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Research article

Effect of ultraviolet radiation on physiological and biochemical properties of yeast *Saccharomyces cerevisiae* during fermentation of ultradispersed starch raw material

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ABSTRACT

Background: Study of correlation between pretreatment of yeast with ultraviolet radiation and efficiency of further fermentation of wort made of ultrafine grain particles to ethanol.
Results: We investigated three races of industrial yeast *Saccharomyces cerevisiae* (native and irradiated by ultraviolet). Physiological properties during fermentation of starchy wort were tested in all variants. It was shown that activation of the yeast by ultraviolet radiation allows to further increase the ethanol yield by 25% on average compared with the native yeast races when using thin (up to micro- and nano-sized particles) or standard grain grinding.
Conclusions: Using mechanical two-stage grinding of starchy raw materials and ultraviolet pretreatment of yeast, the efficiency of saccharification of starch and fermentation of wort to ethanol was increased.

1. Introduction

Important properties in the ethanol industry are intensification of wort fermentation process and to save energy, raw materials, and time. The ethanol yield depends on a number of factors (pH, temperature, etc.) and the physiological state of industrial yeast culture.

In ethanol production from dry milled grain, enzymatic hydrolysis of starch is carried out in two steps: gelatinization with the addition of thermostable amylase resulting in dextrin formation (liquefaction) and subsequent incubation with glucoamylase at lower temperatures to convert dextrins into glucose (saccharification). In industrial settings, the combination of pretreatment time and temperature varies from 165 °C (3–5 min) to 90–105 °C (1–3 h).

In wet milling technologies, granular starch is obtained and then hydrolyzed without heat cooking at 30–32 °C using special enzyme preparations (the so-called granular starch hydrolyzing (GSH) enzymes) [1]. Approaches to GSH enzyme technology application for dry milling have also just begun to develop.

Another problem of the alcohol industry is industrial yeast culture. The general direction of yeast new strain selection is to obtain races that provide the required fermentation rate and alcohol yield at wort fermentation for 3 d or less and resistance to stress factors such as ethanol and dry matter concentration, temperature, and presence of inhibitors [2]. There are a number of basic methods to obtain new races. Classical methods of selection include hybridization, polyploidy, mutagenesis, and genetic engineering [3,4,5,6]. One such mutagenic factor is UV radiation with a wavelength of 254 nm [7,8,9,10]. Depending on the radiation intensity, both activation of yeast (as a protective mechanism against stress) and mutation can occur. Some authors have shown that mutation of various genes occurs under UV exposure [11,12]. It was found that inactivation of genes responsible for radiosensitivity changes the frequency of mutations induced by different mutagenic factors. Therefore, rad2 mutation increases the frequency of UV-induced mutations of resistance to serine and respiratory failure mutations. Moreover, xrs2 and xrs4 mutations reduce the frequency of UV-induced mutations of resistance to serine and reversions to adenine independence by ADE2 gene nonsense mutation. The xrs2 mutation increases the frequency of cytoplasmic mutations, suggesting that the repair system in yeast acts not only in the nucleus but also in the cytoplasm [11,13]. Transcriptome analysis of mutant and wild-type alcohol yeast showed that the ethanol tolerance is caused by increased levels of oxidative processes in the mitochondria, which also stimulates glycolysis [14]. It was also found that several genes were highly expressed only in the ethanol-tolerant

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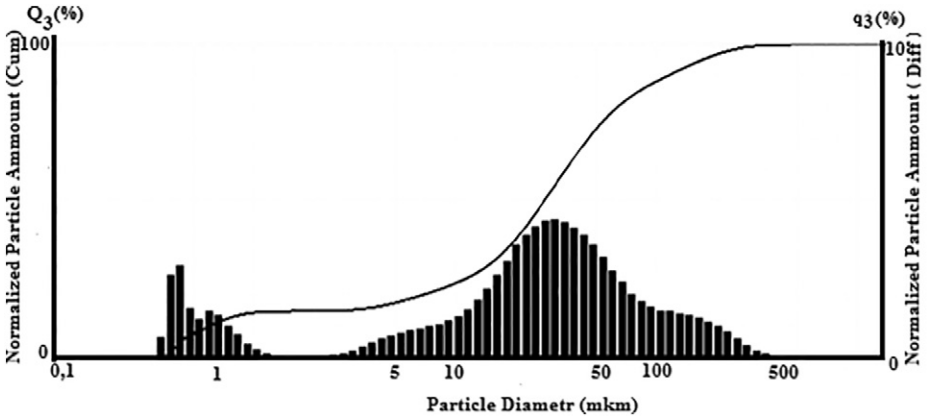

Fig. 1. Size distribution of the obtained ultradispersed raw material particles.

mutant but not in the parent strain. These genes were known to be induced in cells that were exposed to various stresses, such as ethanol, heat, and high osmolarity stress, or at the stationary phase but not log phase. In the ethanol-tolerant mutant, the expression level of these stress-responsive genes was further increased after exposure to ethanol. It was found that substances such as catalase, glycerol, and trehalose, which may have protective roles under stressful conditions, were accumulated in high amounts in the ethanol-tolerant mutant. The ethanol-tolerant mutant also exhibited resistance to other stresses including heat, high osmolarity, and oxidative stress in addition to ethanol tolerance [15,16,17].

In this article, a highly efficient yeast strain for producing ethanol from starch raw materials was obtained by UV radiation. Efficient ethanol producers were selected depending on sugar tolerance, ethanol tolerance, and fermentation activity.

2. Experimental

2.1. Yeast strains

Nine strains of *Saccharomyces cerevisiae* (Safdistil C-70 (Fermentis, France) and two mutant variants, Angel (Angel yeast, China) and two mutant variants, and Oeonoferm (Erbsloh, Germany) and two mutant variants) were used in this study. Mutant variants were selected after UV treatment from among 10 strains of each race of yeast with relatively high ethanol production and high tolerance to ethanol and sugar.

2.2. UV treatment conditions

Yeast culture was inoculated in 10 ml of YPD medium (2% glucose, 1% peptone, and 2% yeast extract) and incubated overnight at 30 °C until cell density reached 2×10^8 cells/ml. The cells were diluted to obtain a final density of 10–100 cells/ml. Then 100 μl of this suspension was taken and inoculated on Saburo agar plate. Exposure was conducted at UV irradiation intensity (wavelength 254 nm) of 200 mW/cm^2 for 5, 10, and 15 min. To stop photoreactions, plates were kept in the dark for 24 h. The colonies were incubated for 3 d at 30 °C.

2.3. Raw materials

We used wheat grain with the following characteristics as raw material: trash admixture 0.22%, humidity 10%, and conventional starch content 59%.

2.4. Preparation of raw materials for fermentation

For wort preparation, grains were ground in two stages [18,19]. In the first stage, grains were ground to a size of approximately 1 mm in a laboratory grain mill LZM-1 (Russia). Further grinding of the grain raw materials was performed in the planetary ball mill Retsch PM 100 (Retsch GmbH, Germany) at 18 g for 30 min. Particle size was determined by the device Laska 1K (Lumex, Russia). Measurement of the actual size of the ultradispersed grain mixture particles showed that the two-stage grinding results in nanometer- (about 10%) and micron-sized (the lion's share from 10 to 200–300 μm) samples (Fig. 1).

Mashes with grain to water ratio of 1:3.5 were prepared using ultradispersed raw materials. For enzymatic hydrolysis, the following commercial enzyme preparations were used: Mezomey-2500 and Glucomey-8000 (Beijing Shifa Multi-Business Agency, China) and Laminex BG2 and Maxazyme NNP DS+ (Genencor International Oy, Finland). The dosage of enzyme preparations was chosen according to the manufacturer recommendation. Pretreatment temperature was 60 °C. Hydrolysis was carried out according to the following scheme: addition of the enzyme preparations Mezomey-2500, Laminex BG2, and Maxazyme NNP DS+ and incubation at 60 °C for 30 min, acidification of the mixture to pH 4.2 with 1 N sulfuric acid, and addition of Glucomey-8000. Saccharification was carried out for 1 h. Soluble material, carbohydrate, and cationic composition and viscosity of the wort were controlled during the experiment.

2.5. Modeling of fermentation conditions

The experiment consisted of two stages: activation of freeze-dried yeast and wort fermentation. Yeast activation was conducted for 24 h at a constant agitation of 150 rpm at 30 °C using wort prepared according to the method described above. Dry yeast was introduced at a concentration of 0.1 g/l.

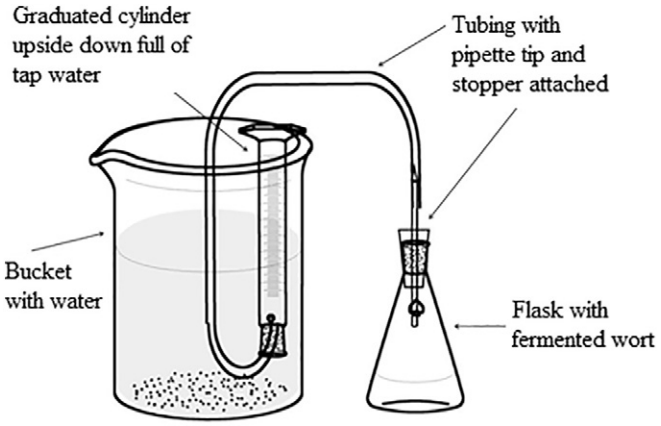

Fig. 2. Device for measurement of fermentation activity.

Table 1
Main technological parameters of fermented wort obtained using native yeast races.

Indicator	Angel	Safdistil C-70	Oenoferm
pH after fermentation	4.43 ± 0.03	4.33 ± 0.06	4.26 ± 0.09
Titratable acidity	0.56 ± 0.05	0.59 ± 0.04	0.61 ± 0.04
Apparent dry matters, %	6.83 ± 0.16	6.8 ± 0.0	6.9 ± 0.0
Unfermented carbohydrates concentration, g/100 cm^3	0.351 ± 0.048	0.326 ± 0.043	0.381 ± 0.003
Dextrin concentration, g/100 cm^3	0.179 ± 0.048	0.195 ± 0.024	0.203 ± 0.023
Starch concentration, g/100 cm^3	0.084 ± 0.036	0.049 ± 0.014	0.051 ± 0.015
Alcohol content in the fermented wort, % vol	6.24 ± 0.33	6.49 ± 0.42	5.92 ± 0.37

Worts were fermented by commercial (native) and mutant yeasts in bioreactors (BIOSTAT® A plus, Sartorius, Germany). Fermentation was performed under anaerobic conditions, stationary, at optimal temperature for 72 h. Activated yeasts were added to wort to a final concentration of 100–120 million cells/ml (cell concentration in initial inoculums was 700–750 million/ml).

2.6. Research methods

The mash viscosity was determined using a viscometer VT-04F (Rion Co., Ltd., Japan) at one rotational speed of the disk. Visible solutes were analyzed in the clear wort filtrate using an automatic refractometer model PTR 46 (Inde Instruments Ltd., UK). For more quantitative analysis of the carbohydrates, HPLC analysis was performed using an LC-20 Prominence (Shimadzu, Japan) HPLC with a SupelcoGel Silica-LC-NH2 column (mobile phase: ACN:H_2O 72:25) and a refractive index detector (RID-10A). Counting and viability analysis of yeast cells was performed using the cell viability analyzer Vi-CELL® (Beckman Coulter, USA). Alcohol accumulation in the wort was determined according to the Compendium of International methods of wine and must analysis [20]. Fermented wort indicators were determined by the anthrone colorimetric method [21]. A eudiometer was used to determine the fermentation activity of yeast (Fig. 2).

All the results obtained in the experiment were subjected to statistical analysis using the PC program Microsoft Excel 2000.

3. Results and discussion

The main reasons for yeast race selection were their close technological properties (Table 1) and the fact that they have traditionally been used in alcohol production.

After UV irradiation, a number of variants were obtained; two mutants for each race were selected. The selection was carried out by resistance to external, so-called exogenous, ethanol. Previously, for the determination of sensitivity point to exogenous ethanol, native (unirradiated) races were plated onto YPD medium containing 10–25% vol ethanol and incubated for 4 d at 30 °C under static conditions. A dramatic drop in the viability of the native strains was revealed after reaching an alcohol concentration of 20% vol (Fig. 3); we used this concentration to study the irradiated variants and compare their viability to that of the original strains.

As shown in Fig. 4, UV irradiation reduced the living cells' quantity more than twice; the surviving yeast cells did not differ from the original in morphology (Fig. 5).

Further, physiological and biochemical properties of the mutants were studied in the grain wort with the following characteristics: concentration of starch 12.7 g/l, visible solids 17%, pH 5.3, fermentable carbohydrates 7.9 g/100 cm^3, and dextrins 4 g/100 cm^3. The qualitative and quantitative composition of the wort sugars was determined (Fig. 6).

Calculations showed that the glucose and fructose peak falling at 5.01–5.4 min corresponds to the sugars concentration 67 mg/cm^3. The concentration of maltose (peak 5.57–6.4 min) was 12 mg/cm^3 and maltotriose (peak 7.02–7.66 min) was 6.4 mg/cm^3. The total amount of fermentable sugars was approximately 85 mg/ml (or about 67% of hydrolyzed starch), which coincides with the data obtained by the anthrone method (the difference is within the error of anthrone method). However, this is not the final concentration of fermentable sugars because hydrolysis of the remaining starch and dextrins continues during fermentation both by yeast (low molecular weight dextrins are degraded by yeast enzymes) and by exogenous enzymes introduced in the saccharification stage.

Furthermore, the concentration of some ions important for the fermentation process was determined in the wort. It is known that potassium and magnesium are macroelements for yeast, while other ions may be present in trace amounts [22]. Therefore, potassium ions promote yeast multiplication and play an important role in oxidative phosphorylation (activate reaction catalyzed by isocitrate dehydrogenase) [23] and glycolysis, where they activate yeast aldolase and participate in phosphoric acid residue transfer by phosphotransferase enzyme from phosphoenolpyruvate to ADP residue during substrate phosphorylation. Potassium specifically stimulates inorganic phosphorus transfer into the cell and together with magnesium is necessary for pyruvate carboxylase action, which is especially important when obtaining the inoculum [24]. Potassium also

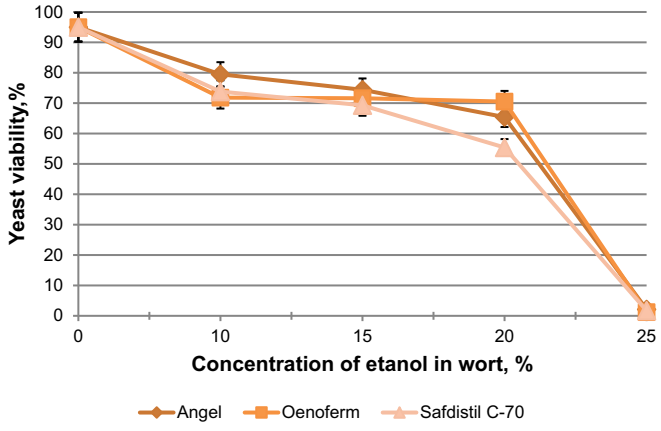

Fig. 3. Viability of native yeast cultures in the presence of exogenous ethanol.

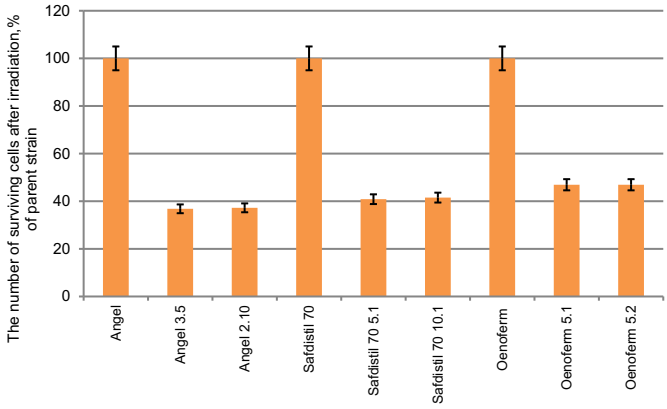

Fig. 4. Percentage of surviving cells after irradiation.

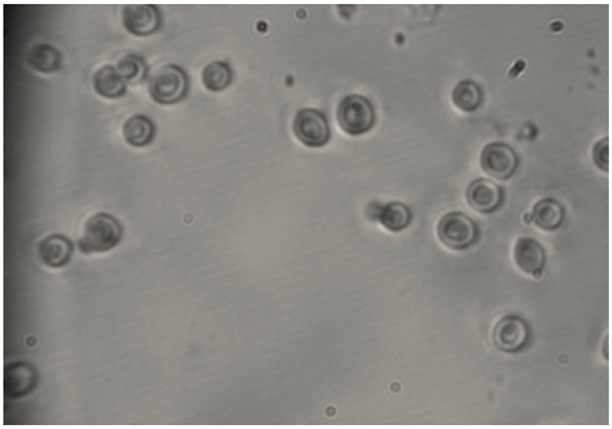

Fig. 5. Irradiated yeast cells (race Oenoferm 5.2).

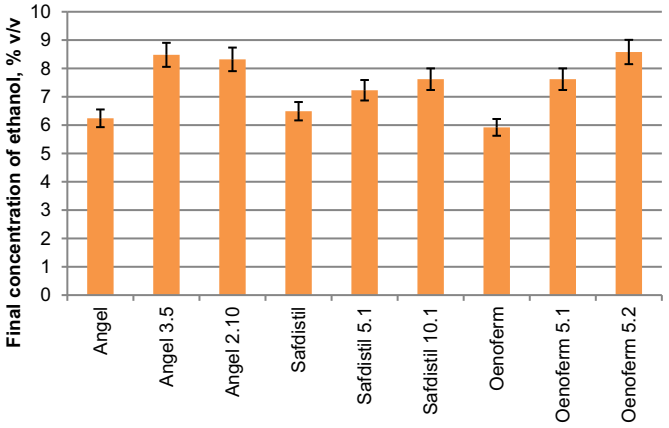

Fig. 7. Final concentration of ethanol accumulated by native and irradiated yeast strains after fermentation of wort prepared from ultradispersed starch raw materials.

increases the rate of glucose consumption by enhancing membrane permeability [25]. Its concentration in the investigated wort was 33 mM, which is within the optimum range. In the study performed by Da Silva et al. [26], it was shown that up to a concentration of 60 mM, potassium increases glucose consumption by yeast.

Magnesium also plays an important role in glucose and amino acids transport: its presence on the inner surface of the cytoplasmic membrane is one of the main conditions for this process efficiency [24]. In addition, magnesium activates a number of glycolysis enzymes, in particular hexokinase (the rate of the first reaction of glycolysis determines the whole process) and enolase [24]. Moreover, magnesium protects yeast cells by preventing an increase in cell membrane permeability under ethanol influence and temperature-induced stress [27]. Its concentration in the wort was 13 mM, which is almost 3 times above optimal [24].

Calcium influences the activity of the aldolase enzyme that catalyzes the formation of two phosphotrioses: 3-phosphoglyceraldehyde and phosphodihydroxyacetone. In cells, calcium exists in both free and bound form and often stabilizes the ribosomes and polysome structures; extracellular calcium can influence the charge of the cell membrane surface [28]. Its concentration in the wort was 4 mM, which is slightly higher than the optimal [29]. It was shown that addition of Ca^{2+} (in the form of $CaCl_2$) to media at optimal concentrations (0.775 to 2.025 mM) results in fermentation process acceleration and accumulation of higher concentrations of ethanol in *S. cerevisiae*, *Saccharomyces bayanus*, and *Kluyveromyces marxianus*. This also increases the alcohol resistance of the strains [30,31,32].

The next step was selection of the optimal culture that meets a number of requirements. The yeast culture should be able to convert the glucose to ethanol, accumulate the maximum amount of ethanol in the medium, and have a high alcohol resistance. Results of the research and calculations are presented in Fig. 7.

Data analysis showed that irradiated yeast has better characteristics in almost all parameters. Alcohol yield was 35–45% higher when using irradiated yeast (near 8.5% v/v of ethanol in wort) compared to using the native strains (only 6–6.5 8.5% v/v of ethanol in wort). Moreover, the pH and temperature optima of the original races and irradiated variants did not differ.

The stability of the newly acquired properties was evaluated by re-determination of resistance of the obtained irradiated variants to exogenous alcohol introduced at a concentration of 20% vol. The

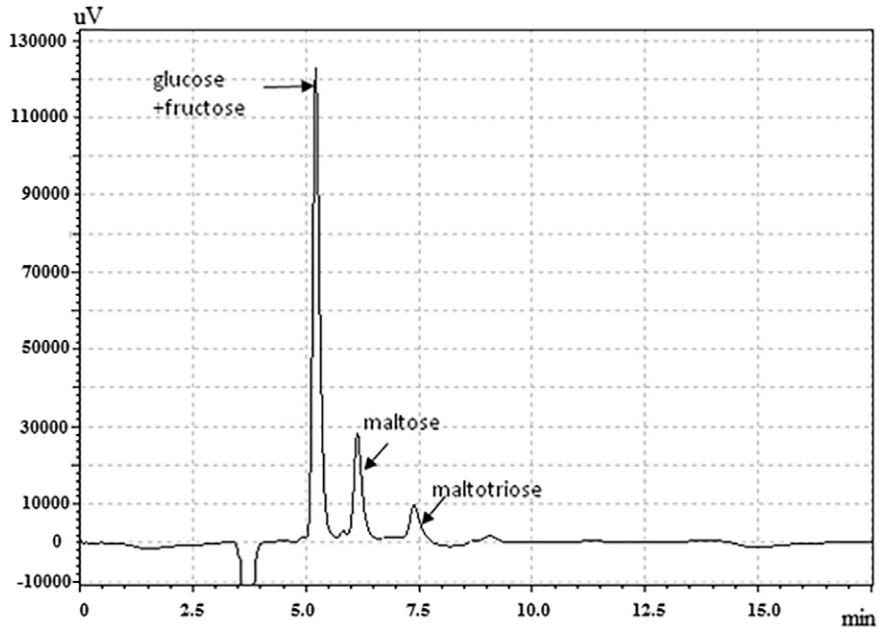

Fig. 6. Analysis of the carbohydrate composition of the wort prepared from ultradispersed starch raw materials by HPLC (device LC20 Prominence (Shimadzu, Japan) column SupelcoGel Silica-LC-NH2).

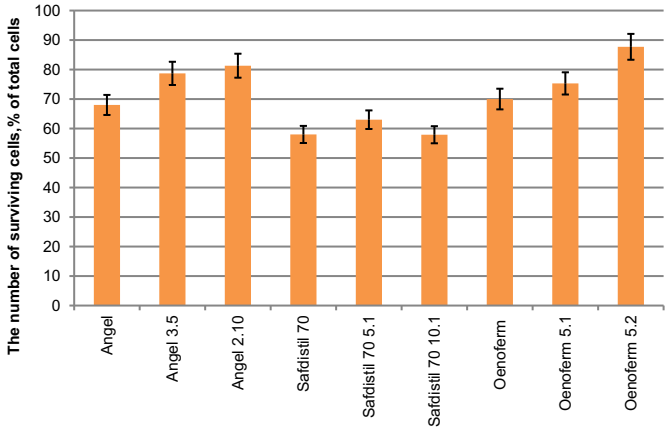

Fig. 8. Viability of native and irradiated yeast strains after incubation in the presence of 20% exogenous ethanol.

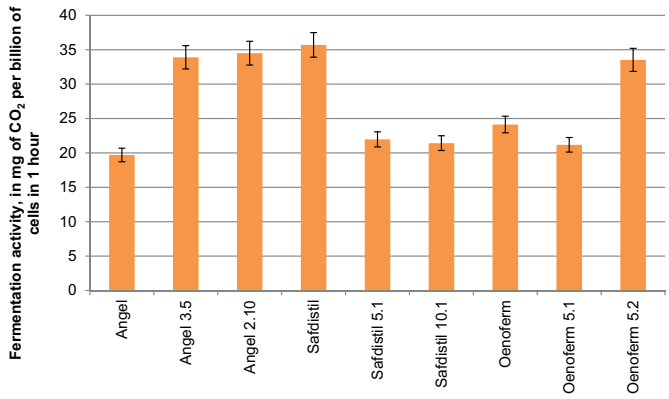

Fig. 9. Fermentation activity of native and irradiated yeast strains.

results are shown in Fig. 8. The fermentation activity of native and irradiated yeasts was also measured (Fig. 9).

As shown in the data presented, the alcohol resistance of the irradiated strains increased, possibly due to yeast mutation during UV irradiation. It is known that UV radiation results in DNA lesions that are both mutagenic and recombinogenic. At high UV doses, most recombination events reflect the repair of two sister chromatids broken at the same position. At lower UV doses, most events involve the repair of a single broken chromatid, and it can cause mutagenesis in different regions [33]. The observed positive effect of the effect of UV radiation is possibly related to the fact that the areas of genes responsible for stress resistance and fermentation of sugars in yeast were affected.

As a criterion of objectivity of the proposed method for determining alcohol resistance, we assessed the correlation between the two datasets, namely percentage of increase in mutant strain viability compared to the original, calculated from the screening results, and

Table 3
Levels of significance.

Level of statistical significance, *P*	Statistical interpretation	Designation in SPSS
$P < 0.001$	Maximum significant	***
$0.001 \le P \le 0.01$	Very significant	**
$0.01 < P \le 0.05$	Significant	*
$0.05 < P \le 0.10$	Weakly significant	
$P > 0.10$	Insignificant	

the alcohol accumulation during wort fermentation. To determine this, we calculated the linear correlation coefficient, i.e., the Pearson's coefficient. Its mathematical expression is represented by [Equation 1]:

$$\mathbf{r}_{XY} = \frac{\mathbf{cov}_{XY}}{\sigma_X \sigma_Y} = \frac{\sum (X-\overline{X})(Y-\overline{Y})}{\sqrt{\sum (X-\overline{X})^2 \sum (Y-\overline{Y})^2}}. \quad \text{[Equation 1]}$$

where.

$\mathbf{cov}_{XY}$ covariance coefficient;
σ_X the standard deviation of random variables X;
σ_Y the standard deviation of random variables Y;
X the arithmetic mean of values of random variables X;
Y the arithmetic mean of values of random variables Y.

The results of the calculation are presented in Table 2. To interpret the significance levels, we used Table 3.

Thus, the correlation is significant at the 0.05 level (two-sided): the event occurred not accidentally with probability 95%. Consequently, we succeeded in obtaining irradiated variants with increased alcohol resistance and performance.

4. Conclusions

The analysis of literature and our experimental data prove the relevance of introduction of thin (up to micro- and nano-sized particles) grinding that allows enzymatic hydrolysis at lower temperatures and atmospheric pressure, which will reduce steam and electricity consumption and increase alcohol yield by reducing sugar losses during raw material cooking. In addition, to increase the alcohol yield, the yeast should be activated, for example, by UV irradiation, which allows an additional 25% increase in the yield on average compared to the native yeast races. Of the studied races, Oenoferm and Angel are optimal for the fermentation of ultradispersed grain raw materials.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Table 2
Coefficient of linear correlation.

Indicator	Array	% of alcohol resistance increase	Alcohol accumulation
Pearson correlation	Percentage of the alcohol resistance increase	1	0.650*
Value (two-sided)			**0.022**
N (number of measurements)		12	12
Pearson correlation	Alcohol accumulation	0.650*	1
Value (two-side)		**0.022**	
N (number of measurements)		12	12

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