Journal of Applied Biology & Biotechnology Vol. 13(1), pp. 83-92, Jan-Feb, 2025
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2024.177031

Optimization of *Bacillus subtilis* PW12 biomass production using RSM: a preliminary study towards single-cell protein production for aquaculture

Merin Gracious*, S Nandakumar

PG and Research Department of Zoology, N.S.S. College, Pandalam, Kerala, India.

ARTICLE INFO

Article history:
Received on: May 10, 2024
Accepted on: October 13, 2024
Available online: November 15, 2024

Key words:
Biomass yield,
Bacillus subtilis MTCC10402,
Central Composite Design (CCD),
Microbial protein,
Plackett- Burman Design (PBD).

ABSTRACT

Bacillus subtilis strains are extensively integrated into aquafeeds, serving either as probiotics or single-cell proteins, due to their proven nutritional advantages for farmed fish. However, low biomass yields and high production costs limit their usefulness as microbial proteins in aquafeeds. Hence, a pioneering effort was made to statistically optimize the growth medium for enhancing the biomass production of a beneficial aquaculture probiotic bacterium, *B. subtilis* PW12, which holds potential for contributing to the production of high-value additives for aquaculture diets. Plackett-Burman Design was used for the primary screening of nutrient components and culture conditions. Four of the eleven variables investigated in the PBD, such as soya peptone, glucose, pH, and inoculum size, had a significant influence on the biomass production of the bacteria. These factors were further optimized by using the Central Composite Design and Response Surface Methodologies. The predicted biomass yield was 14.19 g/L, whereas the obtained biomass yield as dry cell weight was 14.29 ± 0.23 g/L. A glucose and soya peptone-based medium demonstrated efficacy in promoting both growth and nutritional enrichment of the target bacteria. Furthermore, this optimized medium facilitated the attainment of high cell density, a critical factor for the future production of quality microbial products tailored for aquaculture applications.

1. INTRODUCTION

The practice of incorporating beneficial microorganisms into feeds in the form of single-cell proteins and probiotics represents a promising strategy in aquaculture, as it enhances the growth and immunological resilience of farmed fish. Single-cell protein, or microbial protein, refers to the dried cells of bacteria, fungi, algae, and yeast, which has been regarded as a potential substitute for fish meal up to 25-50% in aquaculture diets. Bacterial cells outpace other microbial proteins in terms of their rich crude protein concentration (50-80 wt.%), large amino acid spectrum, carbohydrate, vitamin content, etc. Although bacterial meal serves as a valuable source of protein, the efficiency of its mass production relies on the final product yield; therefore, using cost-effective methods to maximize cellular growth is a crucial prerequisite for microbial protein production [1-3]. A culture medium exacting in terms of the nutritional requirements of the selected bacteria can exert greater influence on its growth, nutrient content, and biomass yield [4]. The nutritional requirements and composition of a bacterial cell are in fact attributed to the species and strain to which it belongs. Since their growth tactics are nutrient-sensitive, finding the optimal growth medium and conditions for the bacterial strain under study is pivotal for obtaining the desired biomass yield with a high crude protein content.

Bacillus subtilis is a common probiotic bacterium used in aquafeeds. They are frequently incorporated into aquaculture diets as live or heat-killed probiotics, and research indicates that they enhance fish nutrition and exhibit potent immunomodulatory effects. [5-7]. Furthermore, different *Bacillus* species have been proven to produce single cell proteins with high crude protein content [8-10]. This microbial protein is highly sought after for its essential amino acid profile, which complies with Food and Agriculture Organization and World Health Organizations standards [2,3,11]. *B.subtilis* PW12 (MTCC 10402) is a non-pathogenic, salt tolerant, probiotic bacterium with anti-microbial activity against aquaculture pathogens. The ability of the bacteria to thrive in normal to higher salinities renders it a valuable water probiotic that can find application across diverse aquaculture sectors spanning freshwater, brackish water, and marine environments [12,13]. The bacterium has been found to have anti-microbial properties, particularly against *Vibrio* species, and the primary compounds produced are anti-microbial chemicals of aquaculture grade, such as *N*-substituted phenazinecarboxylate, propyl/phenethyl 2-oxoacetates [14]. Microbial

*Corresponding Author:
Merin Gracious,
PG and Research Department of Zoology, N.S.S. College,
Pandalam, Kerala, India.
E-mail: graciousmerin@gmail.com

© 2025 Merin Gracious and S Nandakumar. This is an open access article distributed under the terms of the Creative Commons Attribution License -NonCommercial-ShareAlike Unported License (http://creativecommons.org/licenses/by-nc-sa/3.0/).

formulations, comprising *B. subtilis* strains and other bacterial species producing similar antimicrobial compounds, have been developed with elevated nutritional value and antagonistic properties that are intended for incorporation into aquafeeds, aiming to improve fish health and promote growth [15]. Production of high-quality microbial products, however, demands enhancement of microbial biomass production. Since biomass production of *B. subtilis* PW12 has not yet been the subject of extensive research, the current study deals with pioneering efforts on optimizing the medium to maximize the biomass production of the bacteria in batch culture to overcome the low yield challenge associated with the production of microbial proteins. Moreover, the research fosters the opportunity to explore the potentiality of this microbial biomass in aquafeed as a single-cell protein.

Statistical medium optimization using Response Surface Methodology (RSM) is an excellent strategy to formulate nutritionally balanced as well as cost-effective culture mediums for obtaining desired products from microorganisms before stepping into their large-scale manufacture. Statistical medium optimization surpasses conventional strategies such as the one-factor-at-a-time approach in terms of cost, time efficiency, accuracy in result interpretation, and understanding of the interactive effects of variables. This approach combines statistical and mathematical techniques to build models, analyze the impact of numerous independent variables, and determine the optimal values for each variable. In essence, response surface methodology is a combination of steps comprising experimental design, mathematical modeling, and statistical inference that, when used together, enable the researcher to analyze the response that is influenced by a number of variables [16].

Statistical medium optimization studies have successfully yielded various growth-associated secondary metabolites and facilitated the preparation of bio-control formulations utilizing different strains of *B.subtilis*. However, the current investigation aims at optimizing a medium specifically for enhanced biomass production of *B. subtilis* PW12 (MTCC10402) in a batch culture, to unlock its potential in aquafeeds. In the present study, Plackett-Burman design (PBD) was used to select the medium components and culture conditions that significantly affect biomass production. The selected factors were then optimized using RSM with a Central Composite Design (CCD) to achieve a higher biomass yield for the bacterium.

2. MATERIALS AND METHODS

2.1. Microorganism

Bacterial strain *B. subtilis* PW 12 (MTCC 10402) was procured from CSIR- Microbial Type Culture Collection Centre (MTCC), Chandigarh, Punjab, India. The culture was preserved in 40% glycerol and kept at –20°C. The bacterial culture was maintained on nutrient agar slants and plates for routine work. The slants were kept at 4°C and periodically subcultured in nutrient broth medium.

2.2. Preparation of Seed Culture

Pure culture was used to inoculate 100 mL of Tryptic Soy Broth medium contained in a 250 mL Erlenmeyer's flask and cultured in an orbital shaker at 37°C and 150 rpm for 24 h.

2.3. Medium Components and Culture Conditions

Medium components such as glucose, peptone, yeast extract, soya peptone, NaCl, $MgSO_4.7H_2O$, and KH_2PO_4 were chosen, while culture conditions like temperature (°C), pH, agitation (rpm) and inoculum size (% v/v) were selected for the study. The incubation period for the strain was chosen as 48 h following a trial.

2.4. Statistical Experimental Design for Optimization

2.4.1. Primary screening of medium components and culture conditions using Plackett- Burman design (PBD)

Plackett-Burman Design (PBD), based on the first order model [Equation 1], was employed for determining the relative significance of the medium components and culture conditions on the biomass production of the bacteria [17].

$$Y = \beta_0 + \Sigma \beta_i X_i \quad \textbf{[Equation 1]}$$

Where Y is the response, β_0 is the model intercept, β_i is the linear coefficient, and X_i is the level of the independent variable. According to the design, the total number of experimental trials is n+1, where n is the number of variables. Nutrient components such as glucose, peptone, yeast extract, soya peptone, NaCl, $MgSO_4.7H_2O$ and KH_2PO_4, while culture conditions like pH, temperature, inoculum size, and agitation were subjected to screening with 15 experimental runs. Concentration ranges of medium components and culture conditions applied in the PBD were determined by an initial one-factor-at-a-time method (OFAT). The response variable was the biomass yield (g/L) produced after 48 h of culture. Each component in the PBD was examined at two different levels: low (-) and high (+) (Table 1). Centre point (0) replications were done in triplicate.

2.4.2. Medium optimization by Response Surface Methodology (RSM)

A central composite design with five coded levels (-α, -1, 0, +1, and + α) was used to explicate the effects of the factors selected by the PBD on biomass yield. A full factorial design was used in the central composite design, with 31 experimental runs. Biomass yield, measured as dry weight (g/L) obtained after 48 h of cultivation, was considered the response variable. The variables in the CCD were tested at low (−) and high (+) levels, and six replications at center point (0) were

Table 1: Coded and uncoded values of experimental variables used in Plackett- Burman design.

Factor	Name	Coded levels	
		Low level(–)	High level(+)
A	Glucose (g/L)	10	20
B	Peptone (g/L)	10	20
C	Yeast extract (g/L)	10	30
D	Soya peptone (g/L)	10	30
E	NaCl (g/L)	5	10
F	$MgSO_4.7H_2O$ (g/L)	2	4
G	KH_2PO_4 (g/L)	2	3
H	Temperature (°C)	28	37
J	pH	6	7
K	Agitation (rpm)	150	250
L	Inoculum size (% v/v)	5	10

performed (Table 2). The CCD model was explained by a second-order polynomial equation [Equation 2].

$$Y = \beta_0 + \Sigma_i^k \beta_i X_i + \Sigma_{ii}^k \beta_{ii} X_i^2 + \Sigma_{i<j} \beta_{ij} X_i X_j \quad \textbf{[Equation 2]}$$

where Y is the predicted response, β_0 is the offset term, β_i is the *i*th linear coefficient, β_{ii} is the *i*th quadratic coefficient, and β_{ij} is the *ij*th interaction coefficient [17].

2.4.3. Statistical analysis

The statistical design and analysis were performed using Minitab 21. The analysis of variance (ANOVA) was used to statistically analyze the models. The coefficient of determination (R^2) and its statistical significance, determined by the F-test was used to evaluate the statistical quality of the polynomial model equations. The significance level for all statistical tests was set at 0.05. The student t-test was used to assess the statistical significance of the regression coefficients. In PBD, the Pareto chart was used to screen out insignificant variables at a significance level of $p = 0.05$. Contour plots and three-dimensional response surface plots were applied to elucidate the main and interactive effects of the independent variables on the biomass production of *B. subtilis* PW 12. By solving the regression equation and evaluating the response surface and contour plots, the optimum values for the study variables were determined.

Table 2: Coded and uncoded values of experimental variables used in the central composite design.

Factor	Name	Coded levels				
		−α	−1	0	1	+α
A	Soy Peptone (g/L)	5.86	10	20	30	34.14
B	Glucose (g/L)	7.93	10	15	20	22.07
C	pH	5.586	6	7	8	8.414
D	Inoculum size(% v/v)	3.965	5	7.5	10	11.035

α=1.414.

2.5. Evaluation of Biomass Production

Biomass production of *B. subtilis* PW 12 grown in optimized medium was evaluated in a laboratory bioreactor. The dry weight of the bacterial cell biomass was determined by centrifuging 10 mL of culture sample at 5000 g for 20 min, drying at 80°C overnight, and weighing the resulting dry cell biomass in grams per liter.

3. RESULTS AND DISCUSSION

3.1. Screening of Significant Nutrient Components Using Plackett-Burman Design

The Plackett-Burman design is a key tool in screening the effects of the variables on the final response [18]. The biomass yield showed variation across the 15 experimental runs (Table 3). ANOVA results for PBD revealed that only 4 out of 11 variables had a significant effect on the response, i.e., biomass yield. Soya peptone, glucose, pH, and inoculum size had a significant influence on biomass production ($p < 0.05$) [Table 4, Figure 1]. Hence, only these factors were used for further optimization experiments. The polynomial equation for biomass yield is represented by Equation 3.

$$\begin{aligned}\text{Biomass} = {} & -1.64 + 0.0716\ \text{Glucose} + 0.0336\ \text{Peptone} - 0.01532\ \text{Yeast extract} \\ & + 0.06157\ \text{Soya Peptone} + 0.1303\ \text{NaCl} + 0.1527\ \text{MgSO4.7H2O} \\ & + 0.142\ \text{KH2PO4} + 0.0148\ \text{Temperature} + 0.890\ \text{pH} + 0.00407 \\ & \text{Agitation} - 0.1469\ \text{Inoculum size} - 1.256\ \text{Ct Pt}\end{aligned}$$

[Equation 3]

The linear regression coefficient of determination, R^2, was 0.99, which indicated that the predicted model could account for 99.17% of the variability in the experimental data. The model was fit to the data, as the lack of fit was found to be insignificant ($p > 0.05$). Since the curvature was significant ($p < 0.05$), a higher order model was necessary to optimize the levels of significant variables.

The Selection of medium components and their concentrations is fundamental to medium optimization, as the availability of substrates

Table 3 : Design and responses of the Plackett - Burman Design (PBD).

Run	A	B	C	D	E	F	G	H	J	K	L	Biomass yield (g/L)
1	-	+	+	+	-	+	+	-	+	-	-	9.34
2	0	0	0	0	0	0	0	0	0	0	0	7.55
3	0	0	0	0	0	0	0	0	0	0	0	7.06
4	+	-	-	-	+	+	+	-	+	+	-	9.85
5	-	+	+	-	+	-	-	-	+	+	+	7.98
6	+	-	+	+	-	+	-	-	-	+	+	8.36
7	-	-	-	+	+	+	-	+	+	-	+	9.22
8	-	-	+	+	+	-	+	+	-	+	-	9.00
9	+	+	-	+	-	-	-	+	+	+	-	10.45
10	0	0	0	0	0	0	0	0	0	0	0	7.50
11	+	+	+	-	+	+	-	+	-	-	-	8.57
12	-	+	-	-	-	+	+	+	-	+	+	7.33
13	+	+	-	+	+	-	+	-	-	-	+	9.08
14	-	-	-	-	-	-	-	-	-	-	-	6.74
15	+	-	+	-	-	-	+	+	+	-	+	7.58

Table 4: Analysis of variance for the experimental results of the Plackett - Burman Design (PBD).

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model*	12	16.6529	1.38774	19.84	0.049
Linear	11	12.8638	1.16943	16.72	0.058
Glucose*	1	1.538	1.53797	21.99	0.043
Peptone	1	0.338	0.33802	4.83	0.159
Yeast extract	1	0.2815	0.28152	4.03	0.183
Soya Peptone*	1	4.5485	4.54855	65.04	0.015
NaCl	1	1.2727	1.27271	18.2	0.051
$MgSO_4.7H_2O$	1	0.2797	0.27969	4	0.184
KH_2PO_4	1	0.0608	0.06078	0.87	0.45
Temperature	1	0.0531	0.05307	0.76	0.476
pH*	1	2.3745	2.37452	33.95	0.028
Agitation	1	0.4978	0.49776	7.12	0.116
Inoculum size*	1	1.6192	1.61921	23.15	0.041
Curvature*	1	3.7891	3.7891	54.18	0.018
Error	2	0.1399	0.06994		
Total	14	16.7927			
$R^2 = 0.9917$					

*Statistically significant at a probability level of 95%; DF: degree of freedom; SS: Sum of squares; MS: mean square.

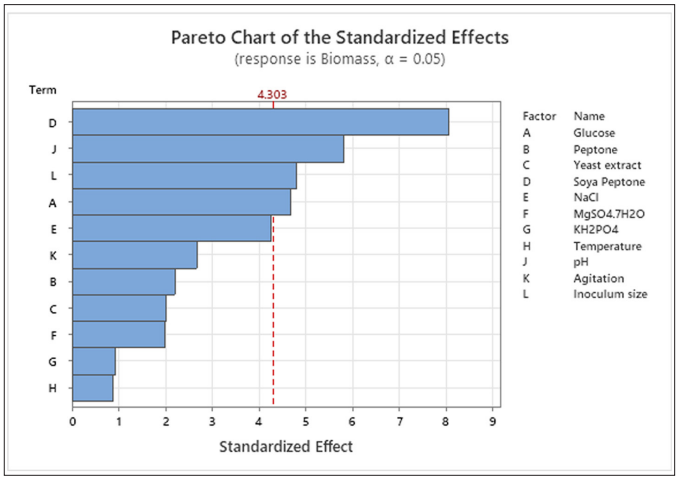

Figure 1: Pareto chart showing standard effects of 11 variables on biomass production of *B. subtilis* PW 12.

influences growth rate, and the limitations of different substrates can significantly affect the nutritional value of microbial biomass [19]. An adequate supply of carbon and nitrogen, together with ideal growing conditions, are the most essential requirements for bacterial growth. The variables investigated in the present study were selected to account for the sources of carbon (glucose), nitrogen (peptone, yeast extract, and soya peptone), inorganic nutrients for the supply of phosphate, sulfate, and minerals (KH_2PO_4, $MgSO_4.7H_2O$, NaCl) and culture conditions (temperature, pH, agitation, and inoculum size). Since no other optimization experiments had been conducted on *B. subtilis* PW12, the medium components and culture conditions for the study were chosen based on prior research on other strains of *B. subtilis*. Prior to screening, OFAT studies provided information on the ranges of concentrations for each individual component that should be used in the Plackett-Burman design.

Screening experiments using PBD showed that soya peptone, glucose, pH, and inoculum size had substantial impacts on biomass yield. The nitrogen source has a key role in determining the rate of bacterial growth [20]. Soya peptone had the highest effect on biomass production by *B. subtilis* PW12. Other nitrogen sources used in the PBD study, such as yeast extract and peptone, had an insignificant effect on biomass production. The effective use of soya peptone or soya protein hydrolysate as nitrogen sources by some strains of *B. subtilis* has been reported, where high cell density biomass production was a prerequisite for the synthesis of certain secondary metabolites and industrially important enzymes [21-23]. Several prior investigations, in contrast to the current study, have utilized other organic nitrogen sources, such as peptone and yeast extract, to produce biomass using some specific strains of *B. subtilis* [24-26]. In the present study, the enhanced production of biomass may be attributed to the complex mixture of amino acids, short peptides, carbohydrates, etc. contained in soya peptone. Despite being a non-essential amino acid, glutamate is the one amino acid that *B. subtilis* prefers [27,28]. According to the study by Leibs et al. [29] the exponential growth phase of *B. subtilis* showed considerable intake of glutamate, aspartate, serine, and alanine from the growth media. Moreover, soya peptone has a high reserve of amino acids and a total glutamic acid concentration higher than other amino acid contents [30]. Together with other growth factors, the presence of these amino acids might have helped the bacteria produce more biomass. Additionally, it is possible that *B. subtilis* may efficiently use soya peptone as it can produce the enzyme protein glutaminase (PG), which has the ability to hydrolyze amides of glutamine in proteins and thereby enhance the solubility of plant proteins. During fermentation, *B. subtilis* may produce extracellular proteases that can self-activate protein glutaminase, which is released in the inactive pro-enzyme form and can facilitate efficient substrate utilization by the bacterium [31].

The effect of glucose was also statistically significant on the biomass production of the bacteria. Previous studies also show that glucose is a preferred carbon source for *B. subtilis* and other *Bacilli* [32-34]. The phosphoenolpyruvate: sugar phosphotransferase system (PTS) in *B. subtilis* aids them to take up and phosphorylate the sugar, and the presence of PTS dependent and independent glucose transporters also facilitates the bacteria in efficiently utilizing glucose as the carbon source [32,35]. Additionally, as glucose is a readily metabolized carbon source, it may accelerate the fermentation process and enhance the production of bacterial biomass. Previous studies have found that glucose was the preferred carbon source for obtaining a high biomass yield in *B. subtilis* strains for the synthesis of several secondary metabolites and enzymes that are linked to high cell densities using batch or fed batch fermentation methods [21,36,37].

Among the culture conditions, pH and inoculum size significantly affected biomass production. Since pH is the primary control of bacterial metabolism, optimum pH is necessary for their maximum biomass production. Besides pH, inoculum size also showed a significant impact on biomass production. As inoculum concentration determines the lag phase of bacterial growth, the addition of adequate inoculum of bacteria at the appropriate developmental stage is important to achieve increased biomass yield [38].

In the present study, temperature and agitation had the least effect on biomass yield among the culture conditions. Previous studies on *B. subtilis* strains suggest that their optimal temperature and agitation ranges are 30°C-37°C and 200–250 rpm, respectively [34,39,40]. The temperature and agitation ranges applied in the present PBD study were ideal for *B. subtilis* PW12 growth. Consequently, the temperature and agitation values in the CCD optimization study were set at 37°C and 200 rpm, respectively.

3.2. Optimization of Biomass Production by RSM and CCD

The Central Composite Design was carried out to optimize the levels of significant variables to maximize the biomass yield. The design matrix and the associated experimental results show that biomass yield varied across the experimental runs (Table 5). The second-order polynomial equation for biomass yield is represented by the following equation [Equation 4].

$$\begin{aligned}\text{Biomass} = {} & -40.96 + 0.4153\ \text{Soya peptone} + 2.129\ \text{Glucose} + 37.21\ \text{pH} \\ & + 0.324\ \text{Inoculum size} - 0.01180\ \text{Soya peptone*Soya peptone} \\ & - 0.02657\ \text{Glucose*Glucose} - 2.638\ \text{pH*pH} - 0.0692\ \text{Inoculum size*} \\ & \text{Inoculum size} - 0.00985\ \text{Soya peptone*Glucose} + 0.0140\ \text{Soya} \\ & \text{peptone*pH} + 0.02488\ \text{Soya peptone*Inoculum size} - 0.1153\ \text{Glucose} \\ & \text{*pH} - 0.02612\ \text{Glucose*Inoculum size} + 0.1369\ \text{pH*Inoculum size}\end{aligned}$$

[Equation 4]

The coefficient of determination, R^2, which was 0.98, indicated that 98.71% of the variability in the response could be elucidated by the model (Table 6). ANOVA was conducted to assess the statistical significance of the polynomial equation, and the results showed that

Table 5 : Design and responses of the central composite design (CCD).

Run	A	B	C	D	Obtained biomass yield	Predicted biomass yield
1	1	−1	−1	1	9.76	9.82
2	−1	1	−1	−1	9.54	9.90
3	−1.414	0	0	0	10.17	9.70
4	1	−1	1	1	12.00	11.65
5	0	0	−1.414	0	7.99	8.12
6	−1	−1	−1	−1	5.89	5.79
7	1	−1	1	−1	7.05	7.32
8	0	1.414	0	0	12.93	12.79
9	0	−1.414	0	0	10.89	10.93
10	0	0	0	0	13.06	13.19
11	0	0	0	0	13.83	13.19
12	0	0	0	0	13.43	13.19
13	0	0	0	0	13.05	13.19
14	0	0	0	0	13.61	13.19
15	0	0	1.414	0	7.94	7.71
16	0	0	0	0	12.53	13.19
17	−1	1	1	1	7.73	8.03
18	−1	−1	1	1	7.35	7.53
19	1	1	−1	−1	9.15	9.00
20	−1	−1	1	−1	5.69	5.69
21	−1	1	−1	1	9.30	9.07
22	−1	1	1	−1	7.52	7.50
23	1	1	1	1	10.04	10.18
24	0	0	0	0	12.63	13.19
25	1	1	−1	1	10.65	10.65
26	1	−1	−1	−1	7.15	6.86
27	0	0	0	−1.414	10.99	11.09
28	1	1	1	−1	7.33	7.15
29	−1	−1	−1	1	6.07	6.26
30	1.414	0	0	0	11.58	11.96
31	0	0	0	1.414	13.75	13.56

Table 6 : Analysis of variance for the experimental results of the Central Composite Design (CCD).

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	14	203.135	14.5096	87.26	0.000
Linear	4	37.249	9.3122	56	0.000
Soya peptone*	1	12.876	12.8757	77.43	0.000
Glucose*	1	8.711	8.7109	52.39	0.000
pH	1	0.411	0.4111	2.47	0.135
Inoculum size*	1	15.251	15.2513	91.72	0.000
Square	4	146.599	36.6498	220.41	0.000
Soya peptone*Soya peptone*	1	13.124	13.1244	78.93	0.000
Glucose*Glucose*	1	4.159	4.1594	25.01	0.000
pH*pH*	1	65.587	65.587	394.43	0.000
Inoculum size*Inoculum size*	1	1.765	1.7647	10.61	0.005
2-Way Interaction	6	19.287	3.2145	19.33	0.000
Soya peptone*Glucose*	1	3.882	3.8819	23.35	0.000
Soya peptone*pH	1	0.314	0.3139	1.89	0.188
Soya peptone*Inoculum size*	1	6.191	6.1914	37.23	0.000
Glucose*pH*	1	5.321	5.3211	32	0.000
Glucose*Inoculum size*	1	1.705	1.705	10.25	0.006
pH*Inoculum size*	1	1.873	1.8735	11.27	0.004
Error	16	2.66	0.1663		
Lack-of-Fit	10	1.237	0.1237	0.52	0.827
Pure Error	6	1.424	0.2373		
Total	30	205.795			
R2 = 0.9871					

*Statistically significant at a probability level of 95%; DF: degree of freedom; SS: Sum of squares; MS: mean square.

the regression was statistically significant ($p < 0.05$). The significance of the regression coefficient of the model reveals that the linear effect of all variables except pH ($p > 0.05$) on the maximum biomass production was significant ($p < 0.05$) (Table 6). However, the squared effects of all the variables were significant ($P < 0.05$) and except for the soya peptone - pH interaction ($P > 0,05$), all other interactions were statistically significant ($P < 0.05$). The lack of fit of the model was insignificant ($P > 0.05$) indicating that the model fits the data. The significant interactions of variables described by the model are shown in contour plots and three-dimensional response surface plots [Figure 2].

Carbon and nitrogen sources are absolutely essential for bacterial growth. In the present study, the interactive effects of glucose and soya peptone also significantly contributed to biomass production. The response surface and contour plots show that the optimal concentrations of glucose and soya peptone were around 14 g/L and 27 g/L, respectively [Figure 2A]. In concordance with the current investigation, a single factor optimization study by Naveed et al. [36] revealed that the addition of 1.5% glucose and 2.5 % soya peptone in the fermentation medium of a newly isolated strain, *B. subtilis* BSN314, caused enhanced biomass production in the bacterium. However, studies by Zhong et al. [21] showed that an optimized medium containing 30.70 g/L of glucose and 2.4 g/L of total nitrogen from soya bean meal hydrolysate was beneficial for high cell density cultivation of *B. subtilis* ZK8 in fed batch fermentation. Investigation of Yue et al. [41] demonstrated that cell concentration of *B. subtilis* ZK-H2 could be increased to $7x10^8$ cfu/mL in an optimized medium containing glucose and soya peptone, where the optimal concentration for glucose and total nitrogen concentration from soybean protein were 21 g/L and 4.0 g/L, respectively. Zhong et al. [42] used soya bean meal hydrolysate and glucose in the medium of a wild type strain, *Bacillus sp*. H-18W, for the production of chiral acetoin, a metabolite associated with fast cell growth. Nguyen [22] used glucose at 5.62 g/L and soya bean peptone at 13 g/L in the optimized medium for higher biomass production by *B. subtilis* Natto, whereas a study by Ullah et al. [43] showed that soya peptone and glucose at 1% w/v in the medium resulted in improved cell growth of a probiotic strain of *B. subtilis* and in contrast to the present study, higher concentrations of soya peptone caused a decline in cell growth. Stamenkovic et al. [33] reported that biomass production of *B.subtilis* NCIM2063 could be enhanced when the growth medium was supplemented with 10 g/L of glucose. Study by Cho et al. [25] demonstrated that supplementation of the initial medium with 10 g/L glucose and 50 g/L peptone in a fed batch culture caused an increase in the cell density of *B. subtilis* however, exhaustion of glucose after 6 h was also reported. Since *B. subtilis* can work on soya-based proteins, Wang et al. [44] included soy bean curd as well as soya peptone at concentrations of 12.2% w/v and 5.7% w/v respectively, in the medium for metabolite production and growth of *B. subtilis*. Study by Yanez et al. [45] reported that even defatted soy flour (40 g/L) could be used in the growth and metabolite production medium of *B. subtilis* CtpxS2-1 and was found to improve bacterial growth. From the present study, it can be concluded that *B.subtilis* PW 12 strain could also successfully utilize glucose at less than 15 g/L and soya peptone at less than 30 g/L in the culture medium for attaining maximum growth.

Soya peptone and glucose had significant interactions with inoculum size as well [Figures 2B, C]. In the current investigation, it was shown that biomass production rose in tandem with inoculum size. A high inoculum concentration can promote growth up to a certain point, but afterwards, it causes the microbial activity to decline because of the depletion of nutrients in the medium. Conversely, a low inoculum concentration decreases the cell concentration, which may affect the amount of bioproduct produced. At lower cell concentrations, it takes a long time to attain optimum growth [46]. The optimum inoculum size in the current investigation was 11.03% v/v, indicating that the soya peptone and glucose-based optimized medium had sufficient nutrients to maintain a higher inoculum concentration to enhance the growth of the bacterium. Study by Huang et al. [47] on *B. subtilis* biomass production shows that 8% v/v of inoculum size may be ideal for achieving optimal production.

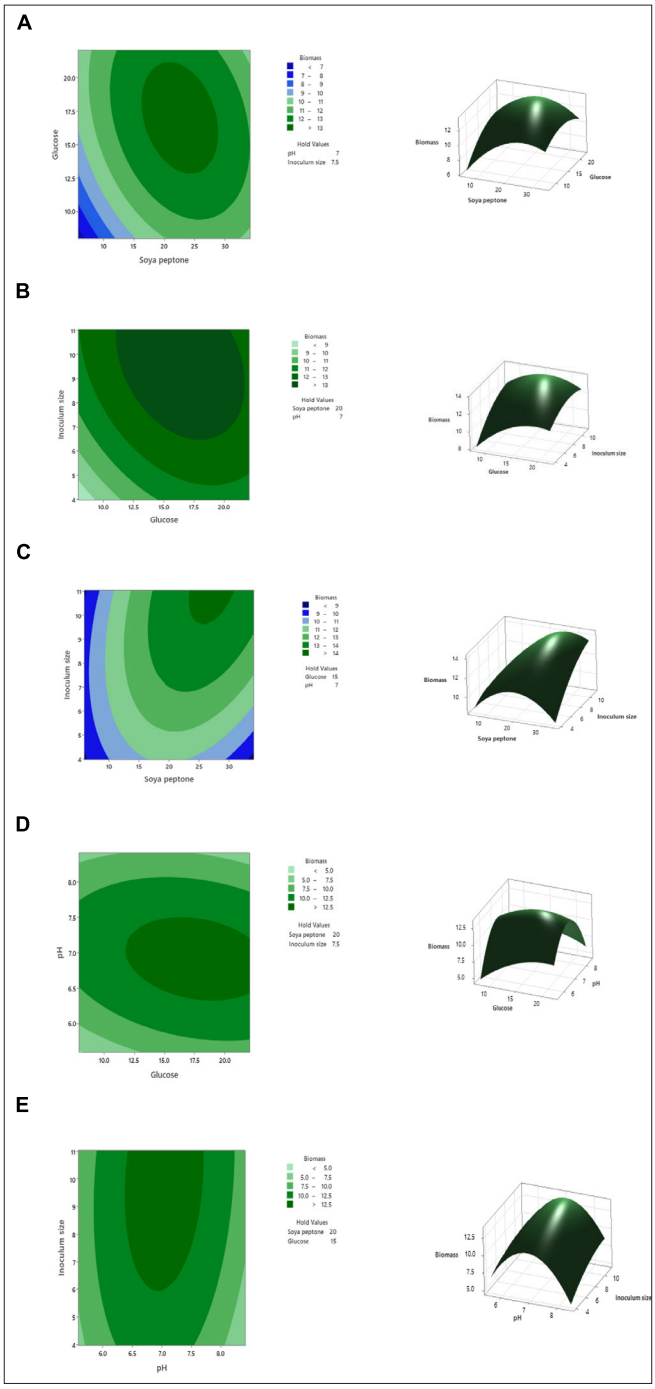

Figure 2: Counter (left) and response surface (right) plots representing significant interaction of variables on biomass yield: **(A)** Glucose vs. soya peptone, **(B)** Inoculum size vs. Glucose, **(C)** Inoculum size vs. soya peptone, **(D)** pH vs. glucose, and **(E)** Inoculum size vs. pH.

Though the linear effect of pH was not significant, its interaction with glucose and inoculum size was significant [Figures 2D, E]. Response surface and contour plots show that maximum production of biomass was obtained at pH 7. The optimal pH for *B. subtilis* strains varies depending on the specific strain and the conditions of the study. In general, the optimal pH for *B. subtilis* strains appears to range from pH 4.0 to pH 8.0, depending on the specific strain and the activity being measured, and previous studies show that most of the *B. subtilis* strains prefer pH 7 for their optimal growth [22,26,36]. Acidic pH may result in rigid and ordered membranes, which can affect the membrane dynamics of the bacterium [48].On the other hand, alkaline conditions may cause membrane disorder [49] and hence maintaining optimal pH is crucial for the membrane properties and thereby the growth of *B. subtilis*. At the optimum pH, glucose at a concentration of 14.07 g/L contributed to the maximum biomass production [Figure 2D]. pH variation during bacterial growth is greatly influenced by the medium composition, particularly the carbon source. Since glucose is a reduced carbon source, more energy may be derived from its oxidation, leading to greater bacterial proliferative potential, and there might be an increased proton flux causing acidification of the medium during the exponential phase of bacterial growth [50]. Therefore, maintenance of optimal pH throughout fermentation is desirable. At the optimal pH, an inoculum size of 11% v/v was significant for enhanced biomass production [Figure 2E].

By using the equation, the optimal concentrations for the variables were predicted. Concentrations of soya peptone (a) glucose, (b) pH (c) and inoculum size (d) obtained from the maximum point of the model were 27.56 g/L for A 14.07 g/L for B, 7.09 for C and 11.03% v/v for D respectively. The model predicted a maximum biomass yield of 14.19 g/L for this point.

In the present investigation, inorganic nutrients, including NaCl, $MgSO_4$.$7H_2O$, and KH_2PO_4 had no significant effect on biomass production. While this does not necessarily negate their importance for bacterial growth, the findings of this study suggest that among the media components, soya peptone and glucose notably influence biomass production. Inorganic nutrients are the most common inclusions in bacterial growth mediums as bacteria require anions like phosphates and sulfates, and cations like sodium, potassium, magnesium, iron, and calcium. These compounds are essential for the synthesis of nucleic acids, proteins, and essential cofactors. The quantity of inorganic salts that must be added to the fermentation medium varies depending on the nutritional requirements of the organism, the composition of the culture medium, and the type of desired end product [51]. In the present study, inorganic nutrients were found to have an insignificant impact on bacterial biomass production. Since this study did not center on the production of specific primary or secondary metabolites by the bacteria, there might be room for flexibility in the required quantities of these inorganic salts, thus making their optimal concentration less strict in the bacterial growth medium. The bacteria were able to proliferate at both low and high levels of the inorganic nutrients applied in the PBD. As a result, in the final optimized medium, the concentrations of these inorganic nutrients were set at the lowest level utilized in PBD as 5 g/L, 2 g/L, and 2 g/L for NaCl, $MgSO_4$, 7.H_2O, and KH_2PO_4, respectively (Table 1). Furthermore, soya peptone, the nitrogen source in the medium, usually contains an ash content of less than 15% which indicates the presence of minerals like calcium, magnesium, and potassium. However, soya peptone is reported to have a very low inorganic phosphorous content [52].The essential minerals and vitamins present in the nitrogen sources might have been utilized by the bacteria for growth.

3.3. Validation of the Optimized Culture Medium

To validate the modeling results, triplicate experiments were done using the optimized conditions. The predicted maximum yield was 14.19 g/L, and the average value obtained in the experiments was 14.29 ± 0.23 g/L, which was in agreement with the predicted yield of the current optimization study. The biomass yield obtained in this investigation was similar to that reported in the study of Ma et al. [23] in which a fed batch fermentation experiment with a mutant strain of *B. subtilis* 168 mut-16# was conducted. Feeding of soya peptone and hydrolyzed starch to the medium increased the dry cell weight of the bacteria, and the highest dry cell weight obtained was 14.3 g/L which was comparable to the findings of the present study. However, a statistical optimization study conducted by Zhong et al. [21] on *B. subtilis* ZK8 reported 77.5 g/L dry cell weight of the bacterium in fed batch culture and 20.38 g/L in batch culture. Yadav et al. [53] used peptone as the nitrogen source in the growth medium of *B. subtilis* and a maximum dry cell weight of 7.5 g/L was obtained after optimization. Koim-Puchowska et al. [27] used yeast extract (4 g/L) and soluble starch (40 g/L) as nitrogen and carbon sources, respectively, for the growth of *B. subtilis natto* BS19 and resulted in a biomass yield of less than 6mg/mL. Studies of Nguyen [22], Stamenkovic et al. [33], Naveed et al. [36], and Ghasemi and Ahmadzadeh [54] reported biomass yields as 3.033 g/L, 6 g/L, 3.81 g/L, and 0.5 g/L, respectively, for different *B. subtilis* strains.

The biomass yield achieved in this study emphasizes the importance of medium optimization in augmenting microbial biomass production. It also suggests the potential scalability of this medium to an industrial level for the production of single-cell protein from *B. subtilis* PW12. Considering the former in regard to manufacturing costs, finding alternatives to the main nutrient of the optimized medium i.e., soya peptone, could prove effective in enhancing the production efficiency of the scale-up process. However, cost issues associated with this nitrogen source have been addressed in some prior research, and cost-effective methods of soya peptone synthesis using enzymatic hydrolysis of soya beans have been proposed [55,56]. Substrates based on soya protein, such as defatted soy flour and soy bean curd, have been used in experiments to produce biomass and metabolites by fermentation with *B. subtilis* [44,45]. Considering the adeptness of *B. subtilis* PW 12 at utilizing soya peptone, delving deeper into the effects of cost-effective nitrogen sources extracted from soybean meal on bacterial growth could offer valuable insights.

Moreover, the most critical juncture of an entire scale-up process is the transition from shake flask to laboratory bioreactor. It is imperative to understand the hydrodynamic behavior of the bioreactor, including mass and heat transfer, mixing, and aeration, especially in aerobic fermentation, to verify the efficacy of the scale-up process. [34] This information is pertinent to the current study as well, so as to achieve yields in the bioreactor that are equivalent to or surpass those in shake flasks under the same conditions.

The goal of the current study was to optimize the biomass yield as well as the nutritional quality of a beneficial probiotic bacterium, *B. subtilis* PW 12 that could be used as a single cell protein or protein supplement in aquafeeds. According to Sakarika et al., the nitrogen content of the culture medium may be positively correlated with the nutritional quality, especially the protein content of the microbial biomass [19]. Since the optimized medium is rich in nutrients, the bacteria may synthesize and accumulate more protein due to their ability to proliferate extensively, resulting in a high cell density. Consequently, the biomass can be utilized as microbial protein in

aquafeeds. The current investigation validates that *B. subtilis* grows optimally in a medium based on soya peptone, and glucose, which are the standard ingredients of microbiological media. Since the medium and culture conditions have been optimized to enhance the growth and nutritional quality of the test bacterium, the growth of other strains of *B. subtilis*, particularly those that can achieve high cell densities and other probiotic strains, can also be evaluated in this optimized medium for increased biomass production. This study has only taken into account the maximal biomass yield; nevertheless, due to the fact that bacterial cells are dynamic, they may produce extracellular and intracellular enzymes, proteins, and metabolites when stimulated by external factors, including medium components or the culture conditions. However, as the study has not examined the production of any particular antimicrobial compound, metabolite, or enzyme by *B. subtilis* PW12 in the optimized medium, future investigations on these facets may assist in evaluating the transferability of the medium to other similar *B. subtilis* strains. Given the under-researched status of *B. subtilis* PW12, thorough exploration is needed for a comprehensive grasp of its metabolic, antibacterial, probiotic, and nutritional properties to probe its potential application in aquaculture and related fields.

4. CONCLUSION

Statistical medium optimization aimed to enhance biomass production of *B. subtilis* PW 12 to find application in aquaculture diets as SCP. The result obtained from the study indicates that *B.subtilis* PW 12 can attain high cell densities, and a batch culture of 48 h is effective for the production of improved biomass yield. Using the model, the optimized values of soya peptone, glucose, pH and inoculum size were found to be 27.56 g/L, 14.07 g/L, 7.09, and 11.03% v/v, respectively, and the predicted biomass yield was 14.19 g/L. Under the optimized conditions, the highest biomass yield obtained was 14.29 ± 0.23 g/L which is comparable to the yield predicted by the response model. Hence, the optimized medium can be used for enhanced biomass production of the probiotic bacterium *B. subtilis* PW 12 for microbial protein production.

5. ACKNOWLEDGEMENTS

The authors acknowledge the financial support of CSIR-HRDG, New Delhi, Government of India, for the award of JRF(NET) and SRF(NET) to Ms. Merin Gracious. The authors are thankful to the Principal, N.S.S. College, Pandalam, Pathanamthitta, for the support. The authors acknowledge research facilities provided by the Department of Biotechnology, Cashew Export Promotion Council of India (CEPCI), Kollam, Kerala, India.

6. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be authors as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

7. CONFLICTS OF INTEREST

The authors declare no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

9. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

10. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript and no images were manipulated using AI.

11. PUBLISHER'S NOTE

This journal remains neutral with regard to jurisdictional claims in published institutional affiliations.

REFERENCES

1. Nasseri AT, Rasoul-Amini S, Morowvat MH, Ghasemi Y. Single cell protein: production and process. Am J Food Technol. 2011;6(2):103-16. https://doi.org/10.3923/ajft.2011.103.116
2. Jannathulla R, Sravanthi O, Moomeen S, Gopikrishna G, Dayal JS. Microbial products in terms of isolates, whole-cell biomass, and live organisms as aquafeed ingredients: production, nutritional values, and market potential—a review. Aquac Int. 2021;29(2):623-50. https://doi.org/10.1007/s10499-021-00644-2
3. Anupama, Ravindra P. Value-added food: Single cell protein. Biotechnol Adv. 2000; \18(6):459-79. https://doi.org/10.1016/s0734-9750(00)00045-8
4. Chisti Y, Moo-Young M. Bioreactors. In: Meyers RA, editor. Encyclopedia of Physical Science and Technology. 3rd ed. Vol. 2. San Diego: Academic Press; 2003:247–71. https://doi.org/10.1016/b0-12-227410-5/00067-3
5. Shah S, Chesti A, Rather M, Hafeez M, Aijaz A, Yousuf I, et al. Effect of Probiotics (*Bacillus subtilis*) on the Growth and Survival of Fingerlings of Grass Carp, *Ctenopharyngodon idella*. Curr J Appl Sci Technol. 2021;10(7):31-7. https://doi.org/10.9734/cjast/2021/v40i1531411
6. Büyükdeveci ME, Cengizler İ, Balcazar JL, Demirkale I. Effects of two host-associated probiotics *Bacillus mojavensis* B191 and *Bacillus subtilis* MRS11 on growth performance, intestinal morphology, expression of immune-related genes and disease resistance of Nile tilapia (*Oreochromis niloticus*) against *Streptococcus iniae*. Dev Comp Immunol. 2023;138:104553. https://doi.org/10.1016/j.dci.2022.104553.
7. Liao Z, Liu Y, Wei H, He X, Wang Z, Zhuang Z, et al. Effects of dietary supplementation of *Bacillus subtilis* DSM 32315 on growth, immune response and acute ammonia stress tolerance of Nile tilapia (*Oreochromis niloticus*) fed with high or low protein diets. Anim Nutr. 2023;15:375-85. https://doi.org/10.1016/j.aninu.2023.05.016

8. Al-Awadhi NM, Razzaque MA, Jonker D, Banat IM, Hamdan IY. Nutritional and toxicological evaluation of single-cell protein produced from *Bacillus Sp*. Kisri-Tm1a in Rats. J Food Qual. 1995;18(6):495-509. https://doi.org/10.1111/j.1745-4557.1995.tb00399.x
9. Kurbanoglu EB, Algur OF. Single-cell protein production from ram horn hydrolysate by bacteria. Bioresour Technol. 2002;85(2):125-9. Doi: https://doi.org/10.1016/s0960-8524(02)00094-9
10. Feliatra F, Mardalisa M, Mukti PR, Feliatra VA, Effendi I. Potential of *Bacillus cereus* SN7 as a single cell protein source. J Phys Conf Ser. 2021;2049(1). https://doi.org/10.1088/1742-6596/2049/1/012024
11. WHO J. Protein and amino acid requirements in human nutrition. World Health Organization technical report series. 2007(935):1.
12. Tank PR, Vadher KH, Patel MP. Isolation of probiotic bacteria from gastrointestinal tract of pacific white shrimp *Litopenaeus vannamei* and antibacterial activity of probiotic bacteria against *Vibrio* spp. J Entomol Zool Stud. 2018; 6:974-78.
13. Tank PR, Vadher KH, Patel MP. Effect of salinity on growth performance of probiotics for its potential use in shrimp aquaculture. J Exp Zool India. 2018;21(2):1111-3.
14. Chakraborty K, Vinaya KK, Antony T, Joy M, Sreemol CK. Marine microbes as a source of antimicrobial compounds. In: Winter School on "Recent advances in bioactive compounds from marine organisms and development of high value products for health management, ICAR-Central Marine Fisheries Research Institute, Kochi; 2018;189-198. Available from: http://eprints.cmfri.org.in/id/eprint/13024
15. Nair VA. Bioprospecting of novel antimicrobial metabolites from *Bacillus subtilis* MBTDCMFRI Ba37 and *Pseudomonas aeruginosa* MBTDCMFRI Ps04 of tropical estuarine habitats of Cochin, India and its application in fish health management [Dissertation]. ICAR-Central Marine Fisheries Research Institute; 2016.
16. Breig SJ, Luti KJ. Response surface methodology: A review on its applications and challenges in microbial cultures. Mater Today Proc. 2021;42:2277-84. https://doi.org/10.1016/j.matpr.2020.12.316
17. Plackett RL, Burman JP. The design of optimum multifactorial experiments. Biometrika. 1946;33(4):305-25. https://doi.org/10.1093/biomet/33.4.305
18. Cavazzuti M. Optimization Methods: from Theory to Design, London: Springer; 2013. https://doi.org/10.1007/978-3-642-31187-1
19. Sakarika M, Kerckhof FM, Van Peteghem L, Pereira A, Van Den Bossche T, Bouwmeester R, et al. The nutritional composition and cell size of microbial biomass for food applications are defined by the growth conditions. Microb Cell Factories. 2023;22(1). http://dx.doi.org/10.1186/s12934-023-02265-1
20. Ashino K, Sugano K, Amagasa T, Ying BW. Predicting the decision making chemicals used for bacterial growth. Sci Rep. 2019; 9(1):1. https://doi.org/10.1038/s41598-019-43587-8
21. Zhong J, Zhang X, Ren Y, Yang J, Tan H, Zhou J. Optimization of *Bacillus subtilis* cell growth effecting jiean-peptide production in fed batch fermentation using central composite design. Electron J Biotechnol. 2014;(3):132-6. https://doi.org/10.1016/j.ejbt.2014.04.010
22. Nguyen Anh T. Optimization of the fermentation medium to receive the highest biomass yield by *Bacillus subtilis* Natto and the initial test of nattokinase yield. IOSR J Eng. 2014;4(12):35-40. https://doi.org/10.9790/3021-04124035040
23. Ma Y, Shen W, Chen X, Liu L, Zhou Z, Xu F, et al. Significantly enhancing recombinant alkaline amylase production in *Bacillus subtilis* by integration of a novel mutagenesis-screening strategy with systems-level fermentation optimization. J Biol Eng. 2016;10(1). http://dx.doi.org/10.1186/s13036-016-0035-2
24. Sreekumar G, Krishnan S. Enhanced biomass production study on probiotic *Bacillus subtilis* SK09 by medium optimization using response surface methodology. Afr J Biotechnol. 2010;9(47):8078-84. http://dx.doi.org/10.5897/ajb10.1283
25. Cho YH, Song JY, Kim KM, Kim MK, Lee IY, Kim SB et al. Production of nattokinase by batch and fed-batch culture of *Bacillus subtilis*. N Biotechnol. 2010;27(4):341-6. http://dx.doi.org/10.1016/j.nbt.2010.06.003
26. Koim-Puchowska B, Kłosowski G, Dróżdż-Afelt JM, Mikulski D, Zielińska A. Influence of the medium composition and the Culture Conditions on Surfactin Biosynthesis by a native *Bacillus subtilis natto* BS19 strain. Molecules. 2021;26(10):2985. https://doi.org/10.3390/molecules26102985
27. Fisher SH. Regulation of nitrogen metabolism in *Bacillus subtilis*: vive la difference! Mol Microbiol. 1999;32(2):223-32. https://doi.org/10.1046/j.1365-2958.1999.01333.x
28. Atkinson MR, Fisher SH. Identification of genes and gene products whose expression is activated during nitrogen-limited growth in *Bacillus subtilis*. J Bacteriol. 1991;173(1):23-7. https://doi.org/10.1128/jb.173.1.23-27.1991
29. Liebs P, Riedel K, Graba JP, Schrapel D, Tischler U. Formation of some extracellular enzymes during the exponential growth of *Bacillus subtilis*. Folia Microbiol. 1988;33(2):88-95. https://doi.org/10.1007/bf02928073
30. Davami F, Eghbalpour F, Nematollahi L, Barkhordari F, Mahboudi F. Effects of peptone supplementation in different culture media on growth, metabolic pathway and productivity of CHO DG44 cells; a new insight into amino acid profiles. Iran Biomed J. 2015;19(4):194. https://doi.org/10.7508/ibj.2015.04.002
31. Yin X, Zhang G, Zhou J, Li J, Du G. Combinatorial engineering for efficient production of protein-glutaminase in *Bacillus* subtilis. Enzyme Microb Technol. 2021;150:109863. http://dx.doi.org/10.1016/j.enzmictec.2021.109863
32. Stülke J, Hillen W. Regulation of carbon catabolism in *Bacillus species*. Annu Rev Microbiol. 2000;54(1):849-80. https://doi.org/10.1146/annurev.micro.54.1.849
33. Stamenković-Stojanović S, Karabegović I, Beškoski V, Nikolić N, Lazić M. *Bacillus subtilis* NCIM2063 batch cultivation: The influence of the substrate concentration and oxygen transfer rate on the biomass yield. Adv Technol. 2020;9(1):44-9. https://doi.org/10.5937/savteh2001044s
34. Stamenkovic-Stojanovic S, Karabegovic I, Danilovic B, Mancic S, Lazic M. High cell density cultivation of *Bacillus subtilis* NCIM 2063: modeling, optimization and a scale-up procedure. J Serb Chem Soc. 2023;88(11):1103-17. http://dx.doi.org/10.2298/jsc230407036s
35. Schilling O, Frick O, Herzberg C, Ehrenreich A, Heinzle E, Wittmann C, Stülke J. Transcriptional and metabolic responses of *Bacillus subtilis* to the availability of organic acids: transcription regulation is important but not sufficient to account for metabolic adaptation. Appl Environ Microbiol. 2007;73(2):499-507. https://doi.org/10.1128/aem.02084-06
36. Naveed M, Tianying H, Wang F, Yin X, Chan MW, Ullah A, et al. Isolation of lysozyme producing *Bacillus* subtilis Strains, identification of the new strain *Bacillus subtilis* BSN314 with the highest enzyme production capacity and optimization of culture conditions for maximum lysozyme production. Curr Res Biotechnol. 2022;4:290-301. https://doi.org/ 10.1016/j.crbiot.2022.06.002
37. Kwon EY, Kim KM, Kim MK, Lee IY, Kim BS. Production of nattokinase by high cell density fed-batch culture of Bacillus subtilis. Bioprocess Biosyst Eng. 2011;34(7):789-93. https://doi.org/10.1007/s00449-011-0527-x
38. Prats C, Giró A, Ferrer J, López D, Vives-Rego J. Analysis and IbM simulation of the stages in bacterial lag phase: basis for an updated definition. J Theor Biol. 2008;252(1):56-68. https://doi.org/doi.org/10.1016/j.jtbi.2008.01.019
39. Mohamed H, Awad MF, Shah AM, Sadaqat B, Nazir Y, Naz T et al. Coculturing of *Mucor plumbeus* and *Bacillus subtilis* bacterium as an efficient fermentation strategy to enhance fungal lipid and

gamma-linolenic acid (GLA) production. Sci Rep. 2022;12(1):13111. https://doi.org/10.1038/s41598-022-17442-2

40. Effiom H, Lennox J. Optimization of independent variables for the production of extracellular alpha amylase by *Bacillus subtilis* IMD34 using Plackett-Burman design. Sultan Qaboos Univ J Sci. 2022;27(2):77-83. https://doi.org/10.53539/squjs.vol27iss2pp77-83
41. Yue H, Zhong J, Li Z, Zhou J, Yang J, Wei H, et al. Optimization of iturin A production from *Bacillus subtilis* ZK-H2 in submerge fermentation by response surface methodology. 3 Biotech. 2021;11(2). http://dx.doi.org/10.1007/s13205-020-02540-7
42. Zhong H, Wang L, Zhao J-y, Xiao Z. Fermentative production of chiral acetoin by wild-type *Bacillus strains*. Prep Biochem Biotechnol. 2019;50(2):116-22. http://dx.doi.org/10.1080/10826068.2019.1666280
43. Ullah A, Sun B, Wang F, Yin X, Xu B, Ali N, et al. Isolation of selenium-resistant bacteria and advancement under enrichment conditions for selected probiotic *Bacillus subtilis* (BSN313). J Food Biochem. 2020;44(6). http://dx.doi.org/10.1111/jfbc.13227
44. Wang H, Sun X, Wang L, Wu H, Zhao G, Liu H, et al. Coproduction of menaquinone-7 and nattokinase by *Bacillus subtilis* using soybean curd residue as a renewable substrate combined with a dissolved oxygen control strategy. Ann Microbiol. 2018;68(10):655-65. Doi: http://dx.doi.org/10.1007/s13213-018-1372-9
45. Yánez-Mendizábal V, Falconí CE, Kanaley K. Production optimization of antifungal lipopeptides by *Bacillus subtilis* CtpxS2-1 using low-cost optimized medium. Biol Control. 2023;185:105306. http://dx.doi.org/10.1016/j.biocontrol.2023.105306
46. Kanwal M, Wattoo AG, Khushnood RA, Liaqat A, Iqbal R, Song Z. Advancements and challenges in production of biosurfactants. In: Inamuddin, Charles Oluwaseun Adetunji, editors. Applications of Next Generation Biosurfactants in the Food Sector, Academic Press; 2023:239-59. http://dx.doi.org/10.1016/b978-0-12-824283-4.00019-8
47. Huang J, Pan Y, Zheng X, Lin H, Lin W, Liu J. Enhanced the biomass of *Bacillus subtilis* cultured in industrial wastewater through statistical methodologies. J Biotech Res. 2020;11:155-68.
48. Park MK, Lee S, Kim YS. Effects of pH and osmotic changes on the metabolic expressions of *Bacillus subtilis* strain 168 in metabolite pathways including leucine metabolism. Metabolites. 2022;12(2):112. http://dx.doi.org/10.3390/metabo12020112
49. Petrackova D, Vecer J, Svobodova J, Herman P. Long-term adaptation of *Bacillus subtilis* 168 to extreme pH affects chemical and physical properties of the cellular membrane. J Membr Biol. 2010;233(1-3):73-83. http://dx.doi.org/10.1007/s00232-010-9226-9
50. Sánchez-Clemente R, Guijo MI, Nogales J, Blasco R. Carbon source influence on extracellular pH changes along bacterial cell-growth. Genes. 2020;11(11):1292. http://dx.doi.org/10.3390/genes11111292
51. Singh V, Haque S, Niwas R, Srivastava A, Pasupuleti M, Tripathi C. Strategies for fermentation medium optimization: an in-depth review. Front Microbiol. 2017;7:2087. http://dx.doi.org/10.3389/fmicb.2016.02087
52. Odds FC, Hall CA, Abbott AB. Peptones and mycological reproducibility. Med Mycol. 1978;16(4):237-46. http://dx.doi.org/10.1080/00362177885380341
53. Yadav J, Balabantaray S, Patra N. Statistical optimization of fermentation conditions for the improved production of poly-β-hydroxybutyrate from *Bacillus subtilis*. Chem Eng Commun. 2017;204(10):1122-8. http://dx.doi.org/10.1080/00986445.2017.1347094
54. Ghasemi S, Ahmadzadeh M. Optimization of a cost-effective culture medium for the large-scale production of *Bacillus subtilis* UTB96. Arch Phytopathol Pflanzenschutz. 2013;46(13):1552-63. https://doi.org/10.1080/03235408.2013.771469
55. Uzeh RE, Akinola SO, Olatope SO. Production of peptone from soya beans (Glycine max L merr) and African locust beans (*Parkia biglobosa*). Afr J Biotechnol. 2006;5(18).
56. Rochmaningsih, Harismah K, Mulyaningtyas A. Optimization of peptone production from soybeans. Proceedings of the 4th International Conference Current Breakthrough in Pharmacy (ICB-Pharma 2022); 2022;295-305. Atlantis Press. http://doi.org/10.2991/978-94-6463-050-3_25

How to cite this article:
Gracious M, Nandakumar S. Optimization of *Bacillus subtilis* PW12 biomass production using RSM: a preliminary study towards single-cell protein production for aquaculture. J App Biol Biotech. 2025;13(1):83-92. DOI: 10.7324/JABB.2024.177031