Scientific Papers. Series E. Land Reclamation, Earth Observation & Surveying, Environmental Engineering. Vol. XIII, 2024
Print ISSN 2285-6064, CD-ROM ISSN 2285-6072, Online ISSN 2393-5138, ISSN-L 2285-6064

OPTIMISATION OF CULTURE MEDIA FACTORS FOR ACTIVE WINE YEAST BIOMASS PRODUCTION

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Abstract

The present work describes the optimization of the composition of liquid culture medium in sub-merged fermentation in batch system by varying 7 factors, according to a Hadamard matrix. The work aimed to determine the optimal values of the varied factors (sugar content, yeast extract, KCl, medium volume (Vw/Vt), inoculation ratio, $MgSO_4 \times 7H_2O$, vitamins) that conducted to a maximum value of the system response, namely the yeast biomass cell weight, (6.61 g) a parameter relevant from technical and economic point of view. The temperature, stirring rate and air flow rate for the Hadamard matrix were maintained constant for each process variant. Applying the process, an active yeast biomass with a protein content of 36.45 g% was obtained. The active yeast biomass of S. cerevisiae PFE-II-3 was used in a further study to obtain wines at the Pietroasa Viticulture and Winemaking Research and Development Station.

Key words*: bioprocess, Hadamard matrix, optimisation, S. cerevisiae, yeast.*

INTRODUCTION

The establishment of the culture media composition and the cultivation conditions in batch fermentation process requires the study of the largest possible number of experimental variants, depending on the environmental factors used. The use of the optimization techniques allows to study a larger number of factors and to determine their interaction, optimizing one or more experimental responses. The factor is a defined parameter (e.g., the constituent of a culture medium, the physical-chemical conditions etc.) that can have an influence on the experimental response (Srivastava et al., 2017; Chai, 2024; Michel et al., 2019; Carsanba et al., 2023). The response is the result of an experiment (e.g., biomass concentration obtained) (Vieira et al., 2013). The purpose of optimization consists in determining the optimal values of the factors leading to a maximum value of the system response, provided that the parameter that quantifies the system response is relevant from technical and economic point of view (Bărbulescu et al., 2010).

Saccharomyces cerevisiae (*S. cerevisiae*) is one of the most intensively studied microorganisms and, as a cell factory, has a long history of successful application in industrial applications (Wronska, 2022; Chuo et al., 2011; Radoi-Encea et al., 2023; Dumitrache et al., 2019; Mihai et al., 2022).

Several optimization techniques are used to find the best operational fermentation conditions, mentioned by different authors in their research

studies: Taguchi design (Barbulescu et al., 2021), Latin square method, one-factor-at-a time (OFAT) experiments (Gonzalez et al., 1995), Response surface methodology (RSM) was performed in the study of the optimization fermentation process by Chen et al. (2022) and Morteza et al. (2016).

During the medium optimization it must be considered that a minimal growth requirement of the microorganism must be fulfilled to obtain a maximum production of metabolite(s) (Singh et al., 2017). By performing a fed-batch cultivation of *K. marxianus* in lactose-based culture medium at varying initial lactose concentrations (10-60 g L^{-1}) at 30°C, pH 5.0, dissolved oxygen concentrations greater than 20%, the study elaborated by Lukondeh et al. (2005) led to a final biomass concentration of up to 105 $g \cdot L^{-1}$.

In general, the carbon and nitrogen sources present in the medium can influence the production of metabolites. Carbon source is the meaningful medium component, as it is an energy source for the microorganisms and plays an important role in the growth as well as in the production of yeast cell biomass. The rate at which the carbon source is metabolized can influence the formation of the yeast cell biomass (Bărbulescu et al., 2022). The selection of nitrogen source and its concentration in the culture media fermentation also have an important role in metabolite production. The microorganism can use both inorganic and/or organic sources of nitrogen (Marwick et al., 1999).

Concerning the potassium source, Dubencovs (2021), determined the optimal component (glucose, phosphorus, nitrogen, and potassium) concentration relations for the *K. marxianus* DSM5422 synthetic growth medium to maintain the mentioned components in appropriate amounts during fed-batch cultivations in order to obtain high cell densities. Potassium is also important players in cell physiology. In yeasts, the plasma-membrane H1-ATPase encoded by the PMA1 gene has an essential role in ion homeostasis (Morsomme et al., 2000).

Regarding the media volume, from the data presented by Frîncu et al. (2022) it is observed that the highest amount of wet and dry biomass was obtained when working on a volumetric ratio of 200 mL / 500 mL.

Regarding the *inoculation ratio*, Vieira et al. (2013) grown yeast in a PDA slant for 24 h at 32°C, followed by resuspension in sterilized water and inoculated at a 10% concentration of the total volume of the fermentation medium for biomass obtainment. The authors incubated the flasks in a shaker for 24 h at 32°C and 150 rpm.

Magnesium as sulphate salts, source, is important for culture media fermentation (Barbulescu et al., 2010). In accordance with the literature, the main factors affecting yeast biomass growth and protein content, are pH, temperature, time of cultivation and requirements for carbon, nitrogen, and vitamins. Barbulescu et al. (2010) in their study supplemented the media culture with a nitrogen source and vitamins for optimal growth. It is known that vitamin is an organic compound that an organism needs in small quantities to properly run its metabolic functions (Wronska, 2022).

Referring to vitamins, biotin and pantothenate are required for the proper growth of brewing yeast strains (Łukaszewicz et al., 2024). Biotin (vitamin B7) is an important nutrient for many fermentations (Han et al., 2019) used 0.002 mg as growth factor for aerobic growth to produce glutamic acid.

The goal of the present work was the evaluation of the influence of different media factors (sugar content; yeast extract; KCl; media volume; inoculation ratio; $MgSO_4 \times 7H_2O$, and biotin) on wet cells weight development (WCW, g/100 mL), using a Hadamard matrix for seven factors.

MATERIALS AND METHODS

Biotechnological process optimization

The purpose of optimization consisted in the determination of the optimal fermentation parameters values in batch system that lead to a maximum value of the system response, which are relevant from technical and economic point of view (Bărbulescu et al., 2010).

The Hadamard matrix used in the optimization of culture media factors for obtaining active wine yeast biomass is presented in Table 1.

A molecularly identified *S. cerevisiae strain, coded as PFE-II-3*, formerly isolated in UASMV Bucharest vineyard, was used for liquid inoculum development.

Table 1. Experimental matrix for K = 7 and N = 8

Exp. variant	Factor							Response
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	
1	+	+	+	-	+	-	-	y_1
2	-	+	+	+	-	+	-	y_2
3	-	-	+	+	+	-	+	y_3
4	+	-	-	+	+	+	-	y_4
5	-	+	-	-	+	+	+	y_5
6	+	-	+	-	-	+	+	y_6
7	+	+	-	+	-	-	+	y_7
8	-	-	-	-	-	-	-	y_8
9	0	0	0	0	0	0	0	Control

The response of the optimisation "y" is represented by a function (polynomial) in the form:

$$y = b_0 + b_1X1 + b_2X_2 + ... b_kX_k$$

where:
- b_0 = the average value of the response "y"
- X_i = value of variable factors (culture medium factors)
- b_i = linear coefficients to be determined (i=1; 2; 3; ... ; k)

we have:

$$b_0 = \sum \frac{y_i}{N} \text{ and } bi = \sum \frac{x_i y_i}{N}$$

where:
- X_i is the transposition of the matrix.

To calculate the linear coefficients (b_i) it is sufficient to assign the sign to each answer (y_i), corresponding sign in the column related to the factors for which we want to calculate the coefficient, then the algebraic sum is made and divided by the number of experiences (N = 8).

The response is represented by a polynomial in the form:

$$y= b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_5X_5 + b_6X_6 + b_7X_7$$

where:

$$b_0 = \Sigma \frac{yi}{8} = \frac{(y1+y2+y3+y4+y5+y6+y7+y8)}{8}$$

The interpretation of the results consists in ranking the factors with a significant influence on the response, depending on their sign:
bi > 0 – positive influence;
bi < 0 – negative influence

$$b_1 = (y_1 - y_2 - y_3 + y_4 - y_5 + y_6 + y_7 - y_8) / 8 \rightarrow \sum_1^8 \frac{x_1 y_i}{8}$$

$$b_2 = (y_1 + y_2 - y_3 - y_4 + y_5 - y_6 + y_7 - y_8) / 8 \rightarrow \sum_1^8 \frac{x_2 y_i}{8}$$

$$b_3 = (y_1 + y_2 + y_3 - y_4 - y_5 + y_6 - y_7 - y_8) / 8 \rightarrow \sum_1^8 \frac{x_3 y_i}{8}$$

$$b_4 = (- y_1 + y_2 + y_3 + y_4 - y_5 - y_6 + y_7 - y_8) / 8 \rightarrow \sum_1^8 \frac{x_4 y_i}{8}$$

$$b_5 = (y_1 - y_2 + y_3 + y_4 + y_5 - y_6 - y_7 - y_8) / 8 \rightarrow \sum_1^8 \frac{x_5 y_i}{8}$$

$$b_6 = (- y_1 + y_2 - y_3 + y_4 + y_5 + y_6 - y_7 - y_8) / 8 \rightarrow \sum_1^8 \frac{x_6 y_i}{8}$$

$$b_7 = (- y_1 - y_2 + y_3 - y_4 + y_5 + y_6 + y_7 - y_8) / 8 \rightarrow \sum_1^8 \frac{x_7 y_i}{8}$$

Depending on the results, and the number of studied factors, the step of the variation can then be modified, and a new matrix of the same type is set up, or following the objectives, other

matrices are used for a final study, observing the interactions between different factors.

The processing of the resulting fermentation medium was performed in 2 steps:

- separation of the yeast biomass by centrifugation at 4500 rpm for 5 minutes;
- purification of the yeast cells biomass using successive washing with sterile water by centrifugation at 4500 rpm.

For the determination of wet weight biomass (WCW) (g/100 mL), 10 mL of samples were centrifugated at 4500 rpm for 5 minutes and then weighed. For the determination of dry cell weight (DCW) (g/100 mL), the resulted wet biomass was subjected to a drying process at a Pheonix BM-60 moisture analyser (Lukondeh et al., 2005). Dry cell weight (DCW) was determined through gravimetric measurement after harvesting, washing, and lyophilization of a 2 mL culture sample (Awad et al., 2019).

Physico-chemical analyses

The determination of the *protein content* for yeast biomass was performed according to the Kjeldahl method. The crude protein is obtained based on the nitrogen content determined by the Kjeldahl method multiplied by the factor 6.25. As equipment a digestion Unit Buchi K-426, a titration unit Titrino Plus 877, a distillation unit KjelFlex 360, and an analytical balance OHAUS AX224M (OHAUS Corporation; New Jersey, USA) were used.

The determination of the *ash content* was performed by calcination at 550-600°C. As equipment, a Nabertherm L9 / 11/ B410 oven (Lilienthal, Germany) and an analytical balance (SHIMADZU ATX224R; Kyoto, Japan) were used.

The *moisture content* was determined through drying the samples at 103°C for four hours. As equipment, a Memmert UN55 drying oven (Schwabach, Germany) and an analytical balance (SHIMADZU ATX224R; Kyoto, Japan) were used.

Total soluble solids (TSS) were used as of-line parameter in fermentation bioprocess.

Microbiological analyses

The determination of *yeast and moulds* was performed in accordance with the standard SR ISO 21527-2:2009 - Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of yeasts and moulds - Part 2: Colony count technique in products with water activity less than or equal to 0.95. As cultivation medium Dichloran Rose-Bengal Chloramphenicol (DRBC) agar was used and the incubation was performed at 25 ± 1^0C for 5 days.

The determination of *E. coli* was performed in accordance with the standard SR ISO 16649-2:2007 - Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of beta-glucuronidase-positive *E. coli* - Part 2: Colony-count technique at 44°C using 5-bromo-4-chloro-3-indolyl beta-D-glucuronide. As cultivation medium tryptone-bile-glucuronide medium (TBX) agar was used and the incubation was performed at 44 ± 1°C for 18-24 h.

The determination of *coliform bacteria* was performed in accordance with the standard SR ISO 4832:2009 - Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of coliforms - Colony-count technique. As cultivation medium crystal violet neutral red bile lactose agar was used and the incubation was performed at 37 ± 1°C for 24 ± 2 h.

Optimizing the culture medium and cultivation parameters for obtaining active yeast biomass of *S. cerevisiae* PFE II-3

Step 1 optimization

Fermentation costs could be reduced by replacing expensive components with cheaper sources, such as food sugar, and/or with increased productivity, and these could contribute to a successful optimization strategy. The strain used in liquid media fermentation in different ratio has been proved to be another valuable optimization strategy toward the enhancement of the product yield (WCW) and the process improvement.

Optimizing the fermentation medium at laboratory level (using Erlenmeyer flasks) involves large number of experiments regardless of the chosen formula and is an open-ended experiment (Table 2).

The data generated by the shaker flask media seldom matches the fermentation behavior at the fermenter level (Kennedy et al., 1994; Kennedy et al., 1999). Vitamin solution - 0.002 g biotin was dissolved in 50 mL sterile distilled water at

50°C for 1 h, with stirring. The inoculation ratio was established based on the previous experiments as the salts, C and N source and vitamin source.

Fermentation using the strain *S. cerevisiae* PFE-II-3 for the of optimization biotechnological process

Obtaining the pure culture of S. cerevisiae yeast strain.

The preinoculum of yeast cultures of *S. cerevisiae* PFE II-3 were grown and developed on YPG (yeast extract, peptone hy-soy, and glucose) (Barbulescu et al., 2024) or YPS (yeast extract, hy-soy peptone, and sucrose) agar slant medium - 48 h at 30°C. This yeast was previously isolated from the Tămâioasă Românească grape by Barbulescu et al. (2024) and identified in 2023 by MALDI-TOF techniques and was validated by PCR molecular technique.

Obtaining the liquid inoculum. The liquid inoculum was obtained in 100 mL of YPG medium/500 ml Erlenmeyer flasks, (yeast extract were purchased from Sigma - Aldrich, peptone hy-soy were purchased from VWR Chemicals, and glucose were purchased from VWR Chemicals), inoculated with 2 tubes of previously developed pre-inoculum and left to incubate for a maximum of 14-16 h, at 240 rpm overnight at 30°C (Figure 1).

The experimental matrix for K = 7 and N = 8 media is shown in Table 3 and the factors tested for optimisation in Table 4.

Figure 1. Experimental variants at the laboratory level

Table 2. Concentration level taken in the experiment - optimization step 1

Media factors		Concentration level (g %)		
		-1	**0**	**1**
X_1	Sugar	7	8	9
X_2	Yeast extract E.D.	0.5	0.7	0.9
X_3	KCl	0.04	0.05	0.06
X_4	Medium volume Vw/Vt ratio	100/500	150/500	200/500
X_5	Vitamin	0.5	1	1.5
X_6	Inoculation ratio (%)	5	10	15
X_7	$MgSO_4 \times 7H_2O$	0.04	0.05	0.06

Table 3. Experimental matrix for K = 7 and N = 8

Optimization factors							**Response (y_i)**
Sugar g%	**E.D. g%**	**KCl g%**	**Media volume from Erlenmeyer flasks Vw/Vt ratio**	**Vitamin g%**	**Inoculation ratio (%)** (v/v)	**$MgSO_4 \times 7H_2O$ g%**	**WCW (wet weight cell) g/100 mL**
X1	**X2**	**X3**	**X4**	**X5**	**X6**	**X7**	
+	+	+	-	+	-	-	**y_1**
-	+	+	+	-	+	-	**y_2**
-	-	+	+	+	-	+	**y_3**
+	-	-	+	+	+	-	**y_4**
-	+	-	-	+	+	+	**y_5**
+	-	+	-	-	+	+	**y_6**
+	+	-	+	-	-	+	**y_7**
-	-	-	-	-	-	-	**y_8**
0	0	0	0	0	0	0	**Control**

Table 4. Media factors tested for optimization

Exp. no.	Exp. variant	Optimization factors							Response	y
		Sugar (g %)	YE (Yeast extract) (g %)	KCl (g %)	Medium volume (mL) Vw/Vt ratio	Vitamin (mL)	Inoculation ratio (%) (v/v)	$MgSO_4 \times 7H_2O$ (g %)	WCW (g/100 mL)	
		X1	X2	X3	X4	X5	X6	X7		
1	**1**	9	0.9	0.06	100/500	1.5	5	0.04	4.02	y1
2	**2**	7	0.9	0.06	200/500	0.5	15	0.04	4.09	y2
3	**3**	7	0.5	0.06	200/500	1.5	5	0.06	2.67	y3
4	**4**	9	0.5	0.04	200/500	1.5	15	0.04	3.58	y4
5	**5**	7	0.9	0.04	200/500	1.5	15	0.06	4.33	y5
6	**6**	9	0.5	0.06	100/500	0.5	15	0.06	3.30	y6
7	**7**	9	0.9	0.04	200/500	0.5	5	0.06	4.11	y7
8	**8**	7	0.5	0.04	100/500	0.5	5	0.04	2.89	y8
9	**Control**	8	0.7	0.05	150/500	1	10	0.05	3.28	**Control**

RESULTS AND DISCUSSIONS

By performing fed-batch cultivation of *S. cerevisiae* PFE-II-3 in a complex medium it was possible to achieve a final biomass concentration of up to 50 g/L. The results for fermentation for obtaining active yeast biomass of *S. cerevisiae* PFE-II-3 are presented in Table 5. The response obtained represented the answer for the following optimization calculation, as is presented in Table 6.

Optimization Step 2

In accordance with the most influent factor from the Table 6 were established the next step of optimisation in the Table 7, based on the results: (u) * (c). Following the optimization method, the variation step of the most influent factor is chosen within a more reduced space compared to the preceding factorial plan. This allows a better settlement of the optimal regions. A new experimental plan was established (Table 7) in which variable factors are: sugar, yeast extract, inoculation ration and vitamin, the rest of the ingredients remaining unchanged.

The results from step one goes to improve the results and that is why in the next step 3 variants were selected which are presented in Tables 8 and 9. For the next step, the liquid yeast inoculum *S. cerevisiae* PFE -II-3 was obtained. The results concerning the pH and TSS, Brix for the fermentation process is described in Table 9. According with the optimization method, the variation step of the most influent factor is chosen in a smaller space compared to the previous factorial plan, and this allows a better placement of the optimal regions.

Table 5. Results for fermentation for obtaining active yeast biomass of *S. cerevisiae* PFE-II-3

Cultivation time (h)	Analyses/ Variants	V1	V2	V3	V4	V5	V6	V7	V8	M
0	**pH**	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	6
	TSS, Brix	8.9	7.4	7.7	8.4	7.2	8.3	9.3	7.4	8.6
16	**pH**	5.1	5.1	5.1	5.1	5.1	5.1	5.1	5.1	4.8
	TSS, Brix	2.5	1.5	1.5	1.7	1.4	1.6	1.4	1.6	1.7
18	**pH**	5	5	5	5	5	5	5	5	4.5
	TSS, Brix	1.3	1.3	1.2	1.1	1.3	1.6	1.3	1.2	1.5
20	**pH**	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
	TSS, Brix	1.3	1.1	1.2	1.0	1.3	1.6	1.3	1.1	1.5

Legend: TSS = Total Soluble Solids

Otherwise: $b_1 = (y_1 - y_2 - y_3 + y_4 - y_5 + y_6 + y_7 - y_8) / 8 \rightarrow \sum_{1}^{8} \frac{x_1 y_i}{8} = 0.13$

$b_2 = y_1 + y_2 - y_3 - y_4 + y_5 - y_6 + y_7 - y_8) / 8 \rightarrow \sum_{1}^{8} \frac{x_2 y_i}{8} = 0.51$

$b_3 = (y_1 + y_2 + y_3 - y_4 - y_5 + y_6 - y_7 - y_8) / 8 \rightarrow \sum_1^8 \frac{x_3 y_i}{8} = -0.10$

$b_4 = (-y_1 + y_2 + y_3 + y_4 - y_5 - y_6 + y_7 - y_8) / 8 \rightarrow \sum_1^8 \frac{x_4 y_i}{8} = -0.01$

$b_5 = (y_1 - y_2 + y_3 + y_4 + y_5 - y_6 - y_7 - y_8) / 8 \rightarrow \sum_1^8 \frac{x_5 y_i}{8} = 0.03$

$b_6 = (-y_1 + y_2 - y_3 + y_4 + y_5 + y_6 - y_7 - y_8) / 8 \rightarrow \sum_1^8 \frac{x_6 y_i}{8} = 0.20$

$b_7 = (-y_1 - y_2 + y_3 - y_4 + y_5 + y_6 + y_7 - y_8) / 8 \rightarrow \sum_1^8 \frac{x_7 y_i}{8} = -0.02$

where:

$b_0 = \sum \frac{y_i}{8} = (y_1 + y_2 + y_3 + y_4 + y_5 + y_6 + y_7 + y_8) / 8 = 4.03$

bi > 5.76

Table 6. Step 2 of optimisation

Environmental factors	X_1	X_2	X_3	X_4	X_5	X_6	X_7
Base level	8	0.7	0.05	150	1	10	0.05
Variation unity (u)	1	0.2	0.01	50	0.5	5	0.01
Linear coefficient (c)	0.13	0.51	-0.1	-0.01	0.03	0.2	-0.02
(u) * (c)	0.13	0.102	-0.001	-0.5	0.015	1	-0.0002

Table 7. Concentration level taken in the experiment - optimization step 2

Media factors		Experimental variants		
		M -V10	**V11**	**V12**
X_1	sugar	8	8.13	8.26
X_2	Yeast extract	0.7	0.802	0.904
X_3	KCl	0.05	0.049	0.048
X_4	Media volume: Vw/Vt ratio	150	149.5	149
X_5	Vitamins	1	1.015	1.03
X_6	Inoculation ratio (%) (v/v)	9	10	11
X_7	$MgSO_4 \times 7\ H_2O$	0.05	0.0498	0.0496

Table 8. The answer of the experimental variants by varying the experimental factors

Exp. variant	Optimization factors							Response
	Sugar (g%)	**Yeast extract (g%)**	**KCl (g%)**	**Media volume (ml) Vw/Vt ratio**	**Vitamin (mL)**	**Inoculation ratio (%)** (v/v)	**$MgSO_4 \times 7H_2O$ (g%)**	**WCW (g/100 mL)**
	X1	X2	X3	X4	X5	X6	X7	
V10	8	0.7	0.05	150	1	9	0.05	4.07
V11	8.13	0.802	0.049	149.5	1.015	10	0.0498	4.63
V12	8.26	0.904	0.048	149	1.015	11	0.0496	4.78

Table 9. Fermentation to obtain yeast biomass of *S. cerevisiae* PFE-II-3

Cultivation time (h)	Analyses	V10	V11	V12
0	pH	6	6	6
	TSS, °Brix	9.3	10.4	11.4
16	pH	4.5	4.5	4.5
	TSS, °Brix	2.3	2.5	3.2
18	pH	4.5	4.5	4.5
	TSS, °Brix	1.8	2.4	2.8
20	pH	4.5	4.5	4.5
	TSS, °Brix	1.8	2.4	2.8

Optimization Step 3

A new experimental plan was established (Table 10), in which variable factor is the media volume Vw/Vt ratio (mL) X_4, and the rest of the parameters are unchanged.

The static cultivation of the microorganism was developed in tubes with a slant medium incubated at 30°C – 48 h, to obtain the maintenance strain and the static *S. cerevisiae* PFE-II-3 culture preinoculum;

Cultivation to obtain the inoculum was carried out in a discontinuous system by shaking 500 ml Erlenmeyer flasks with 149 mL YPG liquid media, in a closed incubator shaking system at 240 rpm, 30°C for 12-18 hours overnight.

The liquid inoculum of *S. cerevisiae* PFE-II-3 was obtained further in a shaker with stirring at 240 rpm, at a temperature of 30°C, overnight.

In the next phase, the fermentation was carried out for step 3 of optimization to improve the concentration of wet weight biomass by growing the inoculation ratio because has the highest positive influence (Tables 10 and 11).

Table 10. The response of media factors for the development of *S. cerevisiae* PFE -II-3 yeast biomass

Experimental variants	Experimental media factors							Answer
	Sugar (g %)	Yeast extract (g %)	KCl (g %)	Media volume Vw/Vt ratio (mL)	Vitamin (mL)	Inoculation ratio (%) (v/v)	$MgSO_4 \times 7H_2O$ (g %)	WCW (g/100 mL)
	X1	X2	X3	X4	X5	X6	X7	
V13	8.26	0.904	0.048	149	1.015	10	0.0496	4.6
V14	8.26	0.904	0.048	149	1.015	15	0.0496	5.24
V15	8.26	0.904	0.048	149	1.015	20	0.0496	5.35

Table 11. Obtaining active yeast biomass for optimization step 3

Cultivation time (h)	Analyses	V13	V14	V15
0	pH	6	6	6
	TSS, Brix	10	10.1	9.1
20	pH	4	4	4
	TSS, Brix	1	1.1	0.7

It is observed that the best experimental variant was obtained for V3 based on the WCW yield.

Biomass study of Torrellas et al. (2023) were performed by using molasses provided by Lessafre Iberica (60 g/L of sucrose for batch experiments, 100 g/L of sucrose for fed-batch phase and 20 g/L in diluted molasses experiments), supplemented with 7.5 g/L $(NH4)_2SO_4$, 3.5 g/L KH_2PO_4, 0.75 g/L $MgSO_4$, and 10 mL/L vitamin solution.

The vitamin solution contained 0.5 mg/L D-biotin, 1 mg/L calcium pantothenate and 1 mg/L thiamine hydrochloride (Torrellas et al., 2023).

The optimized variant V3 has the following performance parameters: 5.35 g/ 100 mL WCW (as active wet yeast biomass) and 1.33 g/100 mL DCW (as active dry yeast biomass).

The obtained active yeast biomass of *S. cerevisiae* PFE II-3 was analyzed from physical-chemical and microbiological point of view (Table 12), and it is observed that the sample was free of microbiological contamination and the cell viability decreases over time after 8-14 months.

Table 12. Physico-chemical and microbiological characterization of active dry yeast biomass for winemaking testing

Sample	***S. cerevisiae* PFE II-3 active yeast biomass**
Physico-chemical analyses	
Protein	36.45 g %
Ash	3.19 %
Humidity	5.62 %
Microbiological analyses	
Yeasts and Molds after optimisation	$3.2x10^8$ UFC/g
Yeasts and Molds after 8-14 months	$3.2x10^4$ UFC/g
E. coli	under 10 UFC/g
Coliforms	under 10 UFC/g

CONCLUSIONS

The technology for the production of the active yeast biomass of *S. cerevisiae* PFE II-3 was optimized using a Hadamard matrix by varying 7 factors:

The best variant was for V_3 which consists in varying of the following process parameters: sugar content - 8.26 g %; yeast extract - 0.904 g %; KCl -0.048 g %; media volume 149.5 mL/500 mL Erlenmeyer flasks, inoculation ratio 20%; $MgSO_4 \times 7H_2O$ - 0.0496 g %; vitamins - 1.015 mL.

It is noticed that the cell viability for the active yeast biomass decreases over time after 8-14 months.

ACKNOWLEDGEMENTS

This work was supported by the project 7PFE/2021 “Circular economy in USAMV farms - whole use of by-products resulting from fermentation processing”, Program 1 - Development of the national research-development system, Subprogram 1.2 - Institutional performance - Institutional development projects - Projects to finance excellence in RDI.

REFERENCES

Awad, D., Bohnen, F., Mehlmer, N. & Brueck, T. (2019). Multi-Factorial-Guided Media Optimization for Enhanced Biomass and Lipid Formation by the Oleaginous Yeast *Cutaneotrichosporon oleaginosus*. *Front. Bioeng. Biotechnol., 7*, 54. DOI: 10.3389/fbioe.2019.00054

Bărbulescu, I.D., Dumitrache, C., Diguţă, C.F., Begea, M., Matei, P.M., Frîncu, M., Mărculescu, S.I., Cîrîc, A.I., Tudor, V., Boroiu, E.M., Teodorescu, R.I. (2022). Evolution at the microfermenter level of the growth dynamics of *Saccharomyces cerevisiae* and *Starmella bacillaris* yeasts with potential for use in winemaking at the Pietroasa Winery. *AgroLife Scientific Journal, 11*(2), DOI: https://doi.org/10.17930/AGL202221

Bărbulescu, I.D., Ghica, M.V., Begea, M., Albu Kaya, M.G., Teodorescu, R.I., Popa, L., Mărculescu, S.I., Cîrîc, A.I., Dumitrache, C., Lupuliasa, D., Matei, F., Dinu-Pîrvu, C.E. (2021). Optimization of the fermentation conditions for brewing yeast biomass production using the response surface methodology and Taguchi Technique. *Agriculture 11*(12), 1237, https://doi.org/10.3390/agriculture11121237

Bărbulescu, I.D., Rusu, N., Rughinis, R., Popa, O., Stefaniu, A. Casarica, A. (2010). Obtaining yeast biomass enriched with copper, zinc and manganese. *Romanian Biotechnological Letters, 15*(1).

Barbulescu, I.D., Teodorescu, R.I., Dumitrache, C., Begea, M., Dragotoiu, D., Frincu, M., Marculescu, S.I., Cîrîc, A.I., Banita, D.C., Tudor, V. & Boroiu, E.M. (2024). Influence of inoculum on the dynamics of *Saccharomyces cerevisiae* yeast biomass development. International Symposium on Beverage Crops Eds.: E. Gómez Plaza et al. DOI 10.17660/ActaHortic.2024.1387.35 Proc. III.

Carsanba, E., Agirman, B., Papanikolaou, S., Fickers, P., Erten, H. (2023). Valorisation of Waste Bread for the Production of Yeast Biomass by Yarrowia lipolytica Bioreactor Fermentation. *Fermentation, 9*, 687. https://doi.org/10.3390/ fermentation9070687

Chai, W.Y., Tan, M.K., Teo, K.T.K., Tham, H.J. (2024). Optimization of Fed-Batch Baker’s Yeast Fermentation Using Deep Reinforcement Learning. *Process Integration and Optimization for Sustainability, 8*(2), 1-17, DOI:10.1007/s41660-024-00406-6.

Chen, J., Lan, X., Jia, R., Hu, L., Wang, Y. (2022). Response Surface Methodology (RSM) Mediated Optimization of Medium Components for Mycelial Growth and Metabolites Production of *Streptomyces alfalfae* XN-04. *Microorganisms, 10*, 1854. https://doi.org/10.3390/ microorganisms10091854

Chuo, H.S.E., Tan, M.K., Tham HJ., Teo, K.T.K. (2011). Optimization of fed-batch baker’s yeast fermentation process using learning algorithm, *Proceedings of 18th*

regional symposium on Chemical Engineering (RSCE2011).

Dubencovs, K., Liepins, J., Suleiko, A., Suleiko, A., Vangravs, R., Kassaliete, J., Scerbaka, R., Grigs. (2021). Optimization of Synthetic Media, Composition for *Kluyveromyces marxianus* Fed-Batch Cultivation. *Fermentation, 7*, 62. https://doi.org/10.3390/fermentation7020062

Dumitrache, C., Matei, F., Barbulescu, D.I., Frincu, M., Tudor, V., Hirjoaba, L.N., Teodorescu, R.I. (2019). Protein sources for animal feed: yeast biomass of beer and/or wine - review. *Scientific Papers. Series E. Land Reclamation, Earth Observation & Surveying, Environmental Engineering, VIII*, 175-182, Print ISSN 2285-6064.

Frîncu, M., Dumitrache, C., Begea, M., Teodorescu, R.I., Diguța, C.F., Baniță, C.D., Tudor, V., Cîrîc, A.I., Mărculescu, S.I., Bărbulescu, I.D. (2022). Active wine yeast biomass obtained through biotechnological process. *Journal of Agroalimentary Processes and Technologies, 28*(1), 20-26.

Gonzalez, R., Islas, L., Obregon, A.-M., Escalante, L., & Sanchez, S. (1995). Gentamicin formation in *Micromonospora purpurea*: stimulatory effect of ammonium. *J. Antibiot., 48*, 479–483. DOI: 10.7164/antibiotics.48.479

Han, X., Li, L., Bao, J. (2019). Microbial extraction of biotin from lignocellulose biomass and its application on glutamic acid production. *Bioresource Technology, 288*, 121523, ISSN 0960-8524.

Kennedy, M., & Krouse, D. (1999). Strategies for improving fermentation medium performance: a review. *J. Ind. Microbiol. Biotechnol., 23*, 456–475. DOI: 10.1038/sj.jim.2900755

Kennedy, M., Reader, S., Davies, R.J., Rhoades, D. & Silby, H. (1994). The scale up of mycelial shake flask fermentations: a case study of gamma linolenic acid production by Mucor hiemalis IRL 51. *J. Ind. Microbiol., 13*, 212–216. DOI: 10.1007/BF01569750

Łukaszewicz, M., Leszczynski, P., Jabłonski, S.J., Kawa-Rygielska, J. (2024). Potential Applications of Yeast Biomass Derived from Small-Scale Breweries. *Appl. Sci., 14*, 2529. https:// doi.org/10.3390/app14062529

Lukondeh, T., Ashbolt, N.J., Rogers, P.L. (2005). Fed-batch fermentation for production of *Kluyveromyces marxianus* FII 510700 cultivated on a lactose-based medium. *J. Ind. Microbiol. Biotechnol. 32*(7), 284-8. DOI: 10.1007/s10295-005-0245-y.

Marwick, J.D., Wright, P.C., Burgess, J.G. (1999). Bioprocess intensification for production of novel marine bacterial antibiotics through bioreactor operation and design. *Mar. Biotechnol., 1*, 495–507. DOI: 10.1007/PL00011806

Michel, M., Meier-Dörnberg, T., Jacob, F., Schneiderbanger, H., and Hutzler, M. (2020). Optimisation of yeast vitality measurement to better predict fermentation performance. *J. Inst. Brew., 126*, 161–167. https://doi.org/10.1002/jib.604

Mihai, C., Frîncu, M., Dumitrache, C. (2022). Isolation and characterization of new yeasts strains from barley samples. *Scientific Papers. Series E. Land Reclamation, Earth Observation & Surveying, Environmental Engineering, XI,* 499-504. Print ISSN 2285-6064.

Morsomme, P., Slayman, C.W. & Goffeau, A. (2000). Mutagenic study of the structure, function and biogenesis of the yeast plasma membrane H1-ATPase. *Biochim Biophys Acta, 1469*, 133–157.

Morteza, M.A., Mohammad, R.F., Mohsen, A., Nasim, H.R., Nasrin, S. (2017). Optimization of Culture Conditions for Enrichment of *Saccharomyces cerevisiae* with Dl-α-Tocopherol by Response Surface Methodology. *Iranian Journal of Pharmaceutical Research 16*(4), 1546-1554.

Rădoi-Encea, R.-Ș., Pădureanu, V., Diguță, C.F., Ion, M., Brînduşe, E., Matei, F. (2023). Achievements of Autochthonous Wine Yeast Isolation and Selection in Romania - A Review. *Fermentation*, *9*(5), 407. https://doi.org/10.3390/fermentation9050407.

Singh, V., Haque, S., Niwas, R., Srivastava, A., Pasupuleti, M., Tripathi, C.K.M. (2017). Strategies for Fermentation Medium Optimization: An In-Depth Review. *Front. Microbiol., 7*, 2087. DOI: 10.3389/fmicb.2016.02087.

Torrellas, M., Pietrafesa, R., Ferrer-Pinós, A., Capece, A., Matallana, E., Aranda, A. (2023). Optimizing growth and biomass production of *non-Saccharomyces* wine yeast starters by overcoming sucrose consumption deficiency. *Front. Microbiol. 14*, 1209940. DOI: 10.3389/fmicb.2023.1209940

Vieira, E.D., Andrietta, MdaG, Stupiello, Andrietta, S.R. (2013). Yeast biomass production: a new approach in glucose-limited feeding strategy. *Brazilian Journal of Microbiology, 44*(2), 551-558, ISSN 1678-4405.

Wronska, A.K. (2022). Engineering biotin synthesis; towards vitamin independency of *Saccharomyces cerevisiae.* Degree granting institution, doctoral thesis, 978-94-6423-708-5, https://doi.org/10.4233/uuid:bd748c70-2cda-4094-945f-0f0577700367