(**a**)

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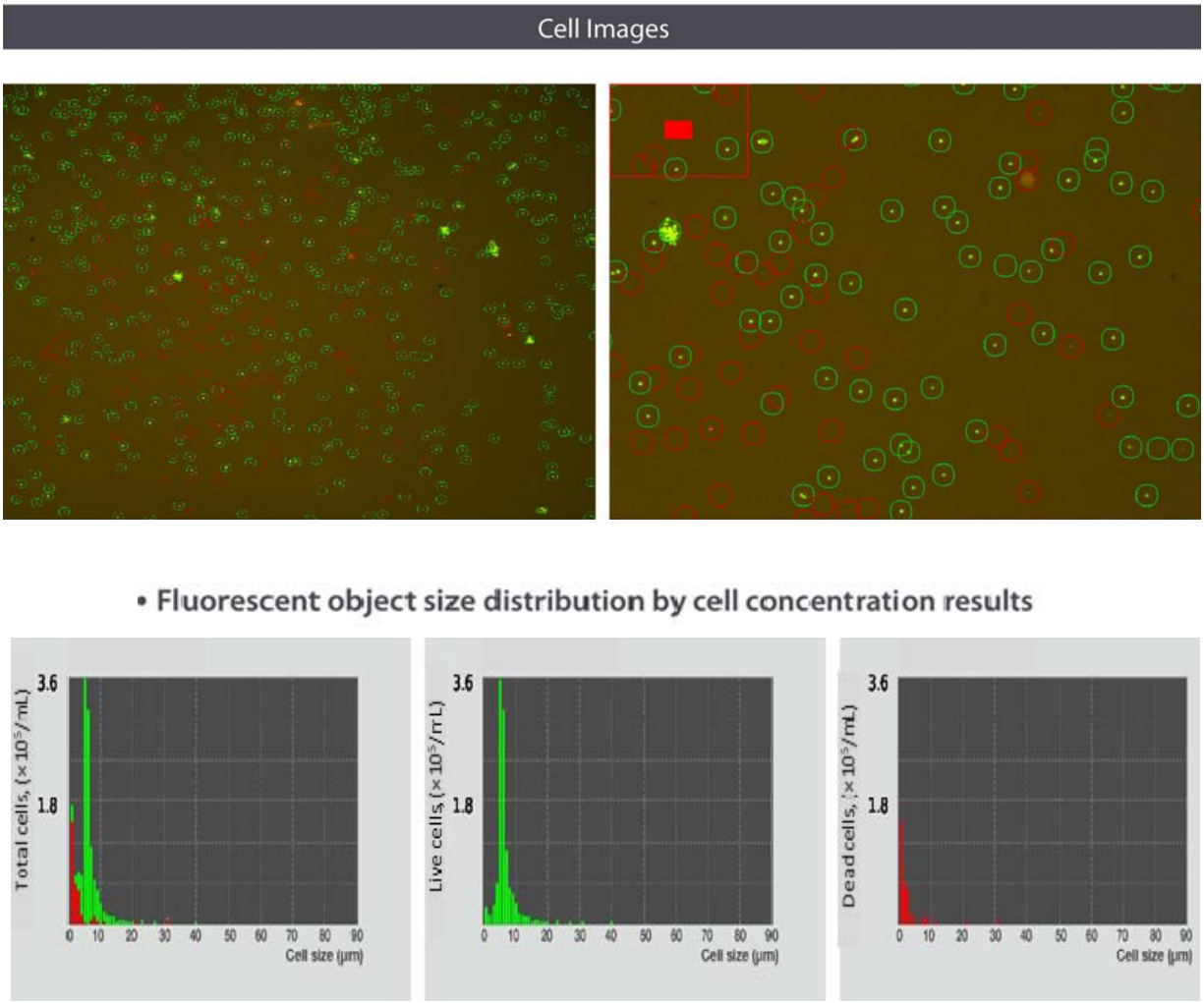
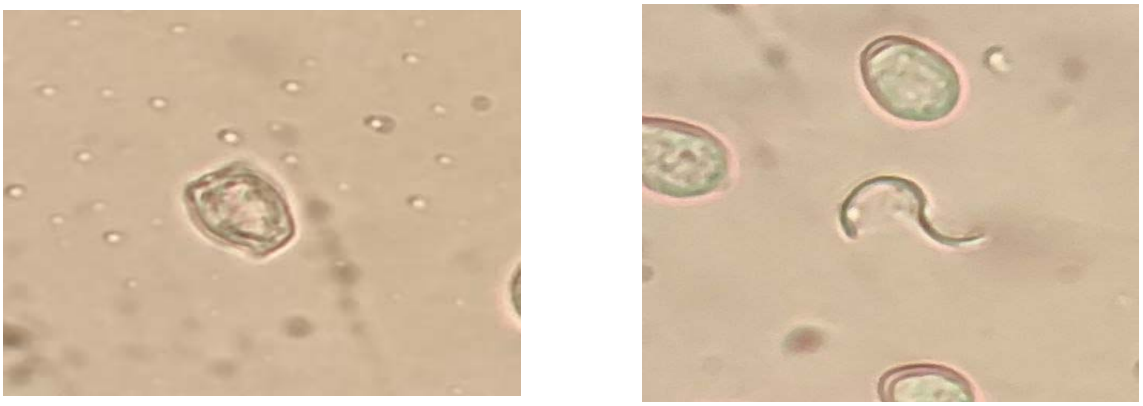



(**c**)

**Figure 2.** Yeast fermentation activity (**a**) and non-viable cell count (**b**) during ultrasound treatment; non-viable cell count (**c**) at 15 W/m$^2$ and a contact time of 20 min (LUNA-II YF™ research protocol) (green - live cells, red - dead cells). In panels (**a**) and (**b**), the values with a statistically significant difference $\leq$0.05 are shown.



(**a**) (**b**)

**Figure 3.** Cells with partially damaged membranes (**a**) and completely damaged cells (**b**) at 15 W/m$^2$ and a contact time of 20 min (×600 magnification).

The fermentation activity of brewer's yeast is an indicator that unites the rate and level of wort sugar fermentation. The latter depends on the activity of enzymes responsible for the transfer of appropriate substrates into the cell and the subsequent utilization of digestible carbohydrates. This research featured the effect of the combined action of ultrasound and a mix of Krebs cycle acids on the complex of yeast cell enzymes that catalyze individual stages of glycolysis and the tricarboxylic acid cycle. The combined effect of physical and chemical factors was evaluated by the changes in the activity of individual yeast cell enzymes. Their list included biocatalysts of the preparatory stage of glycolysis, e.g., α-glucosidase and β-fructofuranosidase that break down maltose and sucrose, respectively, and the zymase complex, which is responsible for the actual alcoholic fermentation. Table 2 shows that the combined action of ultrasound and Krebs cycle acids on yeast increased the enzymatic activity by 138–329% relative to Control 1, by 108–159% relative to Control 2, and by 112–248% relative to Control 3. However, a longer ultrasonic exposure inhibited the activity of the enzymes in the test samples that corresponded with Control 2, especially after 10 min of treatment time.

**Table 2.** Effect of combined treatment of yeast with ultrasound and a mix of Krebs cycle acids on the activity of individual enzymes.

| Enzyme | Activity | | | | | |
|---|---|---|---|---|---|---|
| | C1 | C2 | C3 | TS1 | TS2 | TS3 |
| Zymase complex, μmol glucose/g DM·min | 85.4 ± 2.3 | 135.7 ± 3.4 | 105.1 ± 2.9 | 118.1 ± 2.6 | 146.3 ± 2.1 | 169.8 ± 3.2 |
| α-glucosidase, μmol maltose/g DM·min | 15.8 ± 0.4 | 36.5 ± 1.3 | 21.0 ± 0.8 | 29.8 ± 0.9 | 43.1 ± 1.5 | 52.0 ± 1.6 |
| β-fructofuranosidase, μmolsucrose/g DM·min | 32.5 ± 0.8 | 47.2 ± 1.6 | 39.2 ± 1.4 | 46.7 ± 1.3 | 62.5 ± 1.6 | 75.4 ± 1.5 |

The efficiency of the combined physical and chemical action also manifested itself in a shorter treatment time. The combined treatment inhibited the activity of enzymes over a period that was 6–12 times shorter than in the sample treated with the mix of Krebs cycle acids only.

Along with glycolysis, yeast develops a modified tricarboxylic acid cycle under anaerobic conditions. The tricarboxylic acid cycle functions as its branch oxidizes to α-ketoglutarate and reduces from oxaloacetate to succinate [24,25]. The tricarboxylic acid cycle is the main phase of catabolic processes that provides yeast cells with energy and constructive metabolism. In the latter case, the cycle intermediates (oxaloacetate,

α-ketoglutarate, fumarate, acetyl-CoA, succinyl-CoA) join the gluconeogenesis and biosynthesis of fatty acids, amino acids, pyrimidines, purines, porphyrins, and isoprenoids [24,26].

The activity of enzymes made it possible to assess the functioning of the tricarboxylic acid cycle under the combined treatment. These enzymes were responsible for the following metabolic pathway: isocitrate dehydrogenase, succinate dehydrogenase, and malate dehydrogenase. Acetyl-CoA is the starting substrate for the tricarboxylic acid cycle. Therefore, this research also featured the changes in the activity of some glycolysis enzymes that produce acetyl-CoA. The list included 6-phosphofructokinase, the starting glycolysis enzyme, and the pyruvate dehydrogenase complex, which catalyzes the oxidation of pyruvate to acetyl-CoA.

The data obtained above provided the optimal parameters for the yeast treatment. The ultrasonic treatment lasted 5 min at 10 W/m$^2$, while the dose of the mix of Krebs cycle acids was 1% of the yeast volume. An untreated yeast suspension served as control. Figure 4 shows the changes that occurred in the activity of the enzymes when the yeast incubation medium had been treated with ultrasound and a mix of organic acids. The activity increased by 16–64% with respect to their initial value. Succinate dehydrogenase and 6-phosphofructokinase demonstrated the most pronounced activity change: it increased by 1.5 and 1.6 times, respectively.

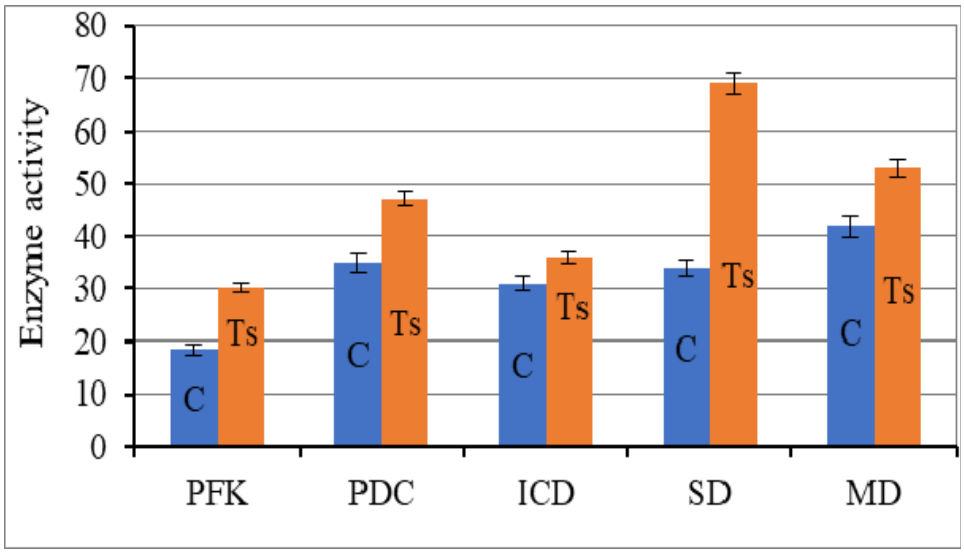



**Figure 4.** Effect of yeast treatment with ultrasound and a mix of Krebs cycle acids on the activity of enzymes: PFK: 6-phosphofructokinase (μmol fructose-6-phosphate/g DM·min·$10^{-2}$); PDC: pyruvate dehydrogenase complex; ICD: isocitrate dehydrogenase; SD: succinate dehydrogenase; MD: malate dehydrogenase (PDC, ICD, SD, MD–μmol NADH/g DM·min); C: control; Ts: test sample. The figure illustrates the values with a statistically significant difference $\leq$0.05.

Therefore, the intensification of the metabolic processes was associated with catabolic metabolism, which released extra energy and developed intermediate products involved in the synthesis of other substances, i.e., amino acids and proteins [8–10,24–26,35,53]. This assumption was confirmed by the results of experimental studies described below.

### *3.3. Effect of Combined Treatment of Yeast with Ultrasound and a Mix of Krebs Cycle Acids on the Assimilation and Synthesis of Nitrogenous Substances*

Yeast uses various sources of nitrogen for protein synthesis, but amino acids are the most valuable ones. External factors have a significant impact on the way cells assimilate the nutrients of the culture medium. Figure 5 illustrates the consumption of amino acid nitrogen from the medium and its content in yeast cells. The first cultivation day saw a significant decrease in amine nitrogen in the medium: it fell by 22% compared to the initial value in the experimental variant and by 12% in the control sample (Figure 5a). Obviously,

the yeast cells assimilated the wort amino acids. The value of this indicator in the microbial mass confirmed this finding (Figure 5b).

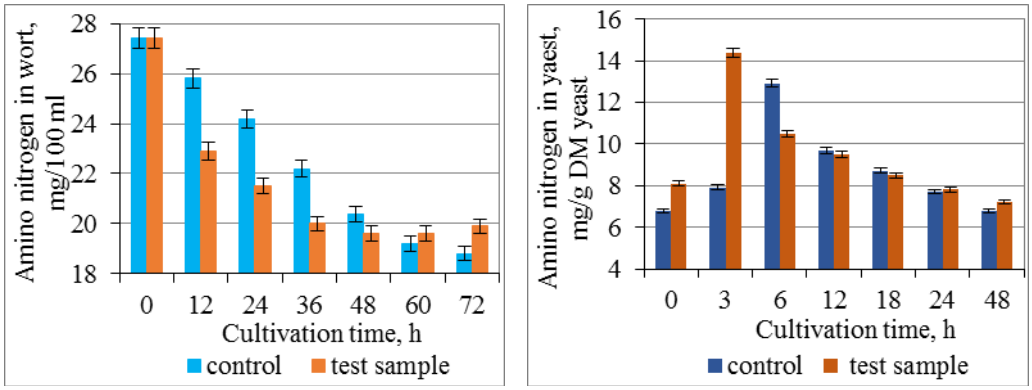



**Figure 5.** Amino acid nitrogen in culture medium (**a**) and in yeast (**b**) in the activated microbial culture. In panels (**a**,**b**), the values with a statistically significant difference $\leq 0.05$ are shown.

During the lag phase and the early logarithmic stage, the yeast consumed amine nitrogen to the maximum. Subsequently, the process slowed down, which was associated with (1) the falling rate of assimilation of amino acids and (2) their possible participation in the synthesis of various intermediates and partial secretion into the medium. The intensified assimilation of low molecular weight nitrogenous compounds also affected the time range: the maximal accumulation of amine nitrogen in the treated yeast cells ($14.4 \pm 0.4$ mg/g yeast DM) occurred three hours earlier than in the control sample ($7.9 \pm 0.2$ mg/g yeast DM).

The amount of new amine nitrogen in yeast cells was, on average, 1.3 times greater than that consumed from the medium. Such an increase happened because of the stimulation of amino acid synthesis. Probably, most amino acids appeared at the expense of endogenous resources of the yeast cell, i.e., the so-called amino acid pool [24,25]. The amino acid composition of yeast in the test and control samples at the onset of the exponential phase revealed a change in the quantitative content, but no qualitative transformation (Figure 6). The total concentration of free amino acids was 1.8 times higher in the test sample than in the control. The following amino acids predominated in the cell biomass in both the test samples and the control: tyrosine (7% of the total), leucine (9%), alanine (14%) and glutamic acid (23%).

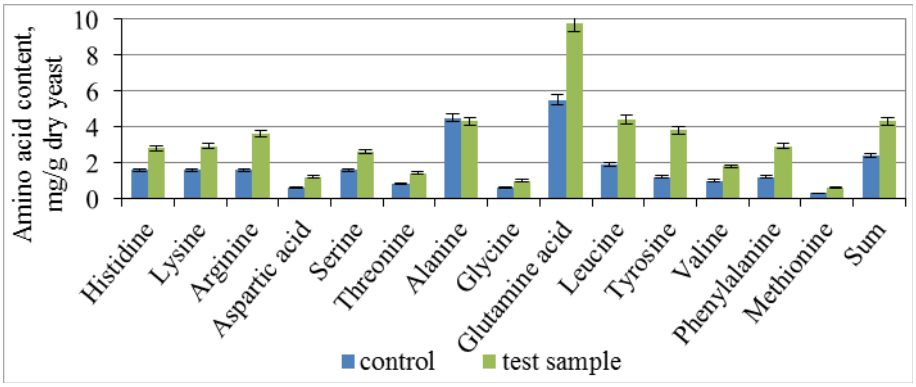



**Figure 6.** Effect of combined treatment of yeast with ultrasound and a mix of Krebs cycle acids on the yeast amino acid composition (the value "Sum" in the figure is represented as the sum value of amino acids$\times 10^1$). The values with a statistically significant difference $\leq 0.05$ are shown.

An extra amount of digestible nitrogenous compounds had a positive effect on the metabolic processes of the yeast cell, particularly on protein synthesis. Figure 7a demon-

strates the intracellular protein in yeast, while Figure 7b illustrates the protein in the culture medium.

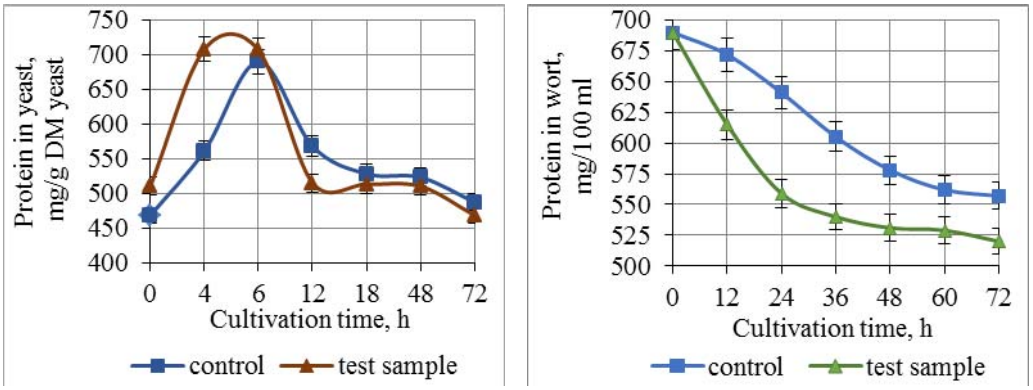



**Figure 7.** Amount of protein in yeast during cultivation (**a**) and in the culture medium (**b**). In panels (**a**,**b**), the values with a statistically significant difference $\leq 0.05$ are shown.

The changes in the amount of intracellular protein in all samples during cultivation followed the same pattern. An increase of 39–47% relative to the initial value during the adaptation phase reached its maximum 1–2 h earlier in the test sample than in the control sample (Figure 7a). We calculated the specific rate of protein biosynthesis in the yeast treated with ultrasound and Krebs cycle acids based on the data illustrated by Figure 7a. In the logarithmic growth phase, it was $(9.2 \pm 0.3) \times 10^{-2}$ $h^{-1}$, which was 1.6 times higher than in the control sample. After this rise, the protein concentration plummeted almost to its initial value. This process was more intense in the test sample. The active consumption of amino nitrogen and the development of new protein obviously happened as a result of the action of the enzymatic system in the yeast cell. Among yeast hydrolases, proteolytic enzymes have a special role in intracellular processes. Their function in the organism is not only a destructive one, but also regulatory [24]. Table 3 shows the changes in the activity of intracellular and extracellular yeast protease that occurred in the test samples and in the control during cultivation.

**Table 3.** Effect of combined treatment of yeast with ultrasound and a mix of Krebs cycle acids on protease activity.

| Proteolytic activity | Sample | Cultivation time, h | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 0 | 12 | 24 | 36 | 48 | 60 | 72 |
| extracellular protease, units/10 $cm^3 \times 10^{-3}$ | Control | - | $0.63 \pm 0.02$ | $1.21 \pm 0.03$ | $1.36 \pm 0.03$ | $1.45 \pm 0.03$ | $0.75 \pm 0.02$ | $0.55 \pm 0.02$ |
| | Test | - | $0.80 \pm 0.02$ | $1.34 \pm 0.03$ | $1.61 \pm 0.03$ | $0.83 \pm 0.02$ | $0.66 \pm 0.02$ | $0.60 \pm 0.02$ |
| intracellular protease, units/g protein $\times 10^{-5}$ | Control | $9.10 \pm 0.35$ | $7.83 \pm 0.30$ | $6.62 \pm 0.30$ | $8.30 \pm 0.35$ | $13.23 \pm 0.50$ | $17.36 \pm 0.50$ | $11.90 \pm 0.44$ |
| | Test | $12.21 \pm 0.40$ | $11.50 \pm 0.40$ | $11.94 \pm 0.40$ | $13.62 \pm 0.50$ | $20.55 \pm 0.60$ | $17.60 \pm 0.50$ | $12.41 \pm 0.40$ |

Table 3 shows that the transformation of enzymatic activity reached its maximum in the case of extracellular proteases after 36 h in the test sample, and after 48 h in the control. In the case of intracellular enzymes, the process peaked 48 and 60 h after the cultivation start. In both cases, the test sample was more active in enzyme activity by 14.5% on average than the control. The process grew less active by the end. The activity of intracellular and extracellular protease was by 4 and 9% higher in the test sample than the control, respectively. A certain correlation occurred between the changes in the activity of extracellular enzymes and the amount of protein substances in the nutrient medium (Figure 7b). Since the extracellular proteases in the test sample were more active, the protein content in the culture medium was 6.6% lower than in the control sample by the end of the fermentation.

The research revealed such phenomena as the changes in enzyme activity and the assimilation and synthesis of nitrogenous substances under the combined treatment. These phenomena become clear if we accept the following position: functionally significant environmental factors affect first the cytoplasmic membrane of the cell and then its internal systems [24]. Several publications [27–31,34,35] report an increase in the activity of biocatalysts (zymase, maltase, β-fructofuranosidase, alcohol dehydrogenase, protease, and peptidase) using various biological objects, e.g., baker's/wine/brewer's yeast, bacteria, grain, and vegetable crops. Their physiological and technological properties improved in the presence of di- and tricarboxylic acids. The effect of ultralow doses of Krebs cycle intermediates on biological objects was described in [32,36]. The authors associated the intensification of physiological and biochemical processes in yeast culture with the transformation of the mitochondrial membrane potential, which boosted the rate of energy release and changed the cell membrane permeability. The use of an acidic complex makes it possible to eliminate the substrate deficiency of the Krebs cycle and increase the bioavailability of its individual representatives for the cell. Probably, the Krebs cycle acids activated the transport system of cell membranes in the yeast suspension medium, thus accelerating the intake of nitrogenous substances appropriate for assimilation, e.g., amino acids, from outside into the cell.

The ultrasound treatment of the yeast culture was also proven to affect the abovementioned processes. When exposed to low-intensity ultrasound, the structure of biomembranes changed and their permeability increased [37,42,47,55,62,63]. As a result, yeast cells obtained more nutrients, and physiological processes accelerated, especially the biochemical reactions that activate certain enzymes. The ultrasound treatment of yeast mass probably freed the cell surface from the substances of the culture medium it adsorbed, thereby improving its permeability.

The quantitative increase in free amino acids could also be explained by the destruction of proteins. As a result, low molecular weight degradation products entered the cultivation medium [64]. These results were confirmed by the data on the content of α-amine nitrogen in the culture medium (Figure 5 a). The nitrogen content of amino acids in the test sample medium was 6% higher than in the control sample by the end of the cultivation process (72 h). The results obtained are confirmed by the studies of other authors. For instance, Huang et al. [22] wrote that the effect of ultrasound treatment on fermented milk stimulated the activity of extracellular proteases of lactobacilli and increased peptide development. Del Fresno et al. [45] reported an increase in the yield of proteins and glucans of the cell wall during ultrasound treatment of wine yeast sediment. Other scientists found an increase in hydrolase after ultrasound treatment of proteolytic enzyme preparations, grain before malting, and yeast before introducing it into the fermentation medium [37,48,53].

The synthesis of high molecular weight cell components depended on the low molecular weight precursors of these compounds. A mix of organic acids in the yeast suspension medium was reported to eliminate the substrate deficiency of the Krebs cycle, which is the central biochemical system of constructive metabolism [32,35]. For instance, oxaloacetic or fumaric acids were involved in the formation of such amino acids as lysine, aspartic acid, threonine, methionine, and isoleucine through a number of intermediate compounds [24,26]. The transamination of aspartic acid with α-ketoglutaric acid led to the development of oxaloacetic and glutamic acids. Aspartic acid served as a precursor of pyrimidines, e.g., uracil, thymine, and cytosine, which are part of the nucleotides, the so-called building blocks of nucleic acids that carry all the hereditary information in the protein.

Nitrogen and carbohydrate metabolism are known to be interconnected [24–26]. The most important reaction in the biosynthesis of amino acids as protein structural components is the reductive amination of the corresponding keto acids. Therefore, stimulation of the activity of key glycolysis enzymes observed in this research most likely increased the development of pyruvic acid, i.e., the precursor for amino acid biosynthesis. The increased protein metabolism and proteolysis in the *S. cerevisiae* biomass can yield bioactive peptides.

## 4. Conclusions

The present study featured the combined effect of ultrasound treatment and a mix of Krebs cycle acids on the *S. cerevisiae* yeast strain. It revealed a number of patterns. The rational ultrasound processing mode was 10 W/m$^2$ with a treatment time of ≤10 min. These parameters increased the fermentation activity of the culture by 94% in relation to untreated yeast at a low count of non-viable cells (≤4%). The joint treatment of the yeast culture with ultrasound and Krebs cycle acids increased the activity of individual enzymes: preparatory glycolysis, glycolysis itself, the Krebs cycle (1.8 times), and proteolysis enzymes (115%). The total content of amino acids increased by 180% compared to the untreated yeast sample.

The regulation of metabolic processes in the *S. cerevisiae* yeast that underwent the combined treatment intensified the nitrogen metabolism and protein proteolysis. In the future, this culture may serve as a source of bioactive peptides.

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