Research Article

Optimization of Biomass and Protein Production by *Saccharomyces cerevisiae* through a Specific Combination of Aerobic-Anaerobic Conditions for Nutritional Applications

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Abstract | Microbial protein or single cell protein (SCP) is a novel protein source anticipated to contribute substantially to future human nutrition. This study aimed to optimize SCP production by *Saccharomyces cerevisiae* applying an enhanced approach within a newly developed device. The growth method included a specific combination of aerobic and anaerobic conditions to maximize SCP yield and quality. The Box-Behnken design (BBD) of the response surface methodology (RSM) was applied to analyze the effects of key process variables (*i.e.*, temperature, aeration duration, and sucrose concentration) on biomass yield and its protein content as responses. The evaluation of variables interactions indicated that the quadratic regression models had strong correlation with experimental outcomes with R^2 value of 0.9987 for both responses. The obtained results demonstrated that aeration duration was the most influential parameter affecting both responses followed by sucrose concentration and temperature. These findings suggest that adequate oxygen availability boosts *S. cerevisiae* metabolism and the optimum conditions for enhanced biomass and protein output were found to correspond to a temperature of 30 °C, aeration duration of 35 h, and sucrose concentration of 20 g/l. Upon applying the suggested aerobic-anaerobic combination method, the biomass yield of *S. cerevisiae* had increased by 18.39 g/l (DW) with 39.15 % (w/w) protein content. This study revealed that aeration is a critical factor for SCP production by *S. cerevisiae* and its protein content. Furthermore, the specific propagation approach of this work can be effectively implemented in industrial production of *S. cerevisiae*, contributing to a sustainable and efficient protein production for nutritional applications. Future research should focus on scaling up the process and exploring its deployment across diverse industrial, microbiological, and biotechnological contexts.

Received | February 04, 2026; **Revised** | April 29, 2026; **Accepted** | May 07, 2026; **Published** | May 20, 2026
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Citation | Safdari, Z., M.K.A. Ariffin, M.T.H. Sultan and E.E. Supeni. 2026. Optimization of biomass and protein production by *Saccharomyces cerevisiae* through a specific combination of aerobic-anaerobic conditions for nutritional applications. *Novel Research in Microbiology Journal,* 10(3): 267-285.
DOI | https://dx.doi.org/10.17582/journal.nrmj/2026/10.3.267.285
Keywords | *Saccharomyces cerevisiae*, Microbial protein, Box-Behnken design (BBD), Process optimization, Food security, Feed industry

Introduction

According to the latest statistics provided by the United Nations, hunger is one of the most urgent global problems affecting more than 800 million people worldwide. The impoverished millions of people globally and the shortage of protein-rich food have triggered researchers to find replacements for the conventional expensive protein sources in animal feed such as fishmeal and soymeal (Escribano, 2016). Accordingly, the production of readily accessible protein products without a negative influence on human life is one of the main challenges in this context (Riches, 2002; Jach *et al*., 2022). Single cell protein (SCP) stands out as an appealing substitute for traditional protein sources in both human diet and animal feed, which can be produced efficiently using various cheap and abundant feedstock to tackle the worldwide food crisis (Tropea *et al*., 2022; Rasool *et al*., 2023; Sekoai *et al*., 2024). This sustainable protein source has the potential to improve food security by supplementing traditional feed sources and reducing reliance on resource-intensive protein production methods. Furthermore, many kinds of yeast and bacterial species such as lactic acid bacteria exhibit many other beneficial properties, including prebiotics, antioxidants, and immunomodulatory effects (Khadem *et al*., 2026). In addition, SCP can be obtained through the bioconversion of decomposable wastes, contributing to waste reduction and recycling efforts, which confers it superiority over the other available choices (Izah *et al*., 2019). Previous studies revealed the distinct advantages of microbial protein in various waste treatment practices (Yang *et al*., 2022; Koukoumaki *et al*., 2024).

Microbial proteins are sourced from a variety of microorganisms, including fungi, bacteria, yeasts, and algae, with yeasts being the most widely embraced among them (Bajić *et al*., 2022). This is primarily attributed to their ability to propagate easily using inexpensive raw materials and their simple harvesting process (Bekatorou *et al*., 2006). Yeasts also offer advantages such as the ability to grow at acidic pH and short growth times leading to a rapid biomass production (Chollom *et al*., 2017). Baker's yeast *Saccharomyces cerevisiae*, a yeast species with distinctive features, plays a crucial role in the diverse biotechnological applications.

Single cell protein production is feasible in controlled environments, ensuring an efficient and scalable final product. The growth rate of microorganisms in a bioreactor may vary depending on the fermentation technique, growth conditions such as availability of nutrients in specific forms and ambiance factors *i.e*., temperature, aeration, pressure, pH, humidity level in the solid culture, and the presence of contaminants or the absence of competitors (Onyeaka *et al*., 2023). With respect to *S. cerevisiae*, multiple studies confirmed that a carbon source concentration and temperature affect the cellular growth decisively (Bertasini *et al*., 2022; Raita *et al*., 2022). Furthermore, *S. cerevisiae* is a facultative anaerobic microorganism and can live both in the presence and absence of oxygen, a feature making it more appropriate for industrial applications (Ishtar Snoek and Yde Steensma, 2007). Generally, effective aeration is a critical component for the yeast propagation process that prevents oxidative stress in cells *via* facilitating the oxygen availability. Oxygen levels in the medium are elevated by aeration and foster the aerobic metabolism, contributing to higher growth rate (Beugholt *et al*., 2023). Bertasini *et al*. (2022) in their experiments on the production of *S. cerevisiae* using the effluent of candies factory reported that aerobic conditions lead to a greater yeast propagation than anaerobic media (Bertasini *et al*., 2022). Another study demonstrated that under aerobic condition, the presence of oxygen in the medium resulted in increased ATP production, which enhanced *S. cerevisiae* metabolism and development (Boender *et al*., 2011). However, it should be noted that the importance of anaerobic conditions, when performed at the appropriate phase and for a defined duration, can be as remarkable as the aerobic conditions (Chopda *et al*., 2015). Firstly, anaerobic condition diminishes other potential aerobic microorganisms' populations, thereby reducing the number of competitors in the medium (Ishtar-Snoek and Yde-Steensma, 2007). As a result, *S. cerevisiae* can expand more effectively in the absence of competitors and more ethanol will be produced through the fermentation process. Subsequently, accumulation of ethanol in the environment exerts toxic effects, leading to the elimination of the remained potential pathogens, while *S. cerevisiae* cells exhibit remarkable tolerance to elevated ethanol concentrations (Mazzoleni *et al*., 2015). This study, building on previous studies, aimed to exploit the advantages of both approaches and improve the process efficiency by integrating the two growth modes within a single system. The proposed method comprised a two-step

process, initially conducted under anaerobic condition and subsequently transitioning to aerobic respiration after a certain period of time, when air was introduced into the system. A distinguished advantage of this approach is that both steps were performed in a single device specifically designed for this purpose under controlled conditions. In this novel device, aeration was provided *via* small nozzles embedded in the walls, allowing direct introduction of air into the medium instead of limiting it to the surface.

Moreover, the successful design of a microbial production process involves optimizing the various parameters affecting the process and the final output (Singh *et al*., 2017). Due to the time-consuming nature of the process, identifying the optimal levels of these critical parameters may be costly and necessitates numerous experiments. These challenges can be addressed by utilizing the response surface methodology (RSM), which is a statistical analysis tool and a validated method in optimizing the bioprocesses (Ferreira *et al*., 2023; Lestari *et al*., 2023). This reliable technique has been employed in many previous studies on SCP production by *S. cerevisiae* to achieve optimal operating conditions (Accettulli *et al*., 2023; Shahzad *et al*., 2024). The commonly applied designs in RSM include the central composite design (CCD) and the Box-Behnken design (BBD) (Said and Amin, 2015). Focusing on the efficiency, multiple previous studies indicated satisfactory results in using BBD for optimization of biomass yield in *S. cerevisiae* (Salem-Bekhit *et al*., 2023; Abdel-Fattah *et al*., 2024). By optimizing the biomass production by *S. cerevisiae* and its protein content using RSM with a Box-Behnken design in a new device, this study improved our understanding in the area of baker's yeast biomass processing, with the aim of producing SCP for nutritional applications.

Materials and Methods

Microorganism culture and maintenance

The inactivated bakers' yeast (*S. cerevisiae*) used in this study was commercialized by "AB Mauri Food Marketing Co., Ltd., China" and procured from a local supermarket of Kuala Lumpur, Malaysia. First, *S. cerevisiae* was immersed in a sterile solution of sugar water (1:4 w/w) overnight. In the next step, it was reactivated on petri plates of Yeast Peptone Dextrose agar medium (YPDA) composed of (g/l): yeast extract 10, peptone, dextrose 20 L, and agar 15 to verify obtaining of pure *S. cerevisiae* colonies (Dunuweera *et al*., 2021). Afterward, they developed pure colonies were maintained on plate count agar (PCA) (Sigma Aldrich, USA) plates and glycerol as stocks.

Experimental set-up

The yeast propagation was carried out in a pilot-scale device in batch mode under submerged fermentation without stirring. The device was designed and fabricated to control the environmental conditions based on the particular experimental method of this study, which t included both aerobic and anaerobic phases during the propagation process. It was equipped with a power supply, and comprised of an insulated vessel, a magnetic door, a temperature control system, and several nozzles (Figure 1). The insulated vessel and magnetic door were designed to prevent contamination and unintended air intrusion into the experimental medium. The temperature control system consisted of a thermostat-controlled heater and an external thermometer. The thermostat constantly monitored the medium temperature, and once the temperature fell below the predefined level (25, 30, and 35 °C), the thermostat instructed the heater to turn on. In contrast, after the temperature reached or surpasses the set point, the thermostat automatically deactivated the heating element. To ensure reliable and precise temperature control, an external thermometer was also utilized. The display unit was installed on the external surface of the device, while the sensor was directly in contact with the yeast production medium. The device was also equipped with an air pump connected to the nozzles through narrow hoses, enabling control over the air flow in the medium. It should be noted that the device was constructed from a fully insulated material and equipped with a heavy, solid magnetic door that ensured an airtight seal when closed. Consequently, these nozzles were the only pathway for air to enter the medium, which were activated according to the experimental design. Furthermore, the nozzles were integrated into the device design to enhance aeration through producing smaller air bubbles under water.

In this study, aeration duration was used as a proxy variable for dissolved oxygen (DO), providing a practical estimate of oxygen availability. All experiments consisted of two phases; an initial anaerobic phase and a subsequent aerobic phase. Each experiment had a total duration of 70 h that

was set for 25-45, 35-35, and 45-25 h time intervals under anaerobic and aerobic conditions, respectively. To provide further explanation, there were three groups of experiments that all began under anaerobic condition for 25, 35, and 45 h, respectively, and then they continued under aerobic conditions for 45, 35, and 25 h in the same order given. In the first experimental phase the air pump was off and at the commencement of the second phase, it was turned on to change the medium from anaerobic to aerobic.

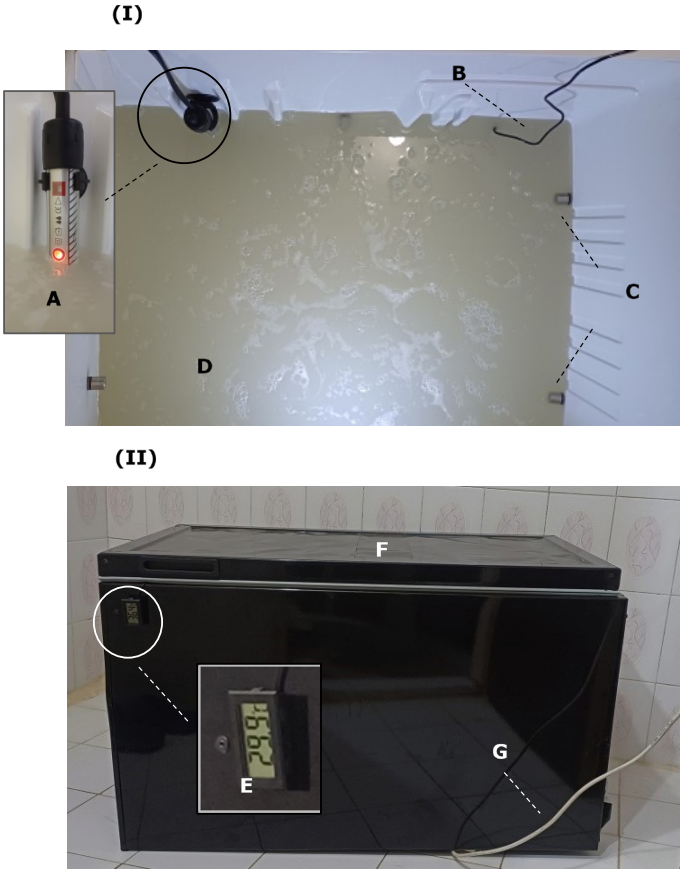

Figure 1: *(I) The inside of the device (Insulated vessel); (A) Thermostat-controlled heater; (B) Thermometer sensor inside the culture medium; (C) Nozzles; (D) Liquid culture medium inside the device; (E) Display unit of the external thermometer, (II) The outside of the device; (F) Device magnetic door; and (G) Power cord connected to the power supply.*

The total volume of the device was 90 l and the volume of medium was 35 l. The medium consisted of $MnSO_4$, KH_2PO_4, $ZnSO_4$, and $MgSO_4$, each at a concentration of 1 g/l. Sucrose was the available carbon source that was mixed at different concentrations (10, 20, and 30 g/l) based on the experimental design. Ammonium sulfate was also added at the concentration of 0.3 g/l as a primary nitrogen source. The prepared medium was adjusted to pH 5.0 and sterilized at 120 °C for 15 min. Starter cultures were prepared using PCA plates and were incubated at 35 °C for 48 h. After incubation, *S. cerevisiae* colonies were/ transferred to 5 ml distilled water under aseptic conditions (Hezarjaribi *et al.*, 2016). Then, 100 ml of the prepared growth medium were inoculated with 1 ml of *S. cerevisiae* suspension containing 1×10^8 CFU/ml and incubated at 35 °C for 48 h. The aforementioned medium was used as a seed culture for inoculating 2 L of the experimental growth medium, followed by incubation at 35 °C for 24 h. The main growth medium was inoculated using the latter culture (Arous *et al.*, 2016). During all steps, the yeast's cell concentration was kept constant to maintain an appropriate inoculation density in the final medium.

Determination of dry biomass yield

To determine the total dry biomass yield, the washed cells obtained from the incubation process were oven-dried (Memmert UN55 Natural Convection Lab Oven, Germany) to a constant weight at 50 °C for 16 h (Thiviya *et al.*, 2022). The weight of produced biomass was measured using a standard precision balance (ViBRA ALE-503 Class II NTEP, Shinko Denshi Co., Ltd., Japan) with an accuracy of 0.001g.

Determination of protein content

The Kjeldah method was applied to measure the protein content of the experimental samples. The first step was digestion and conducted using concentrated sulfuric acid (H_2SO_4) and two copper catalyst tablets. A heat block (Behr K Series Kjeldahl Block Digestion System, Behr Labor-Technik, Germany) was used for this process conducted at 420 °C for 2 h. Then Kjeldahl distillation system (Behr Labor-Technik, Germany) was used for carrying out the distillation and titration steps, after cooling. In the distillation step, boric acid solution was used for capturing the produced ammonia gas. Finally, the titration was carried out using H_2SO_4 as standardized acid solution and the protein content of each sample was calculated using a conversion factor of 6.25 (Shahzad *et al.*, 2024).

Statistical analysis

The experimental data were analyzed using the BBD design of RSM to evaluate the effects of three independent variables (*i.e.*, temperature, aeration duration, and sucrose concentration) on *S. cerevisiae* growth activity and protein production. A quadratic polynomial model was fitted to the experimental results to define the correlation between the desired

responses and the selected parameters. Analysis of variance (ANOVA) was employed to assess the statistical significance and adequacy of the developed models. Additionally, regression coefficients were used to determine the magnitude and direction of the effects of variables and their interactions on biomass and protein production. Response surface plots and contour plots were generated to visualize the interactions between variables and identify optimal conditions. Design-Expert software (version 13.0.5.0, StatEase Inc, Minneapolis, USA) was applied to perform all statistical analysis.

Results and Discussion

Experimental design

In this study, BBD that is one of the most popular experimental designs in RSM was employed to investigate and optimize the effective parameters in *S. cerevisiae* SCP production. Temperature (25, 30, 35 °C), aeration duration (25, 35, 45 h), and sucrose concentration (10, 20, 30 g/l) were chosen as independent variables, whereas the process responses set as the dependent variables were total biomass yield (R1) and the protein content (R2) (Table 1).

A total number of 15 experimental runs including three center points (applied for experimental error appraisal) were performed (Table 1). Each experiment was conducted in triplicate and the mean value was calculated. As the main step of the optimization analysis, a second-order polynomial model was established for each response while the experimental data was subjected to a stepwise regression analysis *via* applying a backward elimination subroutine (only the significant terms with $p < 0.05$ were picked). The analysis of variance (ANOVA) and residual analysis were specified and the correlation (R) and determination (R^2) coefficients were assessed for each regression equation. The relationships between the responses and the experimental variables were visualized graphically by contour and 3-D response surface plots.

Table 1: *Coded levels of independent variables, experimental design matrix using BBD and results.*

Independent variables				**Symbols**		**Level**	
					-1	0	+1
Temperature (°C)				A	25	30	35
Aeration duration (h)				B	25	35	45
Sucrose concentration (g/l)				C	10	20	30
Run	**Variables**			**Responses**			
	Temperature (°C)	**Aeration duration (h)**	**Sucrose Conc. (g/l)**	**Total Biomass (g/l)**		**Protein content (% w/w)**	
				Experimental	**Predicted**	**Experimental**	**Predicted**
1	25	25	20	10.05	9.90	25.75	25.59
2[a]	30	25	30	10.57	10.60	26.13	26.11
3	30	35	20	18.25	18.31	38.75	38.98
4	35	25	20	10.52	10.64	25.43	25.69
5	30	45	10	11.73	11.70	27.3	27.32
6	25	35	30	13.61	13.74	29.47	29.65
7[a]	25	35	10	10.89	11.04	26.48	26.72
8	30	45	30	13.08	13.07	28.78	28.86
9	35	45	20	13.23	13.39	28.88	29.04
10	30	35	20	18.39	18.31	39.15	38.98
11	30	35	20	18.29	18.31	39.05	38.98
12	35	35	30	13.57	13.42	29.19	28.95
13	25	45	20	11.75	11.63	27.35	27.09
14[a]	35	35	10	13.99	13.86	29.65	29.47
15	30	25	10	9.7	9.71	25.29	25.22

Where; [a]: Center point

The general equation of the quadratic model is demonstrated in Equation 1 (Nazari *et al.*, 2017).

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{i \neq j > 1}^{4} \beta_{ij} X_i X_{ij} \quad .....(1)$$

Where; Y = The response, β_0 = The intercept coefficient, β_i = The slope or linear effect of the factor X_i, β_{ii} = The quadratic effect of the factor X_i, β_{ij} = The interaction effect between the input factors X_i and X_j where ε is the residual term.

Statistical analysis and model fitting

Table 1 represents the experimental design matrix comprised of values and levels of independent variables, along with the values of total biomass yield and protein content as responses. The observed considerable changes in SCP production rate by *S. cerevisiae* for different combinations of variables indicated that they remarkably affected the yeast growth. According to the experimental results, the optimum values of temperature, aeration duration, and sucrose concentration were found to be 30 °C, 35 h, and 20 g/L, respectively, to achieve the maximum SCP production and protein content (Table 1). The following equations are the final developed models used to predict the values of total biomass yield and protein content with coded parameters:

$$\textit{Total Biomass Yield (g/L DW)} = +18.31 + 0.6262\,A + 1.12\,B + 0.5650\,C + 0.2525\,AB - 0.7850\,AC - 0.1200\,BC - 2.59\,A^2 - 4.33\,B^2 - 2.71\,C^2 \quad \ldots(2)$$

$$\textit{Protein Content \% (w/w)} = +38.98 + 0.5125\,A + 1.21\,B + 0.6063\,C + 0.4625\,AB - 0.8625\,AC + 0.1600\,BC - 5.15\,A^2 - 6.98\,B^2 - 5.13\,C^2 \quad \ldots(3)$$

Where; A = Temperature, B = Aeration duration, C = Sucrose concentration

In these equations, the positive sign demonstrates the synergistic effect of the variables, whereas the negative ones indicate their opposed effect. The adequacy and statistical significance of the model were evaluated through the analysis of variance (ANOVA) (Celebi *et al.*, 2007; El-Naggar and El-Ewasy, 2017) and the outcomes of ANOVA for the quadratic equation are presented in Table 2.

In RSM, ANOVA plays a crucial role in assessing the importance of the primary effects of the independent variables and the potential interactions among them, which entails fitting a regression model to the data and subsequently conducting F-tests to evaluate the significance of each term in the model. The ANOVA results affirmed the accuracy of the equation and true relationship existing between the response and the significant variables depicted in the equation. The values of F and p serve as indicators of the significance of the coefficient term, with higher F values and lower p values indicating a greater significance (Ramli *et al.*, 2017). In ANOVA, regression coefficients have the essential function in determining the relation between the input variables (factors) and the response variable. They quantify the change in the response variable for a unit change in the corresponding input variable while fixing the other variables constant. The coefficient of determination (R^2) defines the validity of the model in predicting the outcomes of the model or responses. In the context of regression analysis, R^2 is a statistical indicator of how closely the regression line resembles the actual data. The lowest value of R^2 is 0, which means that the points are not defined by the regression, whereas the highest possible value is 1 indicates that a regression line defines all points.

In this study, the coefficient of determination (R^2) for both responses was close to 1 (0.9987 for both biomass yield and protein content), representing the desirable adjustment of the developed models to the experimental data. Additionally, the high values of adjusted-R^2 for total biomass and protein content (0.9963 for both responses) confirmed that the models were highly significant. Since the quadratic models were fitted to a limited number of experimental runs, potential overfitting was assessed. A recorded difference of less than 0.2 between the predicted R^2 and the adjusted R^2 indicated that the predicted R^2 was in proper agreement with the adjusted R^2 for both responses. This reasonable agreement and the non-significant lack of fit tests demonstrated that the models adequately captured the underlying trends without overfitting. *P*-value indicated the probability of obtaining a result as extreme as or more extreme than what was actually observed, assuming that there was no impact or difference (null hypothesis). In a statistically significant test, p-value less than 0.05 means that the model terms are significant (Kim *et al.*, 2003). As shown in Table 2, the p-value for most of the total biomass model terms (A, B, C, AB, AC, A^2, B^2, and C^2), as well as the protein model terms (A, B, C, AB, AC, A^2, B^2, and C^2) were lower than 0.05,

Table 2: *Analysis of variance (ANOVA) for quadratic models for total biomass yield and protein content of Saccharomyces cerevisiae.*

Source	Biomass					Source	Protein				
	Sum of squares	df	Mean square	F-value	*p-value*		Sum of squares	df	Mean square	F-value	*p-value*
Model	124.99	9	13.89	414.98	< 0.0001 (significant)	Model	347.59	9	38.62	416.18	< 0.0001 (significant)
Temperature (A)	3.14	1	3.14	93.76	0.0002	Temperature (A)	2.10	1	2.10	22.64	0.0051
Aeration duration (B)	10.01	1	10.01	299.20	< 0.0001	Aeration duration (B)	11.79	1	11.79	127.00	< 0.0001
Sucrose conc. (C)	2.55	1	2.55	76.31	< 0.0003	Sucrose conc. (C)	2.94	1	2.94	31.68	0.0025
AB	0.2550	1	0.2550	7.62	0.0398	AB	0.8556	1	0.8556	9.22	0.0289
AC	2.46	1	2.46	73.66	0.0004	AC	2.98	1	2.98	32.07	0.0024
BC	0.0567	1	0.0567	1.72	0.2465	BC	0.1024	1	0.1024	1.10	0.3416
A^2	24.74	1	24.74	739.41	< 0.0001	A^2	98.09	1	98.09	1057.00	< 0.0001
B^2	69.35	1	69.35	2072.21	< 0.0001	B^2	179.72	1	179.72	1936.66	< 0.0001
C^2	27.04	1	27.04	808.06	< 0.0001	C^2	97.23	1	97.23	1047.79	< 0.0001
Residual	0.1673	5	0.0335			Residual	0.4640	5	0.0928		
lack of Fit	0.1569	3	0.0523	10.06	0.0918 (not significant)	Lack of Fit	0.3773	3	0.1258	2.90	0.2667 (not significant)
Pure Error	0.0104	2	0.0052			Pure Error	0.0867	2	0.0433		
R^2	0.9987					R^2	0.9987				
Adjusted-R^2	0.9963					Adjusted-R^2	0.9963				
Predicted-R^2	0.9798					Predicted-R^2	0.9821				
Adequate precision	57.6021					Adequate precision	55.3550				
C.V. %	1.39					C.V. %	1.02				
Total	125.15	14				Total	348.05	14			

displaying that the regression equations illustrated the existing correlation between the response parameters and the variable parameters competently.

In the current study, adequate precision which measured the signal-to-noise ratio (S/N) was favorable (greater than four), recommending that the developed models can be used to navigate the design space and predict the responses (Isam *et al.*, 2019). Furthermore, the validity and accuracy of the experimental design as well as the little deviation between experimental and predicted values was revealed by low coefficient of variance (CV) values (1.39 for total biomass yield and 1.02 for protein content) (Isam *et al.*, 2019). This observation also indicated acceptable reproducibility of the experimental measurements. Additionally, a non-significant lack of fit (p < 0.05) for both responses revealed that the models adequately fitted the experimental data (Anuar *et al.*, 2013). These results confirmed the reliability and robustness of the developed response surface models.

Model adequacy analysis

According to the data expanded out on the diagonal lines of the current study there was an obvious nonlinear relationship between the experimental variables, approving the selected quadratic models. In addition, the adequacy of the regression model could be assessed through analyzing diagnostic plots such as normal plot of residuals graph, predicted versus actual graph, residual versus run graph, and residuals versus predicted graph. The residual values appeared to be randomly scattered within the range while comprising low values. As shown in Figure 2A for total biomass yield and Figure 3A for protein content, the data points were close to the linear regression line without deviation, indicating a valid and normal data distribution on the normal plot of the residual graph. The points in the predicted versus actual graph of both responses followed a diagonal line, indicating that the predicted responses matched the observed values (Figures 2B, 3B). The graph of residuals versus

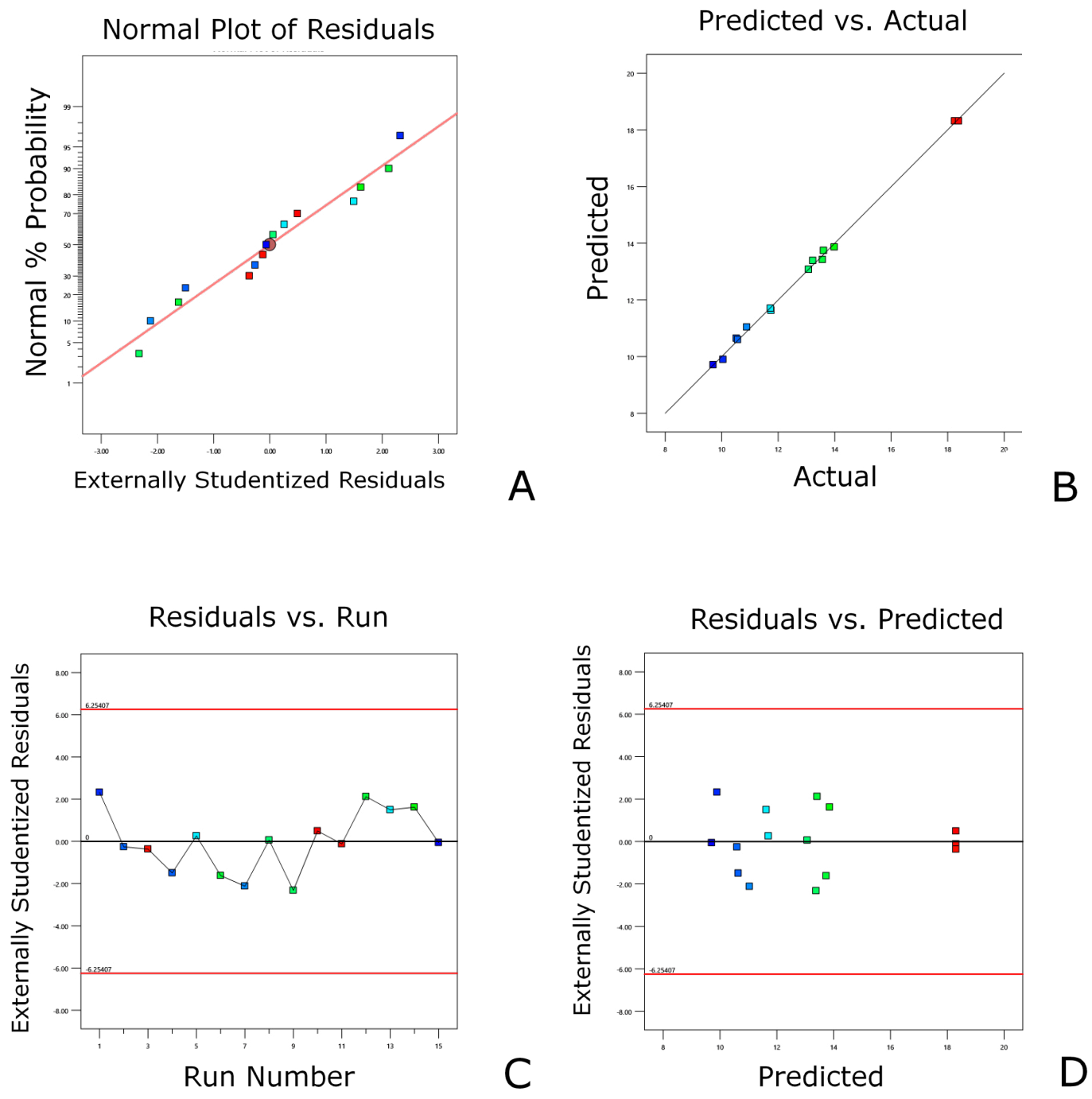

Figure 2: *Diagnostic plots representing the adequacy of the regression model of the biomass production by S. cerevisiae: (A) Normal Plot of Residuals, (B) Predicted vs. Actual, (C) Residuals vs. Run, and (D) Residuals vs. Predicted.*

run shown in Figures 2C and 3C for total biomass yield and protein content, respectively, displayed that all data points were distributed randomly, elucidating the fitness of the models (Flaifel, 2020). The absence of obvious pattern and structure in residuals versus predicted graph is one of the most important criteria in determining the adequacy of a model (Anuar *et al*., 2013), which can be observed in the mentioned graph of this study, demonstrated in Figures 2D and 3D for total biomass yield and protein content, respectively.

Interaction between influential parameters

The experimental results provided substantial perspective on the interaction effects of the key variables on SCP production by *S. cerevisiae* and the protein content. The contour and 3-D response surface plots validated the statistical significant of the optimization strategies as well as the predictive accuracy of the regression models. The combined effects of key variables as well as critical regions of maximum efficiency were illustrated on contour plots (Figures 4 and 5).

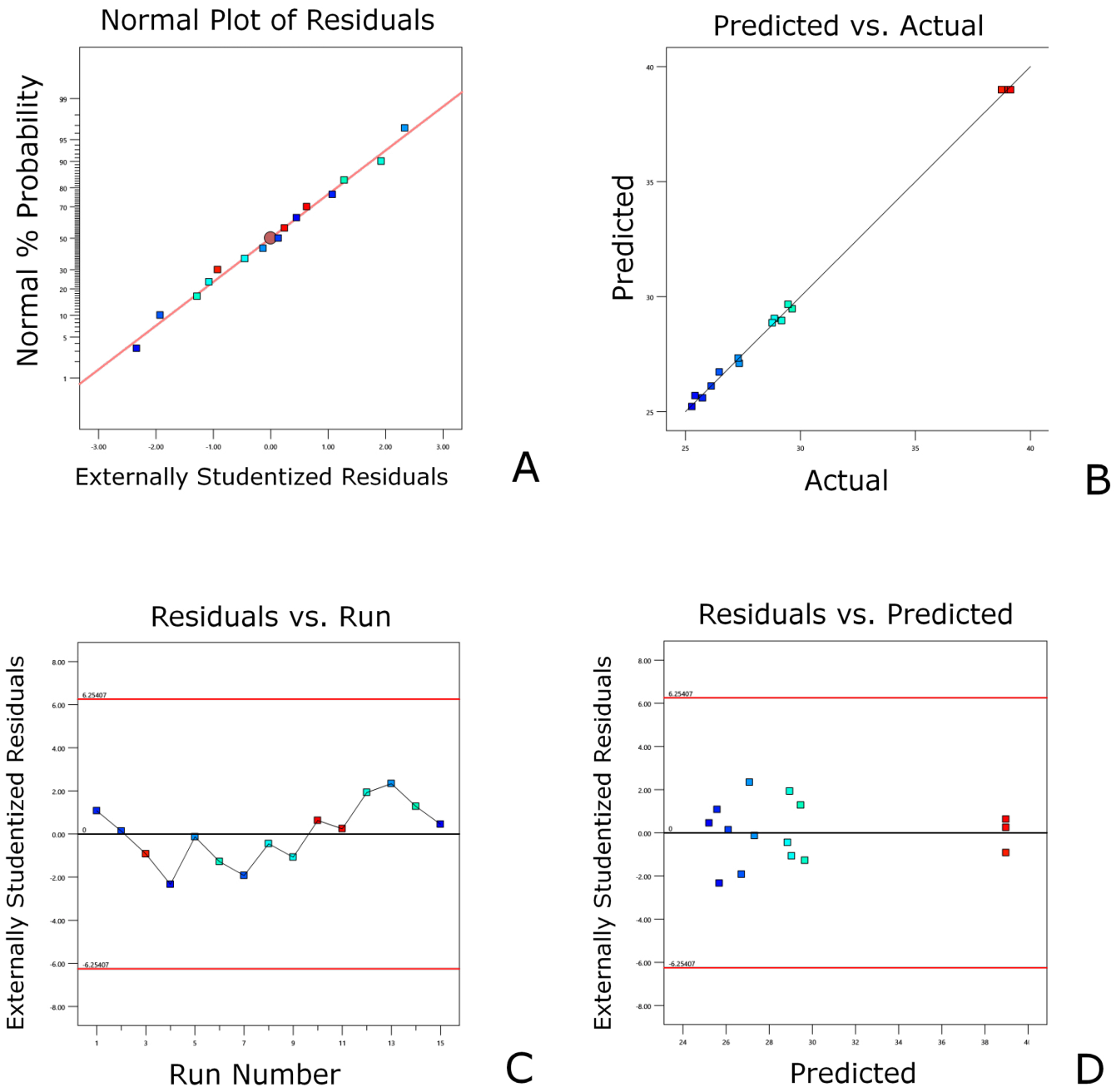

Figure 3: *Diagnostic plots representing the adequacy of the regression model of the protein content of S. cerevisiae: (A) Normal Plot of Residuals, (B) Predicted vs. Actual, (C) Residuals vs. Run, and (D) Residuals vs. Predicted.*

The figures representing the growth rate of *S. cerevisiae* (total biomass) and the protein content of the produced biomass were a function of two experimental variables, while the third variable remained constant at the center point. The first contour plot of both responses (Figures 4A and 5A) showing the interaction between aeration duration and temperature indicated a critical threshold beyond which the response declined. This could be attributed to thermal stress or oxygen saturation effects, as *S. cerevisiae* growth and metabolism was remarkably influenced by the temperature and oxygen level of the growth culture. According to Takagi (2021), the thermal stress affected the metabolic processes and cellular functions of *S. cerevisiae*, leading to growth declines. Generally, the temperature between 28 °C to 33 °C is the preferred temperature range for *Saccharomyces* yeasts growth (Salvadó *et al.*, 2011). Razzaq *et al.* (2020) reported that the maximum protein production by *S. cerevisiae* was observed at 30 °C, where further temperature elevation led to a decrease in final yield. In fact, the results of this study align with the previous findings where contour plots displayed a substantial enhancement in both biomass production and protein content at the temperature around 30 °C (Figures 4A, B and 5A, B).

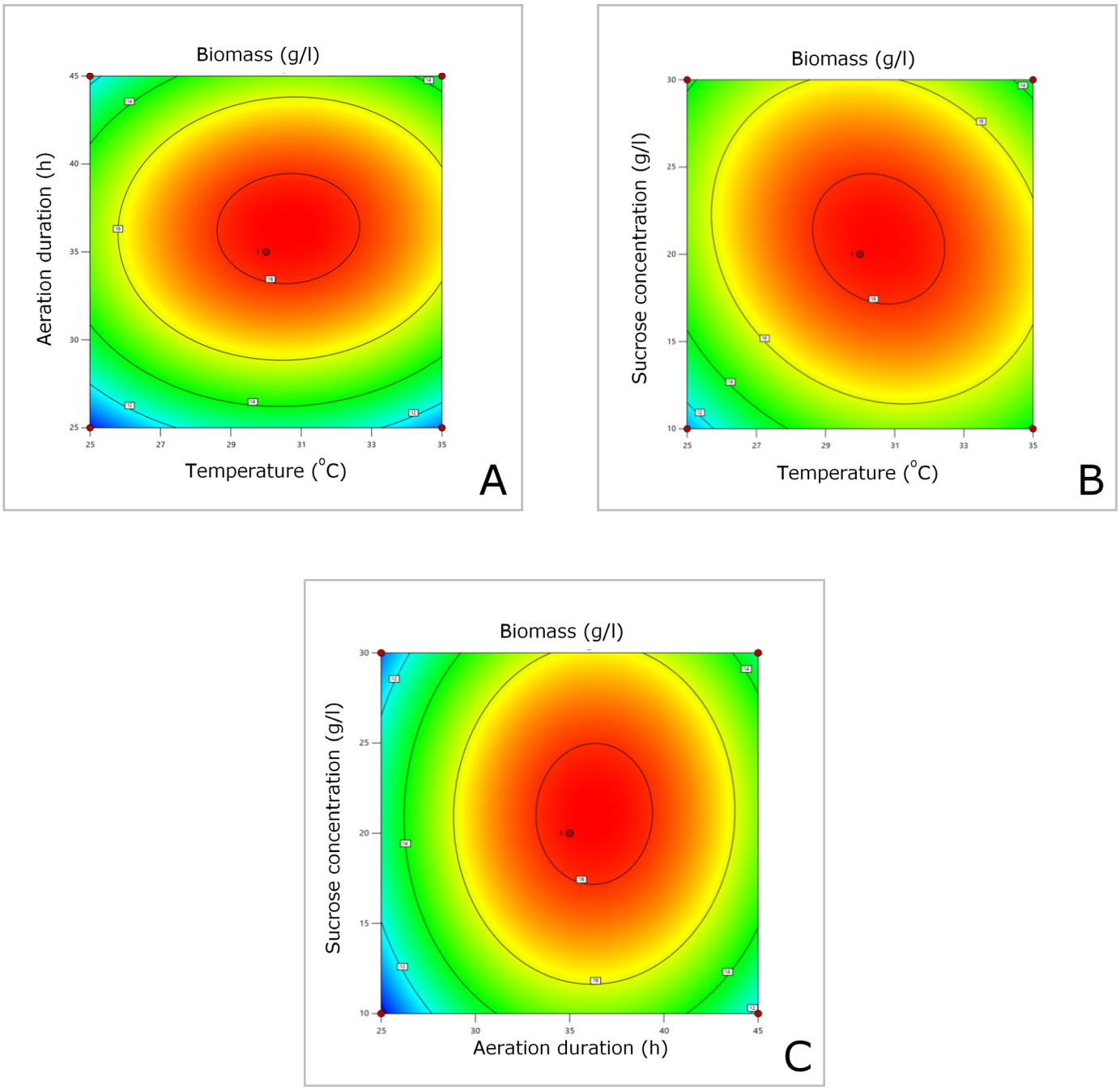

Figure 4: *Contour plots for the mutual effects of interaction between independent variables on biomass production by S. cerevisiae: (A) The mutual effects of interaction between aeration duration and temperature on biomass production with constant level of sucrose concentration at the optimal point, (B) The mutual effects of interaction between sucrose concentration and temperature on biomass production with constant level of aeration duration at the optimal point, and (C) The mutual effects of interaction between sucrose concentration and aeration duration on biomass production with constant level of temperature at the optimal point.*

Depending on the oxygen levels, *S. cerevisiae* expressed different growth behavior. For instance, its specific growth rate is considerably reduced in a microaerophilic condition (low oxygen levels of 2-10%) (Carbó *et al.*, 2015). Conversely, the excessive oxygen level can cause detrimental effects (*i.e.*, imbalanced ion distribution and cell leakiness) (Rintala, 2010). As presented in Figure 5A, C, the protein content of *S. cerevisiae* biomass was also influenced by the oxygen concentration of the growth culture. A previous study revealed that appropriate oxygen levels boosts a metabolic shift in *S. cerevisiae* that regulates the key genes involved in protein synthesis (Liu *et al.*, 2024). As it has been depicted in Figures 4A, C and 5A, C, the maximum level of biomass yield and protein content was observed when the aeration duration was

at an average level of 35 h.

The interaction between sucrose concentration and temperature (Figures 4B and 5B) was a key feature in representing the substrate utilization efficiency. As the contour plots demonstrate, the biomass production and protein content increased with higher sucrose concentration, but only when the concentration was around 20 g/l and within a specific thermal range. Beyond these ranges, excessive sucrose in the medium may inhibit the yeast function because of osmotic stress. Hezarjaribi *et al.* (2016) used sucrose as the main carbon source in their study for SCP production by *S. cerevisiae* and concluded that the enhancement of sucrose concentration increased the microbial protein production, in accordance with what was

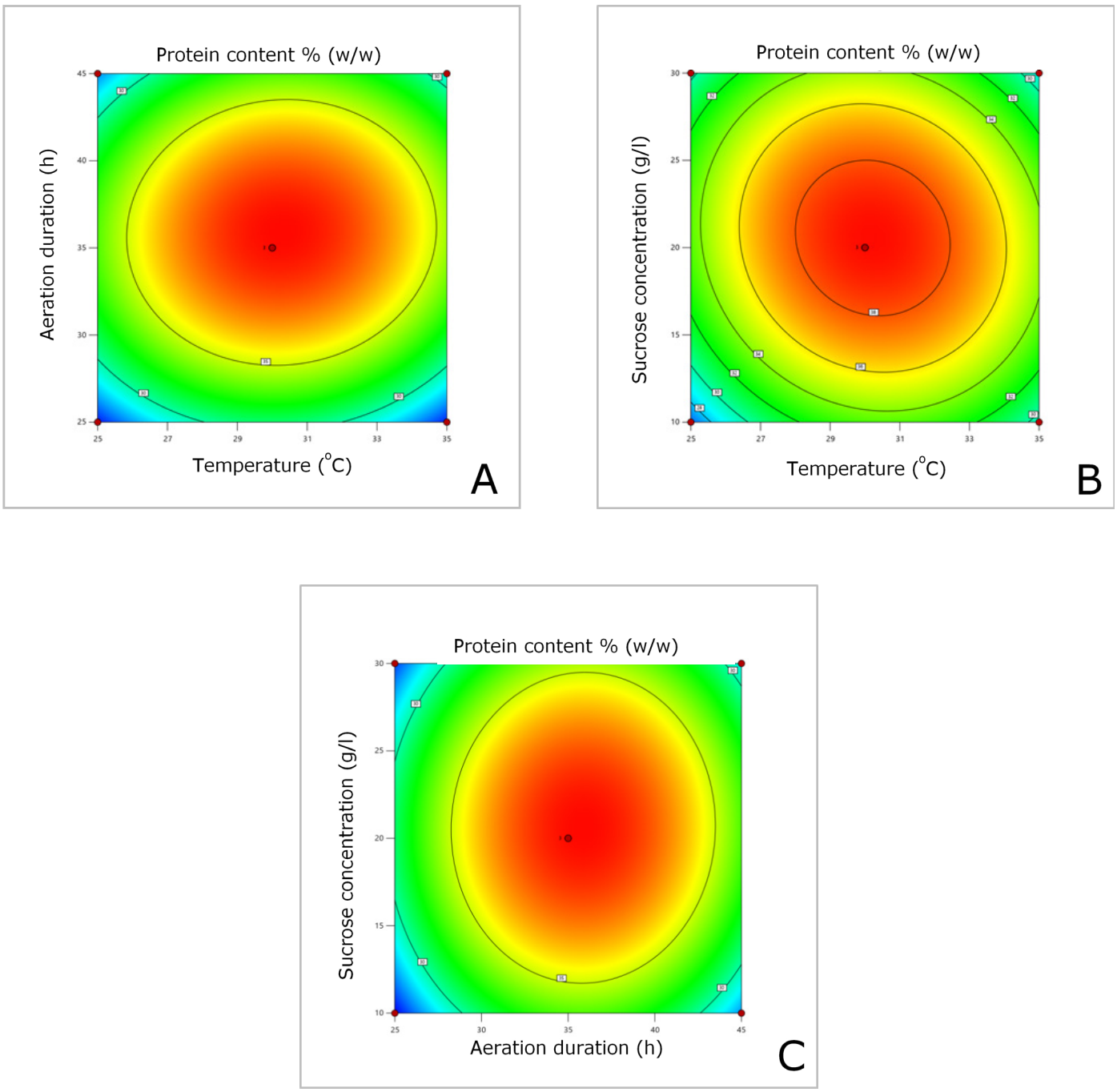

Figure 5: *Contour plots for the mutual effects of interaction between independent variables on protein content of S. cerevisiae: (A) The mutual effects of interaction between aeration duration and temperature on protein content with constant level of sucrose concentration at the optimal point, (B) The mutual effects of interaction between sucrose concentration and temperature on protein content with constant level of aeration duration at the optimal point, and (C) The mutual effects of interaction between sucrose concentration and aeration duration on protein content with constant level of temperature at the optimal point.*

observed in the current study by increasing the sucrose concentration from 10 g/l to 20 g/l. There was certain extent for this enhancement as further increase in the sucrose concentration of *S. cerevisiae* growth culture led to a reduction in biomass production and protein content. This result is in accordance with the previous studies conducted on *S. cerevisiae*, which reported a specific range of tolerable sucrose concentration for this yeast, attributable to sucrose accumulation in the medium and osmotic stress (Kara *et al*., 2017; Olivares-Marin *et al*., 2018).

Finally, the interaction between sucrose concentration and aeration duration is presented in Figures 4C and 5C. These contour plots reflected a distinguished interaction region where biomass yield and protein content were maximized respectively. Both of these graphical visualizations proposed an optimal balance between nutrient availability (sucrose concentration of 20 g/l) and oxygen transfer (aeration duration of 35 h) at the average levels of these experimental variables, ensuring an effective metabolic activity. This metabolic activity is essential for maintaining cellular energy production, as it regulates enzymatic reactions that drive ATP synthesis, substrate utilization, and overall metabolic flux (Lee *et al*., 2023). Adequate oxygen transfer supports the aerobic pathways and increase the process efficiency, while excessive or insufficient oxygen levels may disrupt the metabolic equilibrium, leading to reduced biomass production and protein content. Ribéreau-Gayon *et al*. (2015) also reported that while supplementing the yeast growth culture with sucrose as the main carbon source, a short period of aeration may lead to enhanced growth, notably after the first growth phase.

Effects of process variables on biomass yield and protein content

Saccharomyces cerevisiae growth and metabolism is influenced by the culture medium constituents and various environmental parameters. According to the experimental results of Box-Behnken design, the biomass yield and protein content ranged from 9.7 to 18.39 g/l and 25.29 to 39.15 % (w/w), respectively. The optimum values of both responses were obtained under the independent variables of 30 °C (temperature), 35 h (aeration duration), and 20 g/l (sucrose concentration). From the ANOVA table, there was a close correlation between the predicted and experimental results, where the maximum predicted values of biomass yield was 18.31 g/l and 38.98 % (w/w) for protein content (Table 1). This indicated the adequacy of the developed models. The correlation existing between each of the total biomass production and the protein content with the experimental variables was depicted in 3-D response graphs (Figures 6 and 7). These three-dimensional visualizations effectively demonstrated the orientation of each factor in influencing yeast growth and metabolism and facilitated the analysis of the developed quadratic models. Each plot represented the impact of the two tested variables within their examined ranges, while the third variable was constant at its optimal level.

The primary insight from the obtained graphs is that the optimum biomass and protein production were observed at the center point of the experimental domain of all the three tested variables, indicating that the range of variables was correctly defined. This reflected that the moderate levels of each factor synergistically contributed to the optimal responses, suggesting a balanced interplay rather than a linear or monotonic relationship. Thus, the suitability of the Box-Behnken design has been validated through placing the experimental runs at midpoints of edges (center) strategically to capture the interaction effects effectively.

As the convex surface of the 3-D plots indicates, the deviations from the medium levels in either direction (higher or lower) resulted in a decline in biomass and protein production. For instance, according to the first graph (Figures 6A and 7A), both the total biomass yield and the protein content peaked at intermediate values of temperature and aeration duration, while sucrose concentration was kept constant at the center level. Similarly, in the second graph (Figures 6B and 7B), the highest values of both responses were observed in the middle level of the sucrose concentration and temperature, while the aeration duration was kept constant at the center point. This suggests that an excessive or insufficient heat could limit the yeast growth and protein production, possibly due to the pivotal role of the temperature in metabolism and enzymatic activities of the *S. cerevisiae*. This aligns with the findings of another study performed on this yeast, where the temperatures of 32.3 °C and 45.4 °C have been reported as the highest optimum and maximum temperature for the baker's yeast growth and protein production, respectively (Salvadó *et al*., 2011). The results of the current study also indicated that an optimal temperature of 30 °C was identified

for both responses, while the growth and protein production continued by a temperature around 35 °C (*i.e.*, the highest temperature examined in this study). On the other hand, several studies have reported a remarkable decrease in *S. cerevisiae* growth and protein production at a temperature below 30 °C (Feng *et al.*, 2018; Lip *et al.*, 2020). The optimal temperature of 30 °C most likely reflected the balance that existed between enzymatic activity and thermal stability in yeast metabolism. At higher temperatures, reduced yield may be attributed to protein denaturation and increased cellular stress (Hezarjaribi *et al.*, 2016). From an industrial perspective, this temperature is advantageous as it minimizes the cooling costs while maintaining high productivity.

Regarding the influence of aeration duration on *S. cerevisiae*, as presented in Figures 6A, C and 7A, C, both the biomass and the protein production had maximized in the middle level of aeration duration. This could be attributed to the necessity of an adequate oxygen transfer for aerobic growth of *S. cerevisiae* and its metabolic stability. Insufficient oxygen availability affects the cellular processes and may lead to reduced

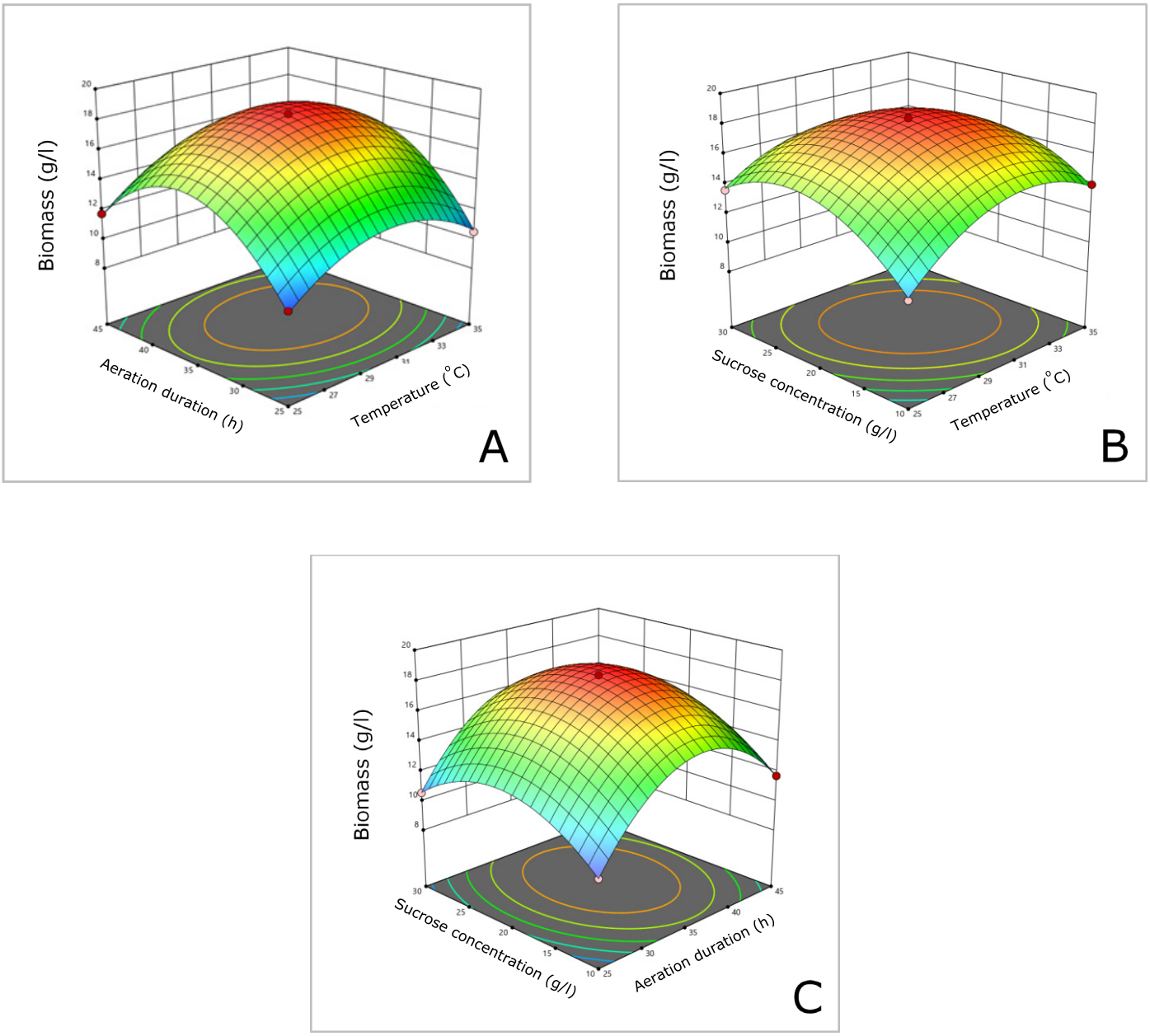

Figure 6: *Three-dimensional response surface plots for the mutual effects of independent variables on biomass production by S. cerevisiae: (A) The mutual effects of aeration duration and temperature on biomass production with constant level of sucrose concentration at the optimal point, (B) The mutual effects of sucrose concentration and temperature on biomass production with constant level of aeration duration at the optimal point, and (C) The mutual effects of sucrose concentration and aeration duration on biomass production with constant level of temperature at the optimal point.*

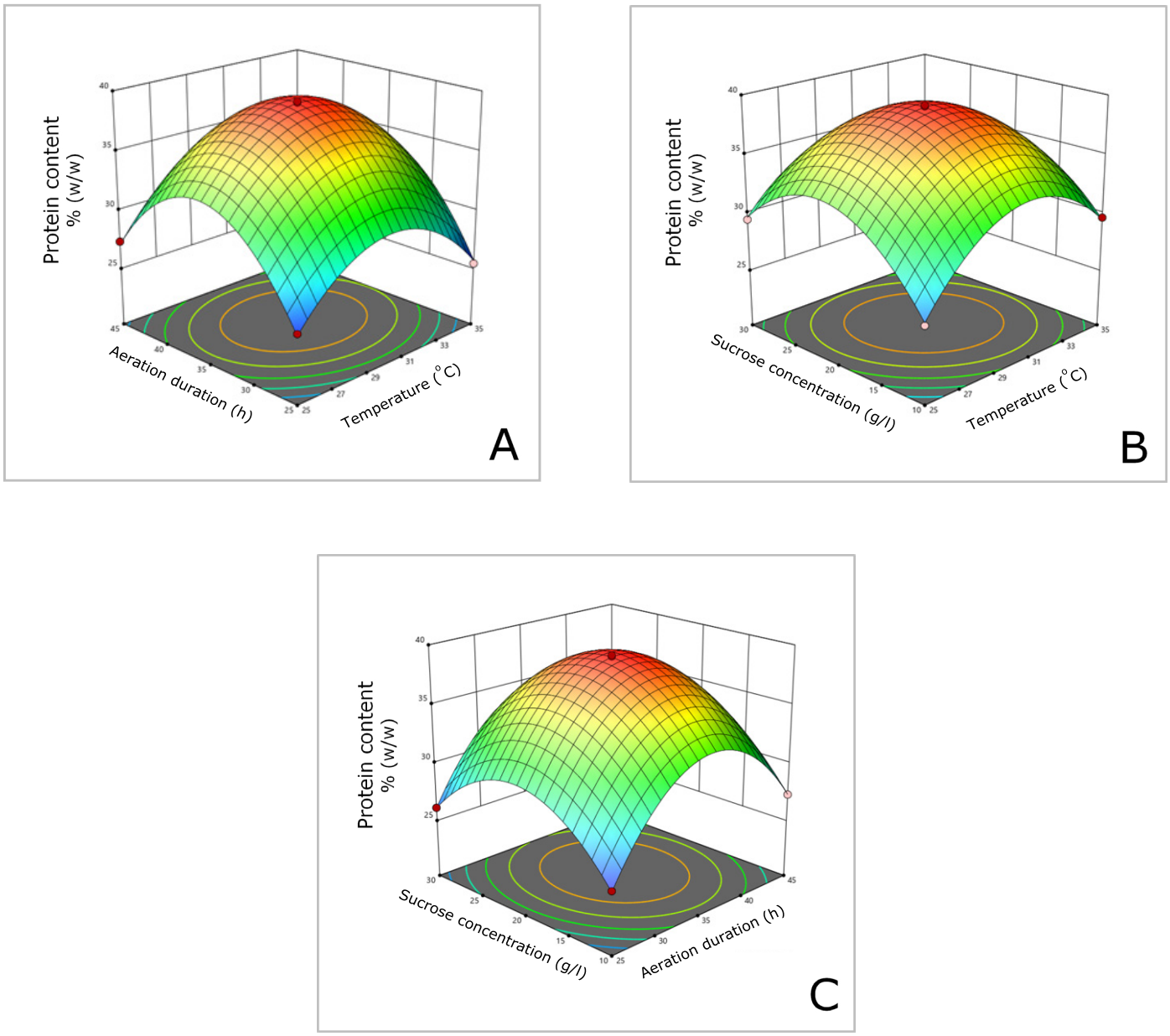

Figure 7: *Three-dimensional response surface plots for the mutual effects of independent variables on protein content of S. cerevisiae: (A) The mutual effects of aeration duration and temperature on protein content with constant level of sucrose concentration at the optimal point, (B) The mutual effects of sucrose concentration and temperature on protein content with constant level of aeration duration at the optimal point, and (C) The mutual effects of sucrose concentration and aeration duration on protein content with constant level of temperature at the optimal point.*

biomass and protein yield. In fact, S. cerevisiae requires a moderate level of oxygen for its optimal growth and protein production (Bisschops *et al.*, 2015; Chopda *et al.*, 2015). Various aeration tests (from micro-aerobic to aerobic cultures) declared that efficient aeration was critical for optimal productivity of protein and biomass production by the baker's yeast in fed-batch processes (Bisschops *et al.*, 2015). This may be assigned to the use of oxygen molecules in the components of the yeast cell membrane, *i.e.*, unsaturated fatty acids and sterols. Furthermore, the chronological lifespan of *S. cerevisiae* is considerably affected by aeration, as inadequate aeration may lead to stress resistance in the stationary phase cultures, causing a reduced lifespan (Bisschops *et al.*, 2015). Although *S. cerevisiae* as a facultative microorganism can perform fermentation and grow anaerobically, its biomass and protein yields are typically lower under anaerobic fermentation conditions compared to respiration. In this study, during the early experimental phase and in the absence of aeration, fermentation was the predominant metabolic pathway. Accordingly, glucose was metabolized *via* glycolysis and converted to ethanol (C_2H_5OH) and carbon dioxide (CO_2),

yielding only two ATP molecules per a glucose molecule. In the late phase, exposure to air triggered a metabolic shift in *S. cerevisiae* and activated several respiratory pathways, including the tricarboxylic acid (TCA) cycle and the oxidative phosphorylation, increasing ATP yield by 38 molecules per a glucose molecule. This metabolic transition may explain the observed differences in product yield under varying aeration conditions, as revealed by the optimization results of the current study (Bisschops *et al*., 2015; Mazzoleni *et al*., 2015).

Despite the fact that yeasts generally benefit from oxygen availability by supporting oxidative protein folding, but extreme aeration can cause damage in their intracellular structures such as DNA and disruption of various cellular functions. Prolonged exposure to high oxygen levels may negatively affect *S. cerevisiae* viability through oxidative stress. Enzyme inactivation, protein carbonylation, and disruption of some metabolic pathways are some of the consequences of this oxidative damage (Morano *et al*., 2012).

Likewise, the correlation between sucrose concentration and either temperature or aeration duration exhibited a dome-shaped response (Figures 6B, C and 7B, C), highlighting that an adequate carbon supply is crucial to optimize the biomass and the protein yield. Maintaining the carbon source at an appropriate level can avoid substrate inhibition or nutrient depletion. Thus, it directly influences the final processes of yields and metabolic efficiency. The optimum sucrose concentration for the growth of *S. cerevisiae* may differ based on the strain and the processing conditions (Shafaghat *et al*., 2010). In the current study, the sucrose concentration was examined in the range of 10 to 30 g/l and the highest value of biomass and protein production was observed at a concentration of 20 g/l. As the plots display, both responses increased with the enhancement of sucrose concentration up to this threshold (20 g/l), beyond which an inhibitory effects occurred possibly due to osmotic stress or substrate saturation. When the concentration of a carbon source in *S. cerevisiae* culture exceeds an optimal level, the resulting hyperosmotic stress may lead to the production of stress-response metabolites and subsequent water efflux and cellular dehydration. Furthermore, prolonged stress disrupts the nutrient uptake by inactivating the related transporters in the process. In addition, the biosynthetic pathways for certain amino acids may be downregulated, reflecting a cellular shift towards stress survival rather than growth (Marques *et al*., 2016).

Overall, the related 3-D plots of both responses confirmed the presence of remarkable quadratic effects, as captured by the second-order polynomial models. The peak observed at the center of these plots indicated that the response was sensitive to deviations from these medium levels, where either an increase or a decrease in any variable may lead to suboptimal biomass and protein production.

Conclusions and Recommendations

This study demonstrated that the propagation of *S. cerevisiae* under a specific combination of aerobic and anaerobic growth conditions can meaningfully influence the final yield and different quality criteria, such as its protein content. The findings indicate that an optimal SCP production by *S. cerevisiae* is a result of balanced correlation between temperature, aeration duration, and sucrose concentration. According to the coefficient of linear terms of ANOVA for both responses, the aeration duration was found to be the most influential parameter affecting the process responses. Thus, maintaining an appropriate oxygen level ensures improvement of bioprocess outputs and metabolic efficiency. Although the oxygen molecules are presented in various cellular structures and low oxygen availability may limit further growth and metabolic stability, high aeration duration can inhibit *S. cerevisiae* biomass and protein production due to oxidative stress.

While the results of the current study were satisfactory for the optimization process of *S. cerevisiae* production at pilot scale, reduced oxygen transfer rate (OTR) and lower volumetric mass transfer coefficients (k_La) can considerably affect the metabolic behavior of the baker's yeast in larger bioreactors. Thus, further studies are recommended to evaluate oxygen transfer dynamics and process performance under industrial scale conditions, exploring the baker's yeast deployment across diverse industrial, microbiological, and biotechnological contexts.

Acknowledgement

This work was supported by Fundumental Research Grant (03-01-18-1940FR) Universiti Putra Malaysia.

Novelty Statement

This study proposes an optimized protocol for *Saccharomyces cerevisiae* propagation in both aerobic and anaerobic conditions, leading to enhancement of metabolic efficiency and protein yield. The obtained results highlight the crucial role of controlled aeration in *S. cerevisiae* growth and are expected to benefit food and feed industry by providing a deeper understanding of the yeast's performance during large-scale fermentation processes.

Author's Contribution

Zahra Safdari: Conceptualization, investigation, methodology, data curation, formal analysis, validation, writing original draft.
Mohd Khairol Anuar Ariffin: Supervision, conceptualization, resource, methodology, validation, reviewing and editing, project administration
Mohamed Thariq Hameed Sultan: Conceptualization, reviewing and editing.
Eris Elianddy Supeni: Reviewing and editing.

Ethical approval
No ethical approval is required.

Funding source
This research did not receive funding from any scientific organization.

Generative AI and AI-assisted technology statement
No generative AI or AI-assisted technology was employed in this study.

Conflict of interests
The authors have declared no conflicts of interest.

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