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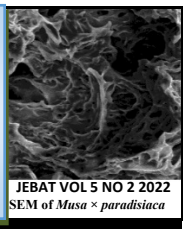

A Predictive Batch Culture Growth and Biosynthesis for *Bacillus cereus* (ATCC 14579) using Response Surface Methodology

Mani Malam Ahmad[1*], Abd.aziz Mohd Azoddein[2], Olusegun Abayomi Olalere[3] and Shuaibu Isa[4]

[1]Department of Biology, Faculty of Science, Kano University of Science and Technology (KUST), Wudil, 3244, Kano, Nigeria.
[2]Faculty of Chemical and Natural Resources Engineering, Universiti Malaysia Pahang (UMP), Lebuhraya Tun Razak, 26300 Gambang, Kuantan, Pahang, Malaysia.
[3]Analytical Biochemistry Research Centre (ABRC), Universiti Sains Malaysia (USM), 11800, Gelugor, Penang, Malaysia.
[4]Department of Microbiology, Faculty of Sciences, Gombe State University, P.M.B 127, Tudun Wada, Gombe State,Nigeria.

*Corresponding author:
Dr. Mani Malam Ahmad,
Department of Biology,
Faculty of Science,
Kano University of Science and Technology (KUST),
Wudil, 3244, Kano,
Nigeria.
Email: mmahmadu@kustwudil.edu.ng, mmahmadu@gmail.com

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ABSTRACT

Optimization studies of *Bacillus cereus* (ATCC 14579) tolerance to stress phenomenon could serve as a basic prerequisite for its utilization in future biotechnological research. Successful cell culture at optimum parametric conditions was found to prepare the cells to withstand upper and lower range values depending on the need and purpose. This study was performed to develop a predictive optimum model for the growth of this mesophilic bacterium, *B. cereus* in batch culture shake flasks. The linear and mutual interactions effects of nutrient availability (4-16 g/L) and composition, temperature (30-40 ºC) and agitation (140 - 200 rpm) collectively termed as growth regulators were sought using central composite face-centred design (CCFD) response surface methodology (RSM). The effectiveness of the independent variables on the dependent variable was weighted and validated using statistical and graphical indices which spelt a suitable predictive model for *B. cereus* growth and biosynthesis. This model provided an efficient and reliable approach for predicting the growth of *B. cereus* as a function of the growth-influencing markers. The results showed that the model term is highly significant at P >0.0001 and a well-correlated adjusted R^2 and predicted R^2 of less than 1.0 (0.9984). Moreover, the coefficient of determination value of only 1.45 % variability as well as agreed predictive (3.01) versus experimental (3.0) values depicted that the hidden (noise) effect was very minimal. Therefore, the model further confirmed the versatility of the isolate to simple growth nutrient within defined optimal physical operational parameters of simple shake flask culture.

INTRODUCTION

The response of microbial cells upon inoculation into a new medium was found to vary significantly in continuous and batch culture systems [1]. The batch culture technique, though not the best option for industrial research; is reliable for its flexibility in studying the bacterial pattern of growth at the abundance and in starvation. In addition, batch culture as well facilitates pilot research to ascertain and prepare an isolate tolerance to a different range of oxidative stresses, and influence of growth markers. the behaviour and growth pattern of *B. cereus* was reported to sharply differ from that of other mesophilic bacteria due to its ability to express a certain gene's coding for sigma factors associated with its maintenance, stress response, and sporulation [2]. The continuous culture approach was known for its perpetual yield throughout due to the abundance of growth regulating factors, although accumulation of metabolic residues was found to affect this trend. In the batch culture however, growth is sustained until the exhaustion of growth markers [1].

According to Maier [3], growth and biomass synthesis in suspending batch culture is measured as a function of time (acclimatization), in addition to the age of the inoculum, substrates composition and availability, temperature, pH and exposure to toxic metabolites. Response to growth influencing factors (stress phenomenon) involved an adjustment to a range of physical and chemical stimulants [4,5,6]. Various research

studies were conducted to ascertain the relative linear effects of these growth factors. Such approaches are presumed to be defective and strenuous as much is needed to access and appreciate the microbial world. The main outstanding drawback to this conventional approach is the persistent nature of offering an explanatory view of the unfolding mechanism, while the current technology has the potential to proffer a predictive view of the mechanisms that enable, the management of a given process, based on needs.

Response surface methodology (RSM), statistical modelling using face-cantered central composite design (FCCCD), provides an insight into the interactions of independent variables, which is a core value for establishing synergism needed in effective biological process modelling [7]. Simple and inexpensive shake flasks experimentation method to perform suspended studies of microbial growth and other bioprocess has been a recognized feasible approach in almost 90% of biotechnological studies when optimization of cultures conditions is in question. Therefore, in the present study, we envisaged using these models (FCCCD and RSM) to identify a better selection and quantitative composition of medium factors. This was achieved by examining the effects of the interaction for optimum values of a factorial combination of nutrient, agitation, and temperature on the growth of *B. cereus* and biomass production. It as well guides the plan of the experiments and the parsimonious analysis of data to gain a maximum amount of information most efficiently usable to propose a workable model for various biotechnological applications of *B. cereus.*

MATERIALS AND METHODS

Media Preparation and Strain Cultivations

This research was carried out with *B. cereus* (ATCC 14579) and its growth media obtained from Merck (Malaysia) Sdn. Bhd as a local agent dealing with the bacteria, sourced from microbiologic, 217 Osseo Ave. North, St. Cloud, USA. Enriched culture media was prepared following the manufacturer's guidelines. Typically, 8g of nutrient broth was dissolved in de-ionized water (di) to a final volume of 1l in a Schott bottle and shaken vigorously until it dissolved. The solution was heated on a hot plate and sterilized in an autoclave (H+P Varioklav Steam Sterilizer ESCO), at 121°C for 15 min; the sterilized media was then placed in a water bath to cool the media to 47°C before pouring into various 20 ml sampling bottles.

The stock cultures of *B. cereus* were maintained throughout the experiment by periodic sub-transfer at least fortnightly on nutrient agar (NA) and stored at 4°C in a refrigerator [8,9]. For preparing preculture, a loopful of cells [10] from a 24 h actively growing culture on a nutrient agar plate was transferred into 20 ml bottles containing freshly prepared nutrient broth (NB) 10% (w v-^{1}) and then incubated at 37 °C for 24 h at a vigorous shaking of 180 rpm using an orbital shaker (B. Braun, German Model). After 24 h, the inoculum was transferred into a 500 ml Erlenmeyer flask containing 150 ml of NB (w/v) with 30% (v/v) of the original volume of the shake flask [11]. The inoculation process was aseptically performed to avoid any contamination. The samples were then placed on a shaker and calibrated for agitation and temperature accordingly for each run. The experiment was run under the selected different ranges of nutrient concentration, agitation, and temperature at different acclimation times. The medium pH was kept constant at a near neutral of 7.0±2 throughout the experiment using 0.5 m buffer.

Analytical Procedure

For growth analysis, 2.5 ml aliquots were withdrawn periodically at fixed intervals throughout the experimental period and monitored turbidimetrically by measuring the optical density (absorbance) of samples. Absorbance was estimated at wavelength 600nm against a blank NB using UV-VIS Spectrophotometer (HITACHI, U-1800, Japan), after appropriate dilution to obtain an OD value of < 0.5 as in Roebuck, [14], modified. Bacterial biomass or cell dry weight (CDW) was determined by dry weight measurement. The sample was centrifuged at 12,000 rpm for 15-20 min in pre-weighed tubes [15]. The supernatant was disposed of, and the pellets were re-suspended in 0.15 m saline solution and centrifuged again as described elsewhere [16, 17] with some modifications. The supernatant was discarded and each tube containing the cell mass was dried at 100 °C for 1 h and weighed to get the dry cell weight. The mass was measured and dried repeatedly until a stable weight was obtained. the dry cell mass density (g/l) was found to follow the following regression equation: x (g/l) = 0.39 (OD600).

Experimental Design

The factors influencing B. cereus growth were screened using the fractional factorial design of experiment (FFDOE) based on the preliminary experiment [16]. Three out of four factors were selected based on their linear and interaction effects on the dependent variable. These model terms were adopted for optimization of B. cereus growth using FCCCD-RSM using design expert software (Stat-Ease, Inc, version 10.0.5.0, Minneapolis, USA). Response surface methodology was used to optimize the identified growth signals (nutrient, agitation, and temperature).

A total number of 20 experimental runs consisting of a different matrix of process parameters of nutrient concentration (A), temperature (B), and acclimatization time (C) at three-coded levels (-1, 0 and +1) were performed. The design consists of a 2*x* full factorial coded notation (-1, 0 and +1), supported by 2*x* axials points (±α, 0, 0), (0, ±α, 0), (0, 0, ±α) and n*c* centre point (0, 0, 0) as described elsewhere (17, 18). The α value was computed as $\alpha = (2n)^{1/4}$ (17), where n is the number of factors. Moreover, the centre runs were used to provide information about the existence of curvature in the system due to experimental error, and repeatability of the data [19]. Whereas the axial point allows gyration of the variance of the model prediction steadily by default in their corresponding centre levels. The independent variables and their levels, as well as the design matrix table for the experiment, were given in **Table 1**.

Table 1. Experimental range and levels of the independent variables for FCCCD.

Terms	Variable	Units	Factor Levels		
			Low (-1)	Center (0)	High (+1)
A	nutrient conc.	g/l	4	10	16
B	temperature	°C	30	35	40
C	acclim. time	h	24	48	72

Statistical Analysis of the model terms

Regression analysis to correlate the impact of independent variables on the *B. cereus* growth was carried using an empirical model utilizing the second-order polynomial equation:

$$Y = \beta_o + \sum_i \beta_i x_i + \sum_{ii} \beta_{ii} x_i^2 + \sum_{ij} \beta_{ij} x_i x_j \quad 1.0$$

where y is the predicted response, β_o is the offset term, β_i is the ith linear coefficient, β_{ii} is the *i*th quadratic coefficient, and β_{ij} the ijth interaction coefficient.

Analysis of variance (ANOVA) was used to test the significance of the model equation and its terms. While the adequacy of the developed response surface model was determined by evaluating the lack of fit and coefficient of determination (R^2), Fisher ratio (F-value), probability and residual. A graphical depiction of the behaviour of the model terms on the response was visualized using 3D surface plots.

Validation of the model

The experiments predicted optimum range values of factors (nutrient, agitation, and temperature) of the software were used to ascertain the validity of the model. This was done through a batch of experimental runs under the predicted points. The experimental results obtained were compared with the predicted values and their fitness was assessed using bias factor (Bf) and accuracy factor (Af) validation indices. According to Giffel and Zwietering, [20], Bf measure how far the distance observed values are above or below the point of equivalence, while Af measure on average the distance between each value relative to the line of equivalence, thus indicating how closely related experimental values are to predict.

RESULTS AND DISCUSSION

***B. cereus* cell biomass synthesis (cell dry weight)**

Fig. 1 shows the finding of *B. cereus* (ATCC 14579) cell dry weight (CDW) for experimental results in g/l. The trend in bacterial cell biomass synthesis is better explained by a calibration curve which is indicated by plotting OD against the cdw. the linear pattern of the plots from this finding fitted well to the supposed relationship between amounts of light absorbance measured at the wavelength (600 nm) of optical density and the increase in cell biomass over time also measured as cell dry weight. Based on the scatter plots; the regression expressed by a correlation coefficient of determination (R-squared) value of 0.9406 was estimated for cell biomass increase for observed values. This result is strengthened by the equation line of best fit, from which the average percentage of OD value that corresponds to maximum CDW (y) could be established. In all the results, cell dry weight was observed to be directly proportional to optical density value. This finding was upheld by [21]. It can be concluded that all the model equations fitted well, indicating a significant positive correlation between cell biomass and optical density with enhanced utilization of growth medium under the physical parameters of shake flasks.

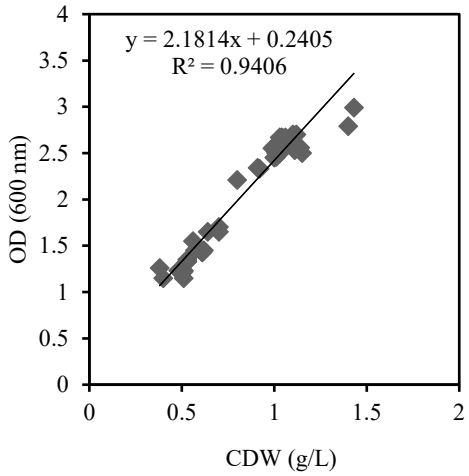

Fig. 1. Standard calibration curves for *B. cereus* cell biomass production.

Model Fitting

The linear, interaction, quadratic, and cubic models were fitted to the experimental data to obtain the regression equations. The enhanced *B. cereus* growth at effective variables ranges was computed from the modified Gompertz function using a nonlinear regression model. The adequacy of the model was evaluated using an ANOVA was presented in **Table 3**. the response and test variables were related to the following response surface quadratic polynomial equation (2.0)

The f value of this model (542.94) indicated a high level of significance (P<0.0001), and the lack-of-fit test was not significant (P>0.05). the coefficient of determination (R^2-values) and values from this study showed R^2 and adj. R^2 values of 0.9980 and 0.9961, respectively. the R^2 value indicated that approximately only 0.2 % of the total variation is not explained by the model referred from **Table 4**. the predicted R^2 of 0.9938 was also in good agreement with adjR^2. furthermore, the probability (P<0.0001*)* for model terms A, B, C, A^2, and C^2 shows their effects of the terms on *B. cereus* growth was highly significant at a 99% confidence level. nonetheless, the impact of AB, AC, BC, and C^2 on the dependent variable was significant but at 95% confidence level. it was also observed that some parameters (B, C, BC, B^2, and C^2), were found to cast their effects in the negative plain. results from the present study indicated that significant growth of roughly 2.61 OD was achieved at average substrates and operational variables of nutrient concentration 10 g/l, temperature 36 ºC and acclimatization time of 48 h at a defined agitation of 170 rpm. however, different growth ranges were obtained from interactions between the independent variables.

Fig. 2 depicted the relationship between predicted and observed values of the response which was remarkably significant at 0.38 % error, indicating good predictability of the model. The blue points showed adequacy at the lower range, while the red and orange colours depicted optimum and highest correlation with minimal deviation from the diagonal line. as can be seen, the predicted values are in agreement with the experimental values of the response variables. The small deflection of the point from straight also indicated that unpredicted possible irregularities (signal-noise-ratio) were negligible.

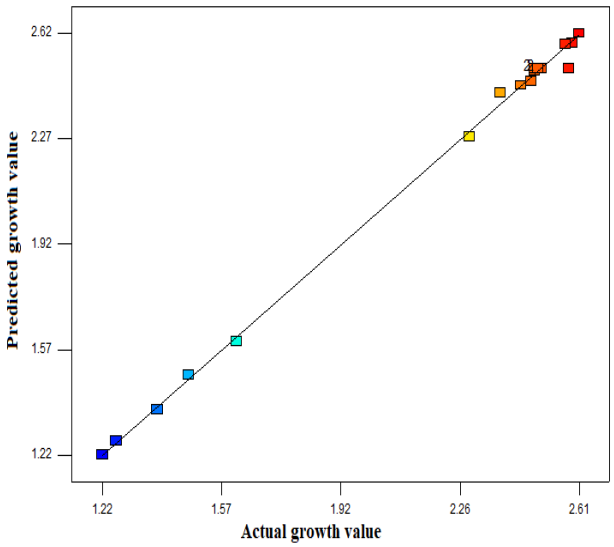

Fig. 2. Predicted versus Observed growth values for *B. cereus*.

Table 2. Design matrix and result for the factors and respective response from experiment using face-centered central composite design (FCCCD).

Run	Type	Factors			Response (B. *cereus* Growth)		
		A (G/L)	B (H)	C (ºC)	Experimental (OD 600 nm)	CDW g/l	Predicted (OD 600 nm)
1	Center	10(0)	48(0)	35(0)	2.50±0.21	0.07	2.50
2	Axial	4(-1)	48(0)	35(0)	1.45±0.12	0.04	1.48
3	Factorial	10(0)	48(0)	35(0)	2.51±0.15	0.07	2.48
4	Axial	16(+1)	48(0)	35(0)	2.61±0.13	0.08	2.62
5	Axial	4(-1)	24(-1)	40(+1)	1.38±0.25	0.04	1.37
6	Factorial	10(0)	48(0)	35(0)	2.59±0.20	0.08	2.55
7	Center	10(0)	48(0)	35(0)	2.48±0.19	0.08	2.49
8	Factorial	16(+1)	24(-1)	30(-1)	2.45±0.17	0.07	2.58
9	Axial	10(0)	72(+1)	35(0)	2.29±0.31	0.06	2.27
10	Axial	4(-1)	72(+1)	30(-1)	1.26±0.26	0.05	1.27
11	Center	10(0)	48(0)	35(0)	2.49±0.22	0.09	2.49
12	Factorial	16(+1)	72(+1)	30(-1)	2.44±0.27	0.07	2.44
13	Factorial	10(0)	24(-1)	35(0)	2.38±0.16	0.07	2.39
14	Factorial	10(0)	48(0)	30(-1)	2.56±0.11	0.07	2.58
15	Center	4(-1)	72(+1)	40(+1)	1.23±0.24	0.04	1.24
16	Factorial	4(-1)	24(-1)	30(-1)	1.56±0.21	0.05	1.60
17	Center	10(0)	48(0)	35(0)	2.50±0.18	0.09	2.51
18	Center	10(0)	48(0)	40(+1)	2.55±0.24	0.08	2.49
19	Axial	16(+1)	24(-1)	40(0)	2.47±0.21	0.07	2.46
20	Factorial	16(+1)	72(+1)	40(+1)	2.49±0.25	0.08	2.50

Table 3. ANOVA for Response Surface Quadratic Model for *B. cereus* growth.

Source	SS	Df	MS	F-value	P-value	Remark	PC[a]
Model	4.74	9	0.53	542.94	< 0.0001	***	
A	3.20	1	3.20	3299.24	< 0.0001	***	67.37
B	0.053	1	0.053	54.88	< 0.0001	***	1.12
C	0.018	1	0.018	19.04	0.0014	***	0.38
AB	0.018	1	0.018	18.59	0.0015	***	0.38
AC	5.0 X10^{-3}	1	5.0 X10^{-3}	5.15	0.0466	**	0.11
BC	0.016	1	0.016	16.68	0.0022	**	0.34
A^2	0.56	1	0.56	573.51	< 0.0001	***	11.79
B^2	0.066	1	0.066	68.04	< 0.0001	***	0.13
C^2	3.37*10^-3	1	3.37 *10^-3	3.47	0.0921	†	0.07
Residual	9.7*10 ^-3	10	9.7*10 ^-3				
Lack of fit	2.98*10 ^-3	5	5.95*10 ^-4	0.44	0.8044	†	
Pure error	6.73*10 ^-3	5	1.35*10 ^-3				
Cor. Total	4.75	19					

*** (Highly Significant), ** (Significant), † (Not Significant), [a] Percentage of Contribution 2.0

$$Growth\ (Y) = 2.50 + 0.57A - 0.073B - 0.043C + 0.048AB + 0.025AC + 0.045BC - 0.45A^2 - 0.15B^2 + 0.035C^2$$

Table 4. The statistical parameters for the developed polynomial model for *B. cereus* growth.

Model	Std. Dev.	F-value	P-value	PRESS	R^2	Adj. R^2	Pred. R^2
Linear	0.30	11.81	0.0002	2.57	0.6889	0.6306	0.4592
2-factor interaction	0.33	0.12	0.9479	9.41	0.6971	0.5574	0.9801
Quadratic	0.031	490.97	<0.0001	0.029	0.9980	0.9961	0.9938
Cubic	0.036	0.40	0.8003	1.13	0.9984	0.9949	0.7613

Interactions Effect of Variables on *B. cereus* Growth

The three-dimensional (3D response surface graphical interpretation of the regression equation was to apprehend the synergistic effects of the variables and locate the significant level of each variable for maximal response. **Fig. 3a** showed a response surface based on nutrient concentration and temperature range which indicated that *B. cereus* growth was sensitive to the interactions effects of the variables. increased growth was observed with variations in nutrient concentration and temperature. However, the sensitivity was significant at the nutrient concentration range of 10-13 g/L and 30-38 ºC temperature, while the optimum range of these parameters that yielded the highest growth stood at 10 g/l and 35 ºC. It was reported elsewhere [22] that *B. cereus* thrives well at a temperature range of 35 to 42 ºC, with 37 ºC being a moderate temperature. this agrees quite well with the finding from this study. Similarly, the interaction effect of nutrient and acclimatization depicted in **Fig. 3b** showed an increase in growth with time up to an optimum period of 48 h, from where the growth appeared to be insensitive and plummeted. it was observed that the initial growth process of *B. cereus* was quite slow and took a little longer time probably due to its phenotype. The interaction effect of acclimatization time and temperature demonstrated in **Fig. 3c** shows that the response was not quite sensitive to the variation of either factor. however, an average growth of 2.30±0.25 od was recorded at an optimum time of 48 h and temperature 36 ºC at a fixed nutrient concentration of 10 g/L.

Model Validation

The reliability of RSM based on goodness of fit requires mathematical evaluation before practical scale-up. An additional four conditions for model validation were evaluated within the range of the experimental design (**Table 5**). Most of the points were relatively close to the 100% correlation ($y=x$) line, indicating the satisfactory performance of the predictive model. The *Bf* of this model was 1.02, within the range considered good and acceptable. However, larger *Af* depicted *a* less accurate

model from the average estimate. Thus, an accuracy factor of two indicates that the prediction is, on average, a factor of two different from the observed value, i.e. either half as large or twice as large, while a value of one indicates that there is perfect agreement between all predicted and observed values. The average *Af* for this study was 1.05, indicating a high degree of accuracy. Therefore, this model can be used as a reference for predicting *B. cereus* growth in a simple and low-nutrient composition medium under optimized process condition of shake flasks. Based on the established model, the optimum *B. cereus* growth was 2.60±0.04 (OD 600 nm) equivalent to 1.04 g/L biomass at 13.9 g/L nutrient, 36 ºC temperature and acclimatization time of 48 hours. Therefore, the finding can be recommended as a reference point for further biotechnological research studies and industrial scale-up.

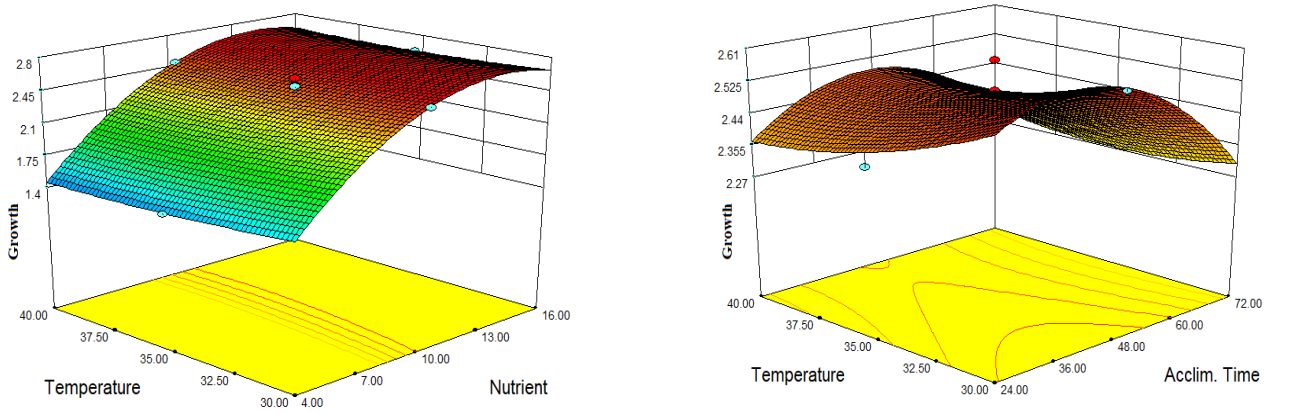

Fig. 4. 3D interactions response surface plots; (a) nutrient versus temperature; (b) nutrient versus, agitation; (c) temperature versus acclimatization.

Table 5. Model validation for experimental and predicted growth for *B. cereus* ATCC 14579.

Run	Variables			Growth (OD 600 nm)				
	utrient	Temperature	Acclimatization	Exp.	Pred.	Pred. /Exp.	Log Pred. /Exp.	Absolute Value
1	13.5	36	40	2.67	2.77	1.04	0.02	0.02
2	13.9	36	48	2.66	2.62	0.98	-0.01	0.01
3	13.5	35	40	2.65	2.86	1.08	0.03	0.03
4	13.8	37	48	2.70	2.65	0.98	-0.01	0.01
							0.01	0.02
Bias factor = (Antilog$_{10}$ 0.01)						1.02		
Accuracy factor = (Antilog$_{10}$ 0.02)						1.05		

CONCLUSION

In this study, the optimum growth conditions of *B. cereus* were facilitated using a face-centred central composite design and response surface methodology. The validity of the model was proven by fitting the values of the variables to the model equation and by carrying out experiments using these values. The optimization of the analyzed response demonstrated that the best results for *B. cereus* growth 2.60±0.04 (OD 600 nm) were obtained with 13.9 g/l of nutrient concentration, at 36 °C temperature and acclimatization time of 48 h. All points were located near the central point of the design, indicating the fitness of the model based on the correlation coefficient index (R^2, adjusted R^2, and predicted R^2) regression analysis. The validation of a model for adequacy between experimental and predicted values using bias and accuracy factor indices revealed an almost 100% correlation ($y = x$) line, signalling the passable reliability of the proposed model.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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