

Article

Bioprocess Optimization by Taguchi Design and Response Surface Analysis for Obtaining Active Yeast Used in Vinification

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Abstract: This study presents the behavior of the identified yeast strain *S. cerevisiae*, isolated from Busuioacă de Bohotin grapes from the Pietroasa winery, during the process of fermentation to obtain dry active yeast biomass for the winemaking process. In this respect, we promoted an optimization strategy for obtaining active dry yeast biomass. The cultivation conditions for micropilot fermentation (temperature, pH, carbon source, and nitrogen source) were selected and designed according to a Taguchi design with four factors and three levels. Reproducibility testing was conducted under specific fermentation parameters: temperature (32 °C), pH (4.5), carbon source (12%), and nitrogen source (0.7%). Following the optimization process, two combinations of cultivation parameters were selected, and one of them, based on the results, was selected for further analysis. Quantitative data were obtained, showing dry yeast biomass (DCW) at 1.39 g/100 mL and protein content at 45.57%. The active yeast was then used in the winemaking process for Tămâioasă Românească and Busuioacă de Bohotin varieties at Pietroasa winery for validation. This optimization aims to facilitate easy and rapid production of fresh wine yeast tailored to the local winemaking practices of Pietroasa winery, with real application potential in other viticultural areas, aligning with the terroir concept.

Keywords: fermentation; autochthonous yeast strain; Pietroasa winery; terroir; statistical experimental design; Taguchi design

1. Introduction

The wine industry is one of the most active food industries worldwide, with wine being one of the most popular beverages, both in rural and urban areas. In particular, wine production in Romania has a tradition that dates back thousands of years. According to the 2023 OIV first wine-production estimates, Romania ranks 6th among EU countries in wine production. Moreover, Romania is considered to be one of the major wine producers, with growing production recorded in 2023 (4.4 million hectoliters, 15% more compared

with 2022, which represents one of the largest increases with respect to 2022 worldwide). In addition, according to OIV, Romania ranks 10th among the major vine-growing countries by surface area for the period 2012–2022 [1,2].

A special place among rosé Romanian wines is occupied by Busuioacă de Bohotin, which stands out for its phenolic quality and aroma [3]. Busuioacă de Bohotin grape variety has been cultivated in Romanian vineyards for a long time, being considered a local grape variety. It likely originates either from ancient Greece, as a close relative of Tămâioasă Românească, another aromatic Romanian grape variety, or from France, from the very similar Muscat violet de Frontignan [3].

Currently, wine production is still based on the use of starter cultures of commercial yeasts, a practice that leads, in most cases, to the reduction of biodiversity in the vineyards. In line with the current approach regarding the exploitation of natural biodiversity to generate artificial diversity, the wine industry adopted the technique of selecting microorganism strains with an appropriate profile for certain specific applications [4–9].

Until recently, the only microorganisms that were given importance in wine production were *S. cerevisiae* and, to a lesser extent, *S. bayanus*. From this perspective, for a long time, the starter cultures for wine production were obtained exclusively based on these yeasts, the other yeasts involved in the wine-production process being given not only secondary importance but even aiming to reduce their influence on the fermentation process. Later, it was found that fermentation is a much more complex process, with the production of wine starting to be no longer viewed simplistically from the unique perspective of the alcoholic fermentation of sugars [6,7,10–12].

S. cerevisiae is generally connected with the production of alcoholic beverages, and for this reason, it is defined as a “domesticated” species used for fermenting high-sugar substrates. *S. cerevisiae* is the main yeast used in winemaking due to its high fermentation capacity, and more than 200 different *S. cerevisiae* strains are currently available commercially, with the strains showing highly diverse fermentation properties [13]. For example, [14] investigated the yeast biota of the winery and showed the constant presence of *S. cerevisiae* and the identification of this species as dominant on winery surfaces at pre-harvest time [15,16] or at the end of the fermentation process [11,12,17,18].

Although the dominant species, *S. cerevisiae* is not the only yeast that contributes to grape must fermentation and wine production [19]. While in most cases, *S. cerevisiae* predominates at the end of fermentation, cases have been observed in which certain non-*Saccharomyces* yeasts persist throughout fermentation and even predominate at its end [11,12,18]. The diversity of yeasts in the epiphytic microflora of grape berries is influenced by several factors, among them the variety of grapes, the degree of maturity at harvest, the climate conditions, the geographical location, the degree of damage to the grape berries [11,20,21]. Currently, it is considered that grape must fermentation is a complex process characterized by the interaction between *Saccharomyces* and non-*Saccharomyces* yeasts [19,22–24].

The wine industry widely uses active dry yeasts, thus obtaining wines of consistent quality. The use of starter cultures has great advantages, such as a predictable technological process. In addition, the most suitable yeasts for the fermentation of wine from a certain viticultural area are those that are isolated from the respective region. These yeasts are considered to have the best ability to bring out the specific characteristics of the respective region [5,25]. For this reason, increasing attention is being paid today to the selection and obtaining of starter cultures based on microorganisms obtained from autochthonous strains (*Saccharomyces* and non-*Saccharomyces* species, used individually or as mixed cultures) that are better adapted to the environmental conditions, to the profile of the grape must, and which can clearly express the “terroir” character and the specificity of the wine [6,7,11,25–28].

Therefore, an increasingly pronounced trend is observed for the development or improvement of fermentation control strategies, for example, related to the reduction of utilization of commercial active dry yeasts and the orientation towards the exploitation of

local diversity, in the sense of using active dry yeasts produced from the local microflora. In addition to the advantages for wine producers, who will obtain wines with a strong personality characterized by a more specific aroma and flavor, this approach also contributes to the preservation of local vineyards and the sustainable development of the wine sector [7,8,10,11,21,29,30].

To obtain new yeast strains with superior winemaking properties, which can allow the production of valuable starter cultures, isolation techniques from natural sources and genetic improvement of isolated strains can be applied [11,17]. According to the already established techniques, the isolated strains are then subjected to preliminary laboratory tests to verify their properties in terms of wine-production potential, followed by fermentation tests at the pilot level, which allow the evaluation of the sensory characteristics of the wines obtained. The yeast strains that successfully pass these tests are then checked from the point of view of stability and additional tests are performed to validate the respective strains that finally allow the obtaining of starter cultures [8,12,29].

The practical solution of production of fresh wine yeast has already been proposed and applied in the wine industry as a viable, practical solution for local winemaking to improve the performance of commercial wineries. This strategy was successfully applied to produce good to excellent wines with lower production costs [30].

However, to validate and upscale an effective fermentation strategy, optimization studies for the production of starter cultures are required. Thus, several optimization solutions were explored based on the metabolism of yeasts, which can be respiratory or/and fermentative, depending on the culture conditions (e.g., pH and temperature), the nature and the characteristics of the substrate (such as the concentration of dissolved oxygen, sugar), as well as on the difference of the behavior of the yeast strains between laboratory and industry environment. Because of the complexity and limited understanding of microbial systems, it is very difficult to perform a mathematical model quantitatively describing these biological systems. [6,12,15].

Optimization represents an essential approach for all processes, including industrial biotechnological applications, to assess and predict the behavior, evolution, and differences between the characteristics of the yeast strains at the laboratory and industry scale. The traditional optimization of a production process is tackled using an experimental design by a statistical approach [31]. Regarding the fermentation processes in the wine industry, the optimization strategies of wine yeasts involve the exploitation of the existing diversity inside the *Saccharomyces* as well as non-*Saccharomyces* genus, and then the creation of new phenotypes [27,32,33], including obtaining yeast biomass as well as its behavior at wine during fermentation.

Besides enological applications, optimization principles and concepts are successfully applied in other food fermentation sectors such as brewing [34,35] or vinegar industry [36].

Konstantins Dubencovs [37] described the optimization of the synthetic growth medium and feeding solution compositions (in terms of carbon, nitrogen, phosphorous, magnesium, and calcium concentrations) for high cell density *K. marxianus* fed-batch cultivations. The media composition based on glucose, urea, and phosphate concentrations was varied, and incubation temperature was established at 30 °C. The laboratory-scale cultivations were performed in a 5.4 L working volume vessel.

The constant parameters for yeast cultivation tested by M. Ciani and F. Comitini (2019) [14] were temperature (at 30 °C) and pH (at 5.5 ± 0.2).

Similarly, Schnierda et al. (2014) [38] used non-*Saccharomyces* wine yeast species *Lachancea thermotolerans*, *Metschnikowia pulcherrima*, and *Issatchenkia orientalis* with different nitrogen and carbon sources on biomass production (yields of 0.7 and 0.8 g⁻¹ were achieved in aerobic bioreactor cultures).

Although the rapid Taguchi methodology has made extensive contributions to the optimization of many industrial applications in terms of robustness and quality, its application as a statistical tool for biotechnological applications such as fermentation and food processing is still limited [31,39–41].

The Taguchi design methodology is extensively used for the optimization of different bioprocesses, including the production of yeast biomass [42–46]. For example, the study performed by Barbulescu et al. in 2021 [40] describes the optimization of the fermentation conditions for brewing yeast biomass production using the Response Surface Methodology and Taguchi Technique.

This paper presents the optimization process for obtaining dry yeast biomass for the winemaking process at Pietroasa winery [47]. In particular, the optimization process of obtaining active dry yeast biomass based on the yeast strain isolated from the Pietroasa area, Buzău County, Romania, and molecularly identified as *S. cerevisiae* is described, followed using this biomass for the production of Tămâioasă Românească and Busuioacă de Bohotin wines.

2. Materials and Methods

The yeast strain *S. cerevisiae* PC3 was isolated from Busuioacă de Bohotin grape pomace from Pietroasa winery and was identified by RFLP analysis of 5.8S-ITS region [48] as *S. cerevisiae*. The species level of the PC 3 strain was confirmed by sequencing the 5.8S rDNA region in both directions using the universal primers ITS1 and ITS4 [49]. The obtained sequences were aligned with Clustal Omega (<https://www.ebi.ac.uk/jdispatcher/msa/clustalo>, accessed on 18 November 2023). The submitted sequences were aligned with different sequences available in the National Center for Biotechnology Information (NCBI) database using the BLASTN tool (<http://www.ncbi.nlm.nih.gov/BLAST/>, accessed on 18 November 2023) based on similarity percentages. The PC 3 strain was identified as *S. cerevisiae* with 99.88% homology to various GenBank sequences. The sequence of the PC3 strain is also available for download under the accession number PP534168.

The yeast strain was maintained as stock culture on a slant tube with YPSM agarized culture medium (yeast extract, peptone hy–soy, sucrose, malt extract, agar–agar).

Agar–agar, sucrose, glucose, and yeast extract were purchased from Sigma–Aldrich, malt extract, peptone hy–soy from VWR Life Science, and KCl, $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, $\text{NH}_4\text{H}_2\text{PO}_4$ from Merck KGaA (Darmstadt, Germany) and AppliChem GmbH (Darmstadt, Germany).

2.1. Biotechnological Investigation

The biotechnological experimental studies include the upstream process (obtaining the preinoculum; obtaining the liquid inoculum; micropilot fermentation), followed by the downstream process (mechanical separation via centrifugation; purification through washing with sterile distilled water; drying through the freeze-drying process).

2.1.1. Obtaining Solid Culture (Preinoculum)

The culture medium used for strain maintenance and preinoculum was YSP based on yeast extract, sucrose, and peptone hy–soy agar, with the following composition: yeast extract—0.5%; sucrose 2%; peptone hy–soy 0.7%; agar 2.5%, sterilized at 115 °C for 10 min.

2.1.2. Obtaining the Liquid Inoculum

Inoculum culture was prepared using the following recipe: yeast extract 0.5%, peptone hy–soy 0.5%, and sucrose 6%. A total of 600 mL (150 mL medium/500 mL flask) of inoculum medium culture was distributed into 4 Erlenmeyer flasks (500 mL each) and sterilized at 115 °C for 10 min. After cooling the medium to room temperature, each flask was inoculated with 2 pre-inoculum static cultures per 150 mL of medium. Subsequently, the freshly inoculated Erlenmeyer flasks were placed in an Optic Ivymen (Barcelona, Spain) incubator shaker and incubated for 20–24 h at 30 °C with a shaking speed of 200–240 rpm.

2.1.3. Micropilot Fermentation

For the micropilot fermentation process, a 4 L Biostat B PLUS fermenter (Sartorius, Göttingen, Germany) was used. The culture media used consisted of yeast extract—0.7%, $\text{NH}_4\text{H}_2\text{PO}_4$ —0.07%, KCl—0.05%, $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ —0.05%, and white sugar—7% was

added to the bioreactor vessel and sterilized together at 121 °C for 20 min. After the sterilization process, the bioreactor containing the culture media was brought to the working parameters: temperature—30 °C, stirring rate—250 rpm, flow of O₂—0.5 L/min. The inoculation ratio with the previously developed inoculum was 15% (i.e., 600 mL inoculum for a bioreactor working volume of 4 L). During the fermentation, the pH level was adjusted with a 5% ammonium hydroxide solution and was automatically controlled at the set point, 1–2 drops of antifoam silicone SNAPSIL FD 10 solution (WWR Life Science, Radnor, PA, USA) were added to break the foam.

2.1.4. Downstream Process

After fermentation completion, the fermented medium was aseptically transferred from the bioreactor vessel into presterilized 1 L flasks. It was then allowed to settle at refrigerator temperature (4 °C) for 24 h before undergoing mechanical separation via centrifugation to obtain the yeast biomass. The purification process involved multiple washes with sterile distilled water at speeds ranging from 3500 to 4500 rpm for 5 min each, using a Sorvall ST 16 Centrifuge (400 mL × 4) (Thermo Fisher Scientific Inc., Waltham, MA, USA). The freshly obtained biomass was then subjected to drying through a freeze-drying process.

The yeast biomass samples were frozen in the freezer at −80 °C for 24 h, followed by the freeze-drying program (Table 1).

Table 1. Freeze-drying process stages.

	1. Freezing	2. Sample Loading	3. Freezing	4. Main Freeze-Drying	5. Main Freeze-Drying	6. Main Freeze-Drying	7. Main Freeze-Drying	8. Main Freeze-Drying	9. Final Freeze-Drying	10. Final Freeze-Drying	11. Final Freeze-Drying
Temperature (°C)	−40	−40	−40	−40	−40	10	20	35	30	30	35
Pressure (mbar)	0	0	0	0.1	0.1	0.1	0.1	0.1	0.1	0.01	0.01
Time (minutes)	90	15	210	15	285	600	480	720	15	45	420

After completing the freeze-drying cycle, the samples were placed in polyethylene bags with plastic zippers (Figure 1) and stored at room temperature in a dark and dry place.



Figure 1. Yeast biomass after freeze-drying process.

2.2. Experimental Design to Optimize Yeast Production

The influence of cultivation parameters as operational conditions on some yeast characteristics was studied. In this respect, biomass production was conducted using a fractional factorial experimental Taguchi design involving 4 factors at 3 levels each. The four independent variables (X_i) selected were temperature (X_1), pH (X_2), carbon source (X_3), and yeast extract (X_4). The coded levels for each independent variable were 1 for low level, 2 for medium level, and 3 for high level, respectively. The response parameters or dependent variables (Y_i) set up were dry biomass, DCW (g/100 mL), and protein (%).

The independent and dependent variables in coded form, together with the corresponding constraints imposed by obtaining certain characteristics of Y_1 and Y_2 , are given in Table 2.

Table 2. Independent and dependent variables; experimental conditions in the fractional factorial plan with 4 factors at 3 levels.

Independent Variables	Coded Symbol	Levels of Variation in Coded and Physical Form		
		Low (1)	Middle (2)	High (3)
Temperature, T (°C)	X_1	28	30	32
pH	X_2	4.5	4.7	4.9
Carbon source, SC (%)	X_3	12	14	16
Yeast extract, ED (%)	X_4	0.5	0.6	0.7
Dependent variables	Coded Symbol	Constraints		
Dry biomass, DCW (g/100 mL)	Y_1	Maximize		
Protein content, PC (%)	Y_2	Maximize		

Different routines of the Statistica StatSoft Release software v10 were used for the experimental data analysis. Our previous studies extensively detailed the steps involved in the optimization analysis [40,50–53]. Briefly, to determine the quadratic polynomial equations for the system responses, the “stepwise regression” routine was used with the “backward elimination” subroutine, which involves the automatic elimination of statistically insignificant terms ($p > 0.05$). The validation of the reduced regressional models was carried out by testing the correspondence of the model (goodness of fit), respectively, by analysis of variance (ANOVA) and residual analysis. The relationship between each dependent variable and the independent variables was further investigated through the response surfaces methodology, respectively, through 3D graphics. The Taguchi technique, expressed by the signal-to-noise performance indicator, was applied as the final stage of the optimization process to establish the combinations of the influencing factors that sharpen product quality and process robustness.

2.3. Winemaking Process for Tămâioasă Românească and Busuioacă de Bohotin

The grapes, harvested into bins/trailers of 2 tons each, were transported to the wine-making line. Upon unloading in the reception bunker, the grapes were directed to the destemming machine and crushed. Following this process, the grape must passed through the tube-in-tube heat exchanger, where the entire mass reached a temperature of 12–14 °C, before being transferred to the pneumatic press. After pressing, sulfur dioxide (SO₂) was added for oxidative protection, and the grape must was transported to a stainless steel vessel for clarification with the aid of deburring enzymes. Subsequently, the clarified grape must was transferred to special micro-sample vessels (8-L vessels). For the alcoholic fermentation of grape must, active yeast biomass obtained from the yeast selected from Pietroasa winery, commercial yeasts, and organic nutrients were used. A total of 3 types of wine were made for each type of grape must: Busuioacă de Bohotin (BB PC3, BB Control, BB TC) and Tămâioasă Românească (TR PC3, TR Control, TR TC). The samples labeled as “PC3” utilized the newly isolated and identified *S. cerevisiae*-PC3 strain (accession number PP534168), while the “Control” samples underwent wild fermentation (without yeast addition), and the “TC” (technological control) samples were fermented with commercial yeasts. After fermentation (2–3 weeks) at an ambient temperature of 15–19 °C within the production facilities at the Pietroasa winery, the wines were conditioned, transferred into 750 mL bottles, and stored at ambient temperature.

2.4. Analytical Investigation

2.4.1. Determination of Dry Matter Content

Biomass obtained by freeze-drying in the experimental optimization matrix was analyzed for total dry matter content and moisture using a Pheonix thermobalance following the experimental procedure outlined by Barbulescu et al. in 2021 [40].

2.4.2. Determination of Protein Content

Protein content was determined using a EuroVector EA3100 CHNS. Well-homogenized yeast biomass samples were heated in a high-temperature furnace in which combustion occurs rapidly, at a temperature above 900 °C in the presence of pure oxygen. It produces mainly water, carbon dioxide, and nitrogen as several oxides (NyOx). This gas mixture passes through a reduction chamber containing heated copper. This stage converts nitrogen oxides into elemental nitrogen and collects excess oxygen. The gases are passed through a column where they are separated, and the total nitrogen and carbon content is measured by a thermal conductivity detector [54]. A conversion factor of 6.25 was used to calculate the protein content based on nitrogen determination ($N \times 6.25$).

2.4.3. Ethanol Content

The determination of the alcoholic strength by volume (% vol) of wine was performed by the OIV-OENO 601B-2021 [55] method, using a hydrostatic balance. As equipment, an oenological electronic distilling unit (Super D.E.E., Gibertini; Milano, Italy) and a hydrostatic balance (Densi Alcomat, Gibertini; Milano, Italy) were used.

2.4.4. Yeast Biomass Viability

Yeast biomass viability was performed using the methodology described by Barbulescu et al. in 2021 [40].

2.5. Sensory Characterization

Tasting is one of the most relevant ways to appreciate the quality of wine and was conducted for each wine individually according to the current OIV specifications [55], as well as standards ISO 8589:2010 and ISO 3591:1997 [56,57]. The jury was informed of the details of the tasting, the type, and number of samples to be analyzed, and were aware of possible alcohol ingestion. The sensory characteristics of the experimental wine samples were evaluated by a professional panel of 20 experimented tasters represented by qualified laboratory personnel and researchers, men and women aged between 25 and 65. All panel members are experienced in sensory evaluation and authorized to participate in wine competitions. Considering the large number of samples, the tasting was organized in two sessions to ensure the objectivity of the results. The most important characteristics of the wines were analyzed and noted in the tasting sheet. Visual characteristics were those that differentiated the wines based on color and clarity. Olfactory characteristics are those that classify the impact of volatile substances, considering the intensity and overall quality of the aroma. Finally, taste is noted for the most key descriptors, such as aromas, intensity, bitterness, and acidity. These are also found in the form of general notes that can encompass multiple factors that together can create a favorable perception of the wine.

3. Results and Discussion

The stages of the fermentation process at the micropilot level include the preparation of the static culture and liquid inoculum, followed by micropilot fermentation and downstream processing. The static culture was prepared using a specific culture medium, while the liquid inoculum was developed through a controlled incubation process. Subsequently, micropilot fermentation was conducted in a Biostat B PLUS fermenter under conditions defined by the experimental matrix. Throughout the process, parameters such as temperature, stirring rate, and oxygen flow were monitored and adjusted as specified by the experimental matrix. The downstream process involved transferring the fermented

medium into flasks, followed by mechanical separation via centrifugation to obtain yeast biomass. The yeast was washed and freeze-dried to create a stable product suitable for use in winemaking. Figure 2 illustrates the entire biotechnological process of obtaining dry active yeast biomass.

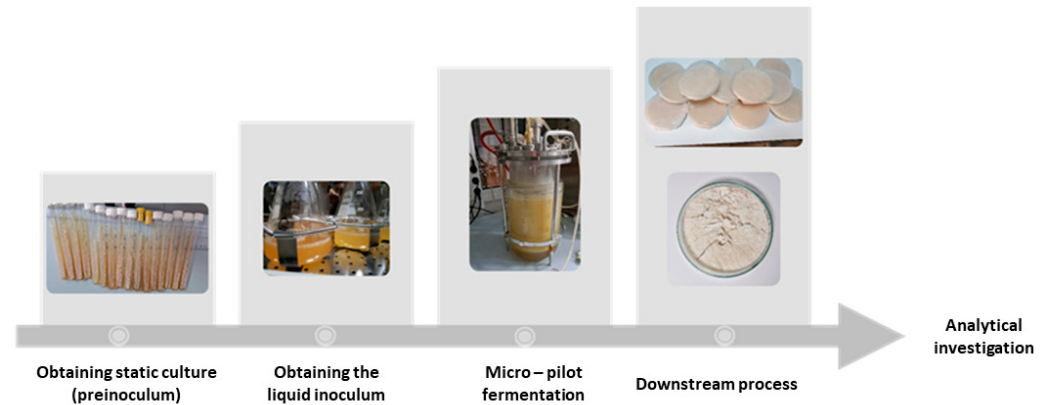


Figure 2. Biotechnological process for yeast biomass.

The experiments from the Taguchi experimental matrix were subjected to an optimization technique based on the classical design of experiments combined with the analysis of response surfaces and the Taguchi technique to determine the influence of some cultivation parameters as operational parameters on dry biomass and protein (Table 3). This optimization approach was successfully used by the authors at the laboratory scale in the field of drug delivery systems [51,52,58,59], at the pilot level [40], as well as at the industrial level [50] to obtain the best values, and the most stable, robust and insensitive to the noise factor responses for different physical–chemical and biopharmaceutical parameters or operational conditions in various (bio)technological processes [51].

Table 3. Experimental trial design: fractional matrix 3^4 –coded and real values of independent variables; observed and predicted system responses.

Exp. No.	Independent Variables (Coded Level/Real Level)				System Responses			
	X_1	X_2	X_3	X_4	Y_1 -DCW (g/100 mL)		Y_2 -Protein (%) *	
	T (°C)	PH	SC (g/100 mL)	YE (g/100 mL)	Obs.	Pred.	Obs.	Pred.
1	1 (28)	1 (4.5)	1 (12)	1 (0.5)	1.16	1.18	48.47	48.43
2	1 (28)	2 (4.7)	2 (14)	2 (0.6)	0.51	0.49	50.47	50.21
3	1 (28)	3 (4.9)	3 (16)	3 (0.7)	0.93	0.95	47.82	47.80
4	2 (30)	1 (4.5)	2 (14)	3 (0.7)	1.11	1.06	47.23	47.61
5	2 (30)	2 (4.7)	3 (16)	1 (0.5)	0.56	0.57	47.98	48.14
6	2 (30)	3 (4.9)	1 (12)	2 (0.6)	1.01	1.02	49.21	49.27
7	3 (32)	1 (4.5)	3 (16)	2 (0.6)	0.98	1.01	45.18	44.92
8	3 (32)	2 (4.7)	1 (12)	3 (0.7)	1.05	1.07	44.15	44.01
9	3 (32)	3 (4.9)	2 (14)	1 (0.5)	1.33	1.29	41.24	41.35

Legend: T—temperature; SC—sugar content; YE—yeast extract; DCW—Dry cell weight. * calculated using nitrogen to a protein conversion factor of 6.25.

To establish the reduced quadratic polynomial equations for the system responses, stepwise regression analysis was performed with the “backward elimination” subroutine. The quantitative effect of the independent variables (in the linear and quadratic form) and their interaction with system responses is demonstrated by the regression models from Equations (1) and (2).

$$Y_1 = 257.553 - 101.860X_2 - 2.739X_3 + 9.997X_2^2 - 4.915X_4^2 + 0.199X_1X_4 + 0.572X_2X_3 \quad (1)$$

$$Y_2 = 195.203 - 21.265X_3 - 0.293X_1^2 + 0.954X_1X_3 + 3.897X_1X_4 + 15.615X_2X_4 - 12.051X_3X_4 \quad (2)$$

As both responses are to be maximized the synergistic effect is given by the positive coefficients and the antagonistic effect by the negative ones. Thus, the independent variables X_2 and X_3 in linear form and variable X_4 in quadratic form are negatively influencing the response Y_1 , while variable X_2 in quadratic form and the interactions between X_1 and X_4 and X_2 and X_3 have a positive influence on the same response. Concerning Y_2 , the positive effect on this response is given by the interactions between X_1 and X_3 , X_1 and X_4 , and X_2 and X_4 . The negative influence is given by X_1 in quadratic form and the interaction between X_3 and X_4 .

The predictive power of these models was assessed by correlation coefficient (R) and determination coefficient (R^2) values, ANOVA analysis of variance and residual analysis (in graphic and analytical form). The values of the correlation coefficients 0.9941 and 0.9973, respectively, show a good correlation between the observed and predictive values. The determination coefficients are 0.9883 and 0.9947, showing that only 1.17% and 0.83% of the total system response variation are not explained by the reduced regression models.

The results of the ANOVA test are presented in Table 4, demonstrating the statistical significance of the regression models.

Table 4. Analysis of variance for reduced polynomial regression models.

Responses	Sources of Variation	Sum of Squares	df	Mean of Squares	F	p
Y_1	Regression	0.567	6	0.094	28.260	<0.05
	Residual	0.007	2	0.003		
	Total	0.574	8			
Y_2	Regression	64.837	6	10.806	62.63	<0.05
	Residual	0.345	2	0.172		
	Total	65.181	8			

The residual analysis presented in Table 4 also shows a good correlation between the experimental and theoretical values and is sustained by the linear distribution from Figure 3a,b. The residual analysis is also supported by the design normality validated through normal probability plots of residuals, with almost a linear distribution trend of the responses, indicating the design robustness (from Figure 4a,b).

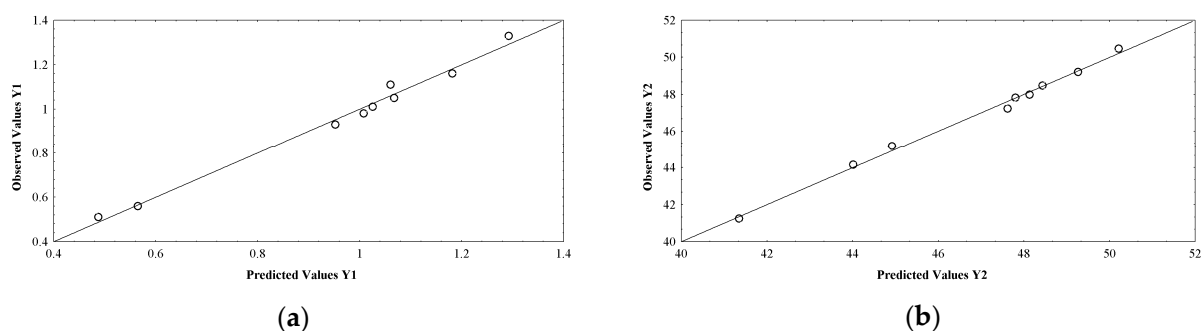


Figure 3. (a,b). Plots showing the correlation between experimental and theoretical values for (a) dry biomass (g/100 mL) and (b) protein content (%).

The statistical tests performed indicate a good predictive power of these reduced regression models, as well as the possibility of their use in the determination of dry biomass and protein and for other combinations between the process variables represented by the cultivation parameters.

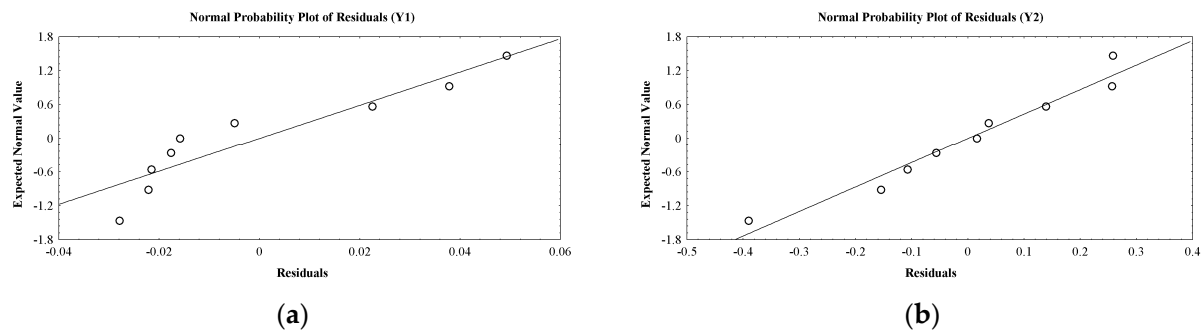


Figure 4. (a,b). Plots showing the correlation between expected normal values and residuals for (a) dry biomass (g/100 mL) and (b) protein content (%).

The relationship between each wine yeast response and the cultivation parameters selected to obtain certain types of biomasses is further illustrated using response surfaces that allow visualization of the effects of the independent variables on the dependent variables in three-dimensional space. (Figures 5a–f and 6a–f).

From Figure 5a, it can be seen that high values of temperature and pH cause an increase in the percentage of dry biomass, reaching a maximum at 32 °C and pH 4.9. Specifically, the Y1 value increases by 1.15 times compared to the scenario where both formulation factors are at their minimum values. This suggests that *S. cerevisiae* yeast responds positively to increased temperature and pH within the studied limits. This behavior can be attributed to the optimal enzymatic activity and metabolic rates that occur at these specific conditions. Enzymes involved in yeast metabolism are sensitive to temperature and pH, which likely explains the observed increase in biomass.

The combination of carbon source and temperature formulation factors leads to moderate variations in the Y1 response. At high temperatures and low carbon sources, biomass is maximized at 1.05%, while at low temperatures and high carbon sources, biomass decreases to 0.93%, indicating a 21% decrease. This relationship, as shown in Figure 5b, indicates a combined influence of these two factors. While temperature plays a dominant role, the carbon source concentration also significantly influences yeast growth, serving as the primary substrate for energy and biomass production, and its availability can impact the efficiency of metabolic processes.

At the temperature variation between the minimum and maximum level and keeping the yeast extract at the minimum level, important variations of Y₁ are recorded, the values passing from 1.16% (minimum temperature) to 0.56% (average temperature) and, respectively, to 1.33% (maximum temperature), i.e., a 2.07-fold decrease followed by a 2.38-fold increase (Figure 5c). The significant fluctuations in biomass observed with variations in temperature and yeast extract indicate that yeast extract supplies crucial nutrients, such as amino acids and vitamins, which are vital for yeast growth.

Figure 5d shows that a low value of dry biomass (0.51 g/100 mL) is obtained for medium values of pH and carbon source, while the maximum percentage of Y₁ (1.33%) is obtained for maximum values of pH and average carbon source values (138% increase). At the same time, a greater influence of pH on the percentage of dry biomass is observed compared to the carbon source, suggesting that yeast enzymatic systems have a higher sensitivity to changes in pH. Maintaining an optimal pH is crucial for proper enzyme activity and nutrient solubility, which in turn promotes yeast proliferation.

From Figure 5e it follows that the independent variables pH and yeast extract have a similar influence on the percentage of dry biomass, the minimum values of Y₁ being recorded for average values of X₂ and X₄, respectively. The minimum and maximum values of the parameter X₂, in combination with the minimum and maximum values of the parameter X₄, result in high amounts of dry biomass, with Y₁ varying between 0.93% and 1.33% (43% variation).

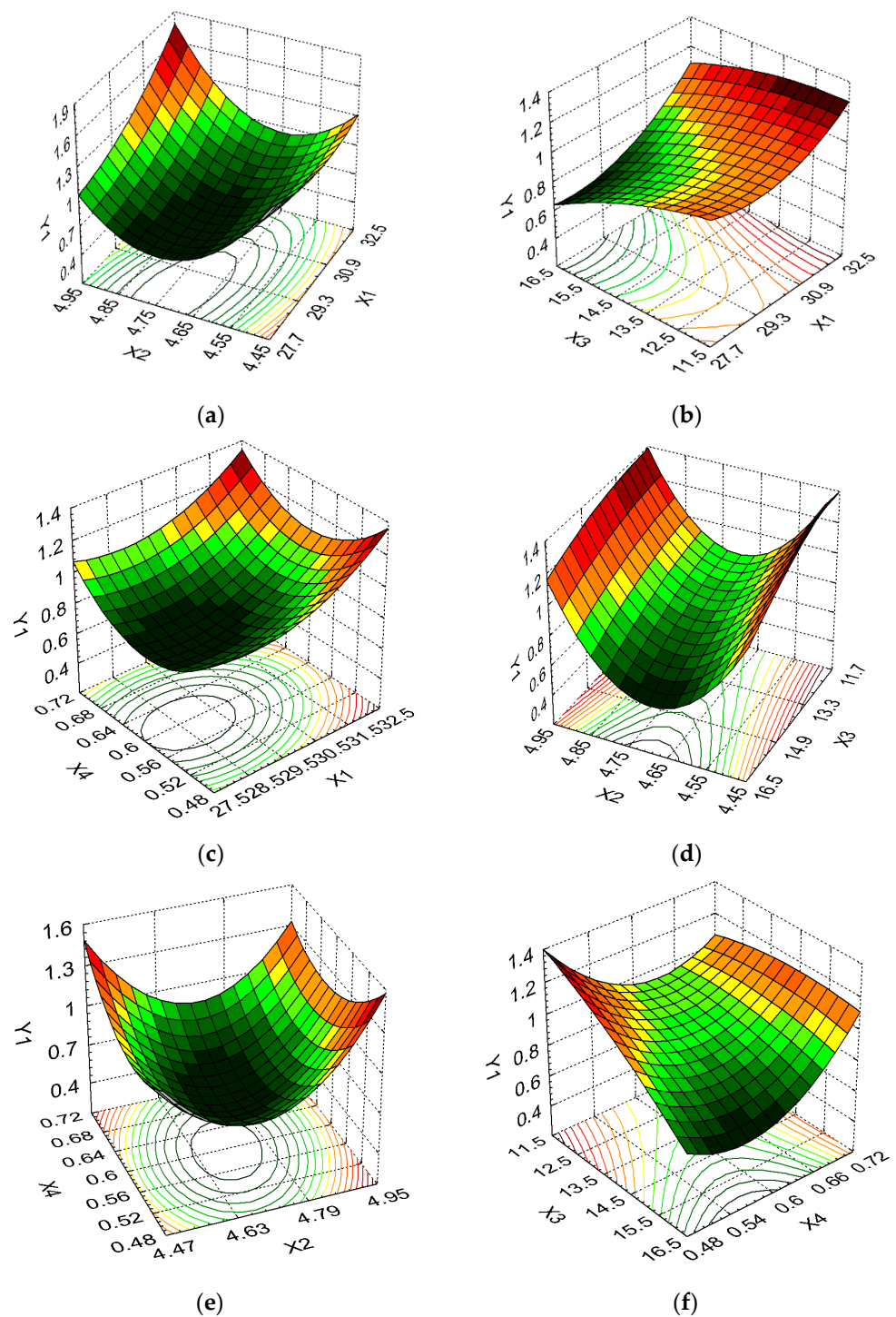


Figure 5. Three-dimensional response surface and contour levels indicating the effect of different cultivation parameters on dry biomass response (Y_1): (a) temperature (X_1) and pH (X_2); (b) temperature (X_1) and carbon source (X_3); (c) temperature (X_1) and yeast extract (X_4); (d) pH (X_2) and carbon source (X_3); (e) pH (X_2) and yeast extract (X_4); (f) carbon source (X_3) and yeast extract (X_4). The dark red color refers to the maximum values for the dependent variable Y_1 , and the green color to their minimum values.

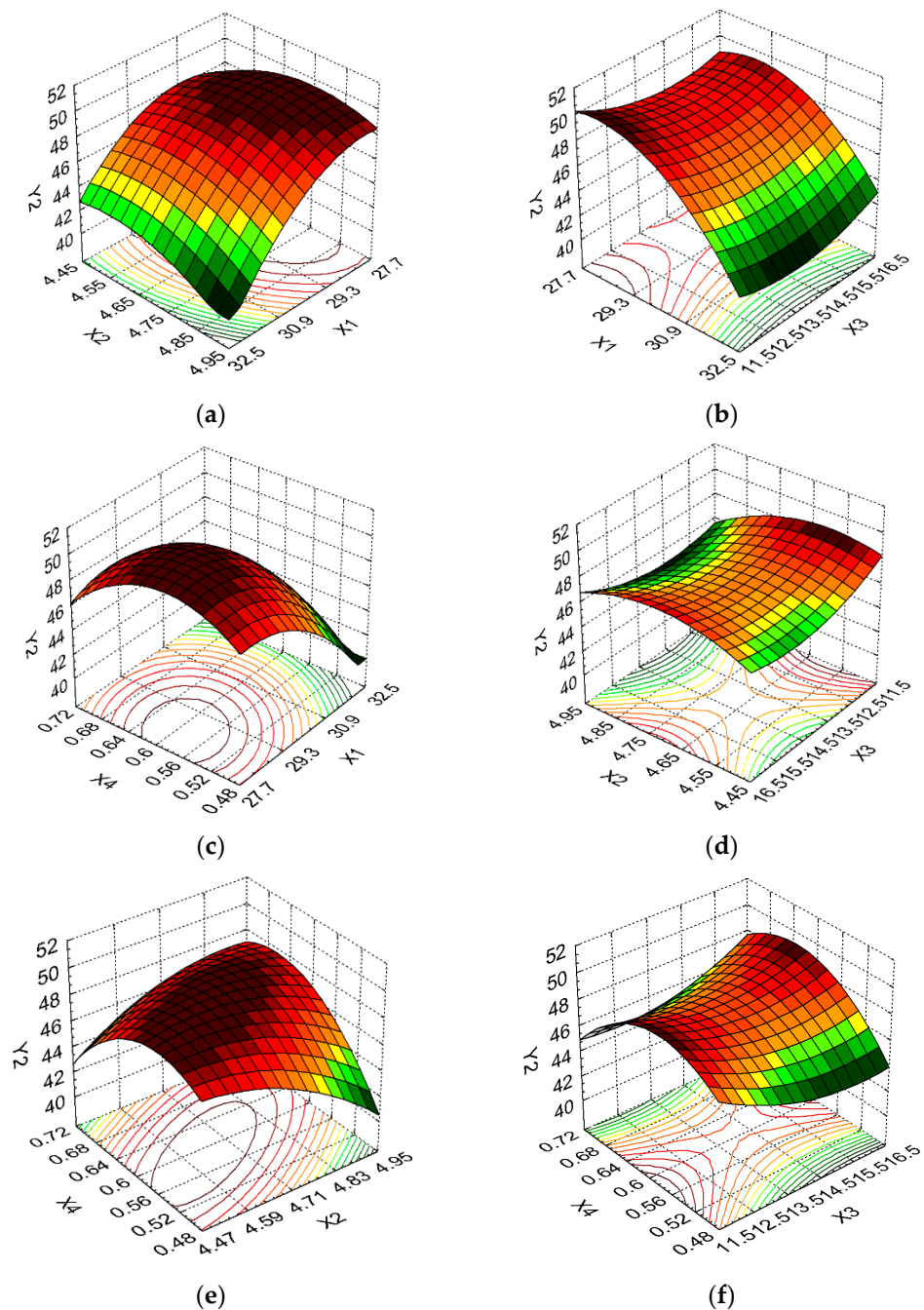


Figure 6. Three-dimensional response surface and contour levels indicating the effect of different cultivation parameters on the protein content response (Y_2): (a) temperature (X_1) and pH (X_2); (b) temperature (X_1) and carbon source (X_3); (c) temperature (X_1) and yeast extract (X_4); (d) pH (X_2) and carbon source (X_3); (e) pH (X_2) and yeast extract (X_4); (f) carbon source (X_3) and yeast extract (X_4). The dark red color refers to the maximum values for the dependent variable Y_2 , and the green color to their minimum values.

A similar influence of the combination of independent variables pH/carbon source on dry biomass is also noted for the pair of independent variables carbon source/yeast extract (Figure 5f). The Y_1 value thus increases from a minimum value of 0.51 g/100 mL when X_3 and X_4 are at the medium level of variation to a value of 1.11% (118% variation) for the carbon source at the medium level of variation and the extract of yeast at the maximum level of variation. This indicates a complex interaction between these two factors, both being crucial for optimal yeast growth.

The protein content is strongly influenced by temperature, the aspect resulting from Figure 6a,b. Thus, high values of protein content are noted at the minimum temperature variation level for both the combination with the cultivation parameter pH and the cultivation parameter carbon source. An increase in temperature from the minimum to the maximum level of variation when the pH is kept at the medium level of variation leads to a significant decrease in Y_2 , from 50.47% to 44.15% (decrease of 12.52%), a phenomenon also observed with an increase in temperature and a carbon source at the minimum level of variation (decrease of 10.28% in protein content, from 49.21% to 44.15%). The decrease in protein content with increasing temperature may suggest that higher temperatures might denature proteins or disrupt protein synthesis pathways.

For the combination of temperature formulation factors and yeast extract, when X_1 is kept at the minimum value, Y_2 shows an increase of 4.12% (from 48.47% to 50.47%) for the variation of X_4 from the minimum level to the medium level of variation, followed by a decrease of 5.25% when X_4 passes from the medium level to the maximum level of variation (as shown in Figure 6c). These indicate that optimal nutrient availability is crucial for protein synthesis. At lower temperatures, yeast extract likely provides sufficient nutrients to support high protein synthesis.

A moderate decrease in protein content is noted for the variation of carbon source between the minimum and maximum when the pH is kept at the minimum (1.07-fold decrease from 48.47% to 45.18%), as shown in Figure 6d. The moderate influence of pH levels and carbon sources on protein content highlights the necessity of preserving balanced conditions to achieve optimal protein synthesis. Deviations to extreme values in these parameters may result in less-than-optimal production of proteins.

Regarding the combination of formulation factors pH and yeast extract (Figure 6e), an opposite behavior can be observed when one of the formulation variables is kept at the minimum or maximum level, and the other varies between the two extreme values. For example, for X_4 at a minimum level and X_2 variation between minimum and maximum levels, the protein content drops from 48.47% to 41.24% (14.92% decrease), while for X_4 at a maximum level and X_2 variation between the minimum and maximum levels, the protein content increases from 47.23% to 47.82% (1.25% increase).

An increase in protein content is favored by the maximum level of the yeast extract and the variation of the carbon source between the minimum and maximum levels (Figure 6f), Y_2 having values between 44.15% and 47.82%, i.e., an increase of 8.31%.

To obtain the proper combination of crop parameters leading to the targeted responses—dry biomass and protein content—least affected by noise factors, the signal-to-noise ratio (S/N), a performance indicator proposed by Taguchi, was determined in the final stage of the optimization process. The signal represents the desired value of the independent variable, and the noise is the unwanted variability that causes the quality of the optimization process to decrease. The higher its algebraic value, the better the performance of the process. Taking into account the constraints imposed (Table 3), the “larger-the-better” S/N ratio was selected for all response variables [40,50–53].

The influence of the controlled factors on the S/N ratio for each response, resulting in the most stable and robust optimal combination of operational conditions, is shown in Table 5.

The graphical transposition of the effects of the controlled factors on the S/N ratio for each dependent variable, highlighting the optimal combination of process variables, is shown in Figure 7.

From Table 5 and Figure 7a,b, it can be seen that temperature has a significant effect on the S/N ratio for both dry biomass and protein content, higher in the case of Y_2 . X_2 and X_3 have a moderate influence on Y_1 and a low influence on Y_2 , while X_4 has the lowest influence on Y_1 but a moderate one on Y_2 . Thus, in the case of dry biomass, the effect size of variable X_1 on the S/N ratio is 70.71% greater than that of variable X_4 , 23.35% greater than that of variable X_3 , and 163.0% greater than that of variable X_2 , while in the case of protein content the effect size of variable X_1 on the S/N ratio is 370.73% greater than that

of variable X_3 , 208.80% greater than that of variable X_2 and 45.66% greater than that of variable X_4 .

Table 5. Coded optimal combinations of cultivation parameters, the sizes of their effects on the signal-to-noise ratio for the system responses, and the expected S/N values.

Control Factors (Input Variables)	Y_1		Y_2	
	"Larger-the-Better"	Effect Size	"Larger-the-Better"	Effect Size
X_1	3	1.632	1	0.386
X_2	1	1.396	2	0.125
X_3	1	1.323	1	0.082
X_4	3	0.956	2	0.265
S/N ratio expected (dB)	4.584		34.259	

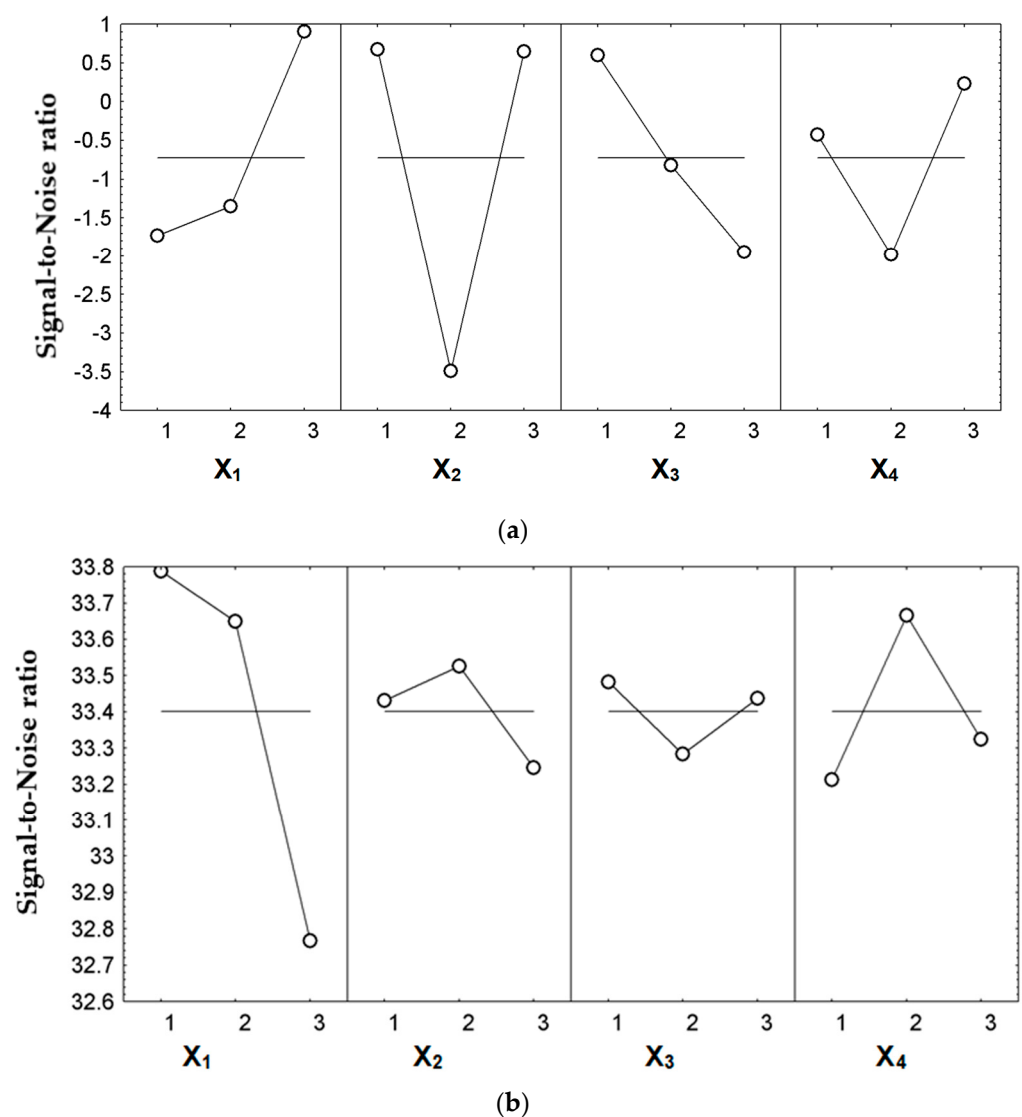


Figure 7. Effects of controlled factors on S/N ratio for the dependent variable: (a) Y_1 —dry biomass (%); (b) Y_2 —protein content.

By applying the Taguchi technique as the final stage of the optimization process, two combinations of cultivation parameters were selected that lead to obtaining dry biomass, respectively, to a protein content as little as possible affected by noise factors.

These combinations of cultivation parameters were not included in the fractional Taguchi experimental matrix. They are coded according to the working temperature in the form D32-3:1:1:3 corresponding to a temperature of 32 °C, pH of 4.5, carbon source of 12% and yeast extract of 0.7% (32:4.5:12:0.7) for dry biomass, respectively, D28-1:2:1:2 corresponding to a temperature of 28 °C, pH of 4.7, carbon source of 12% and yeast extract of 0.6% (28:4.7:12:0.6) for the protein content.

Furthermore, the wine yeast biomass was obtained at the micropilot level, applying the previously established operational conditions. For the wine yeast coded D32, 1.39 g/100 mL dry biomass and 45.57% protein content were obtained, and for the wine yeast coded D28, values of 0.71 g/100 mL for dry biomass and 48.85% protein content, respectively.

Yeast moisture of the freeze-dried cells was less than 1% *w/w*.

The protein content in the yeast biomass was between 41.24% and 50.47% [60]. Active yeast biomass is obtained in smaller quantities and with a slightly lower protein than beer yeast biomass [38].

Table 6 presents the viability values obtained for the fermentations conducted. Much higher values can be observed for the two validation fermentations (FV1A and FV1B), corresponding to experiments with codes D32 and D28.

Table 6. Yeast biomass viability from the experimental variants.

No. of Sample	Experimental Variants	CFU/mL
1	F01	0.8×10^{11}
2	F02	0.5×10^{11}
3	F03	0.4×10^{11}
4	F04	0.5×10^{11}
5	F05	0.8×10^{11}
6	F06	0.7×10^{11}
7	F07	0.7×10^{11}
8	F08	0.6×10^{11}
9	F09	0.5×10^{11}
10	FV1A	1.3×10^{11}
11	FV1B	1.2×10^{11}

Based on the optimization results and taking into account cell viability, the following option (D32 with 1.39 g/100 mL dry biomass and 45.57% protein content) was selected as the optimal technological variant for the next experimental stages.

Table 7 presents the alcohol content of the obtained wines. The results obtained show that the newly isolated yeast strain *S. cerevisiae* PC3 can ferment sugars, leading to a higher alcohol content compared to wild fermentation (Control) or Technological Control (TC).

Table 7. Alcohol content (% *v/v*) of wine samples.

Variety	Coding	Alcohol Content (% <i>v/v</i>)
Busuioacă de Bohotin rosé wine	BB PC3	14.6
	BB Control	13.1
	BB TC	11.1
Tămâioasă românească white wine	TR PC3	14.6
	TR Control	13.7
	TR TC	13.9

In Figure 8, it is evident how BB TC wine tends to lean more towards the ester side. The aromas found in BB TC wine are much more intense and tend toward floral and tropical fruit notes, as well as ripe fruit. BB PC3 wine tends to be much more neutral and discreet in aromas.

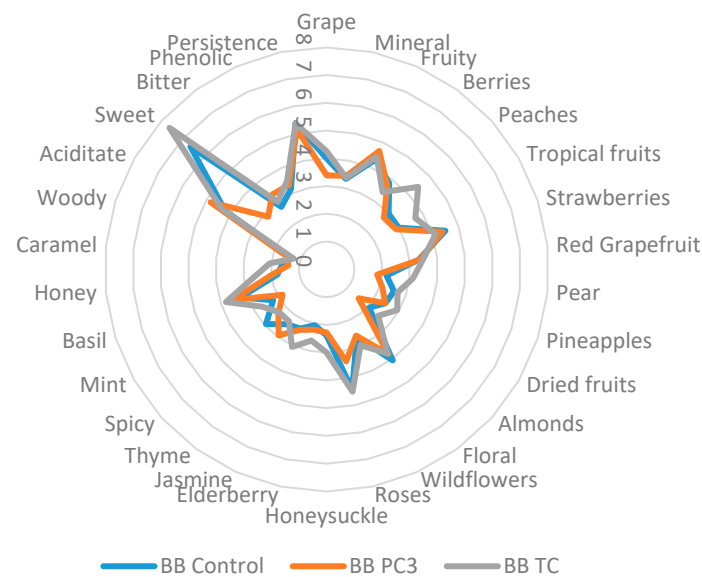


Figure 8. Sensorial evaluation of Busuioacă de Bohotin wines.

For the Tămâioasă Românească wines, commercial yeast used for Tămâioasă Românească wine reflects the same situation as at Busuioacă de Bohotin and tends to offer a wine that is much richer in aromas, which are perceived more clearly by the tasting panel (Figure 9). Crafted specifically to have strong and clean scents, the selected commercial yeasts have led TR TC wine towards the fruity tropical zone. TR Control wine and TR PC3 wine were much more reserved in aroma intensity but provided an additional structure and body to the wine obtained.

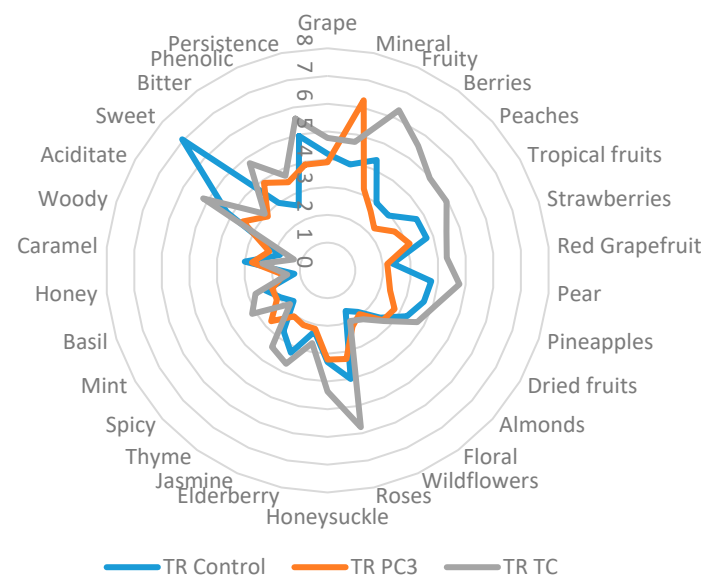


Figure 9. Sensorial evaluation of Tămâioasă Românească wines.

4. Conclusions

The optimization of the process of obtaining active yeast biomass was performed in the present work by fine-tuning some fermentation process parameters and nutrients using Taguchi techniques.

The best solution to reach high yeast biomass concentrations in the reaction medium was identified for the combined optimizing parameters process temperature (32 °C), pH (4.5), carbon source (12%), and nitrogen source as yeast extract (0.7%). The indigenous yeasts biomass obtained through the application of the experimented optimal cultivation

conditions presented a more reserved and neutral aroma and taste but have added structure, body, and a smooth character, in comparison with the commercial yeasts that have contributed to the intensification of the aromas both in the nose and on the palate, providing freshness and a variety of floral and fruity aromas. The results confirmed the oenological potential of *S. cerevisiae* (accession number PP534168) to produce Tămâioasă Românească and Busuioacă de Bohotin wines at the micropilot scale.

Further studies are required to explore the potential of active optimized biomass in the operational environment and to validate its utilization in winemaking, which could be an advantage for small wineries to keep unchangeable the characteristic aroma for each region. Finally, the results of this study have the potential to facilitate easy and rapid production of fresh wine yeast tailored to the local winemaking practices of Pietroasa winery, with real application potential in other viticultural areas, aligning with the terroir concept.

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