https://doi.org/ 10.33472/AFJBS.7.1.2025.1-16

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

OPTIMIZING MEDIA FORMULATIONS FOR RECOVERY: A DESIGN OF EXPERIMENTS STUDY ON RESCUING STRESSED ESCHERICHIA COLI

Baswa Jithendra Kumar[1], R. Gyana Prasuna*[2]

[1]Research Scholar, Department of Microbiology & Food Science and Technology, GITAM Institute of Science, GITAM Deemed to be University, Visakhapatnam-530045, Andhra Pradesh, India

[2]Research Supervisor, Department of Microbiology & Food Science and Technology, GITAM Institute of Science, GITAM Deemed to be University, Visakhapatnam-530045, Andhra Pradesh, India.

Corresponding author: gravi@gitam.edu

*Corresponding author:

Dr. R. Gyana Prasuna

Mail id: gravi@gitam.edu

Mobile No: +91-9703273038

Article History

Volume 7,Issue1, 2025

Received:05 Dec2024

Accepted :05 Jan2025

doi: 10.33472/AFJBS.7.1.2025.1-16

Abstract

The work optimised selective media to fine-tune culture media for stressed bacteria resuscitation in a cost-effective and potent way. Resuscitating exhausted microorganisms is crucial in microbiology. Stress alters metabolism, membranes, and cell walls, limiting culturability. In microbiology, biotechnology, and medical research, *Escherichia coli* (*E. coli*), a model organism, must recover successfully. Revitalization requires optimising nutrients, pH, osmotic balance, and antibiotics in the recovery medium. This optimisation mimics natural circumstances, encourages target microorganism growth, and improves research efficiency, cost, and sustainability. We found that Pancreatic Digest of Casein (A), Papaic Digest of Soybean (B), Sodium Chloride (C), and Dextrose Monohydrate (D) help stressed bacteria recover. Our Box-Behnken Design with twenty-nine experiments produced polynomial equations, ANOVA, and 3D response surface graphs. ANOVA confirms that these models accurately predict CFUs (Y1) and % recovery (Y2).We optimised these aspects using desirability. The ideal factor levels are: A: 3.90 mg/ml Pancreatic Digest of Casein, B: 1.90 mg/ml Papaic Digest of Soybean, C: 2.50 mg/ml Sodium Chloride, D: 0.90 mg/ml Dextrose Monohydrate. Experimental confirmations confirmed these parameters. The procedure's accuracy in determining optimal media composition is shown by the close match between anticipated and observed values. Optimisation of recovery medium composition is crucial for reviving stressed microorganisms. Improves research efficiency, cost-effectiveness, and environmental responsibility while increasing target microorganism output. It is vital to microbiological research and supports many scientific fields.

Key words: Colony count, experimental design, growth factors, nutrient ratios, osmotic balance, recovery efficiency.

1. Introduction

In microbiology and biotechnology, recovering and studying stressed bacterial strains, especially *Escherichia coli*, is crucial. These strains face many stresses, including antibiotics and severe environments[1]. These stresses can change bacterial morphologies, making routine culture and study difficult. Optimising the selected recovery medium composition is essential to revive and study stressed *E. coli* populations[2].

Gram-negative bacterium *E. coli* are common and essential for biological study. Its versatility and prevalence in diverse settings, including the gut, make it an attractive research subject[3, 4]. However, stresses like antibiotics or poor environmental conditions can radically change E. coli, causing changed growth rates, morphology, metabolic pathways, or antibiotic resistance. Stress-induced changes make traditional culturing difficult and require specialised recovery measures[5, 6, 7].

Stressed bacteria need carbon, nitrogen, vitamins, minerals, and energy from culture media to revive. These nutrients power bacterial metabolism and growth. For bacteria to recuperate from stress, they need a rich and balanced food supply[7].

Culture media can selectively resuscitate specific bacterial species or strains, which is essential for separating germs from mixed populations. Selective media may contain antibiotics or growth factors that promote certain microorganisms while inhibiting others[8]. Bacteria are pH-sensitive, hence culture media buffers must keep pH steady. In stressed cells, pH regulation is lost; hence bacterial growth requires pH consistency[9].

Some bacteria need specific growth factors or co-factors. These elements can be added to culture media to help stressed bacteria recover, which is helpful for fastidious bacteria with specific nutritional needs[10]. Bacteria growth and recovery depend on the culture medium and ambient factors such oxygen levels (aerobic, anaerobic, microaerophilic). Stress-adapted bacteria may respond differently to oxygen or other environmental elements, making culture conditions crucial for their reawakening[11].

When stressed, bacteria become viable but non-culturable, yet optimised culture media can help them become culturable. This is crucial in environmental and clinical microbiology for accurate bacterial population assessment[12, 13]. Creating a selected recovery medium for stressed *E. coli* strains is crucial. Stressed or antibiotic-resistant *E. coli* strains should thrive in such a medium while non-stressed or susceptible bacteria die[14]. Provide a supportive environment for stressed strains to recuperate and reveal their unique traits for further investigation.

Traditional "one-factor-at-a-time" (OFAT) culture media development strategies are common. One substance or factor is altered while all others remain constant. This method can show how amino acids and vitamins affect culture growth, but it has limits[15]. The OFAT technique doesn't account for factor interactions, a major drawback. Complex interactions can occur between culture media components. For instance, one nutrient may boost or hinder another's use. Understanding these interactions is important because they affect bacterial growth and recovery[16, 17].

Advanced methods like Response Surface Methodology (RSM) can overcome these constraints and help researchers comprehend cultural media factor interactions. RSM is a reliable statistical and mathematical experiment design and analysis method. It's useful for optimising complex operations and understanding variable interactions[18].

Multiple experimental designs are used in RSM to optimise processes and study variable correlations. Design depends on factors, resources, and system complexity. RSM adjusts process-influencing factors within set ranges. It shows how changes in these parameters (independent variables) affect response variables (dependent variables) using statistical models, frequently quadratic equations. These models explain variable relationships and forecast ideal conditions[19].

The goal of RSM is to find the best settings that maximise or minimise the response variable. To find these perfect settings, optimisation algorithms like the desirability function are used. RSM uses analysis of variance (ANOVA) to determine the importance of each element and its interactions to identify the most important factors. Graphs like contour and surface plots help RSM visualise the response surface. These plots identify ideal circumstances and explain variable relationships[20].

Box-Behnken Design (BBD) is the ideal way for optimising selective media in experimental design, where many methods are accessible. It saves resources by requiring fewer experimental runs than full factorial designs[21]. This is crucial when resources are limited. Culture media optimisation often involves moderately many factors, making the BBD ideal. Its ability to accommodate various parameters while retaining a manageable number of runs makes it practical and resource-efficient[22].

Additionally, RSM's main goal of determining optimal circumstances is achieved by the BBD. The BBD efficiently captures complex factor-response connections using statistical models, frequently quadratic equations[23]. It is important for fine-tuning culture media to maximise bacterial recovery by maximising or minimising response variables, a vital component in stressed bacteria resuscitation [24, 25].

As part of RSM, the BBD uses ANOVA to evaluate each factor and its interactions. This crucial phase in culture medium optimisation identifies the most important parameters for reviving stressed bacteria under stress-induced conditions, simplifying decision-making. Finally, BBD contour and surface charts improve clarity. Visual aids help understand complex variable interactions and find ideal settings, which is crucial when optimising selected media[24]. In light of these advantages, the BBD is used to optimise selective media, a cost-effective and powerful way to fine-tune culture medium for stressed bacteria resuscitation[25].

2. Materials and Methods

2.1 Test Microorganisms

The American Type Culture Collection (Rockville, Md.) provided *E. coli* ATCC 8739 mother cultures, which were cultured on Trypticase soy agar (TSA) slants at 48°C (Becton Dickinson Microbiology Systems, Cockeysville, Md.) and preserved in 1% peptone water with 40% v/v glycerol at -80°C. Oxoid™ Lab-Lemco Beef Extract Powder was acquired from ThermoFisher Scientific, Mumbai, India. Sigma-Aldrich Chemicals Private Limited, Bangalore, India, supplied yeast extract, meat (bacteriological) and casein (cow's milk) peptone. S.D. Fine Chemicals, Mumbai, supplied Potassium Dihydrogen Phosphate, Disodium Hydrogen Phosphate Dihydrate, Sodium Chloride, Peptone (meat or casein), Polysorbate-80, dextrose, Sodium pyruvate, Pancreatic Digest of Casein, Papaic Digest of Soybean, and Agar Soluble.

2.2.Nutrient Broth for Initial Culture Preparation

Nutrient broth is a flexible liquid medium used in microbiology to prepare and grow bacterial cultures. Rich in nutrients, it supports the growth of several bacteria[26]. Nutrient broth is made by dissolving selected components (Table 1) in distilled water and correcting pH to 7.4 ± 0.2 at 25 °C. The mixture is autoclaved at 121°C for 15 minutes.

Table 1. Composition of nutrient broth

S.No.	Component	Concentration (gm/litre)
1	Lab-Lemco Beef Extract Powder	1.0
2	Yeast extract	2.0
3	Peptone from meat	5.0
4	Sodium chloride	5.0

2.3. Standard Growth Media for Maintenance and Propagation of *E. Coli*

E. coli cultures are often maintained in Luria-Bertani (LB) agar or broth. *E. coli* and other bacteria thrive on LB, a rich, nutritious medium[27]. Dissolve Table 2 components in 1000 cc of distilled water to make Luria-Bertani broth. The solution pH was adjusted to 7.4 ± 0.2 using 1M NaOH. The mixture is autoclaved at 121°C for 15 minutes. Adding 1.5 % agar yields Luria-Bertani agar. Agar was mixed with a little distilled water in another container. To completely dissolve the agar, the mixture was boiled while stirring. Then, the tryptone, yeast extract, and NaCl flask received the agar solution. The fluid was carefully swirled to distribute agar evenly. The solution pH was adjusted to 7.4 ± 0.2 using 1M NaOH. Suitable containers received the agar solution. The mixture is autoclaved at 121°C for 15 minutes. Keep LB agar plates and broth cool, dry, and out of direct sunlight.

Table 2. Composition of Luria-Bertani broth

S.No.	Component	Concentration (gm/litre)
1	Peptone from casein	10
2	Yeast extract	5
3	Sodium chloride	10

2.3.Maintenance and Propagation of *E.Coli*

Maintaining the colonies on Trypticase soy agar (TSA) and storing them in 1% peptone water with 40% v/v glycerol at -80°C ensured their viability. Regular sub culture required moving a tiny volume of frozen stock into new growing media like LB broth or agar to maintain *E. coli* genetic stability and vigour. This method ensured culture survival and genetic uniformity. The subcultured *E. coli* were incubated at 37°C until a lawn or colonies appeared. Some subcultures were frozen at 4°C for a week. Luria-Bertani (LB) broth or agar, recognised for fostering *E. coli* growth, was used. A small part of the *E. coli* culture was injected into the growth medium using aseptic methods. *E. coli* grows best at 37°C, thus the infected media was incubated there. By monitoring liquid medium turbidity or agar plate colonies, *E. coli* growth was closely observed. A little piece of the culture was transferred to a new medium for proliferation. This procedure kept the culture developing for future trials and analysis.

2.4.Preparation of Stressed Cells

Microbiologists stress *E. coli* to study their responses to stresses. Antibiotic stress illuminates resistance pathways. A penicillin solution of 4 μg/L was produced with sterile water[28]. Six millilitres of *E. coli* from the stock solution were injected into each antibiotic solution at a concentration of 10 × 102 cells/mL aseptically. Thus, 1 cfu/mL was the final concentration. The mixture contacted for 30 minutes. PBP was aseptically added to the mixture. After 30 minutes, 100 mL of inoculation samples were aseptically collected for bioburden testing. All subsequent testing was done in triplicates for accuracy and consistency.

2.5.Selective Recovery Media with Varying Compositions

Our comprehensive study identified selective media, essential to microbiological research. To investigate selective media for specific objectives, we reviewed the literature and conducted experiments. Their ability to isolate certain bacterial strains and repel non-target species was tested in real life. This method identified several customised media, as shown in Table 3. Each selective media element promotes desired bacteria and inhibits unwanted ones. Pancreatic Digest of Casein supplies amino acids and peptides needed by many bacteria, including E. coli. Papaic Digest of Soybean provides amino acids and peptides for bacterial development. Osmotic equilibrium and water flow into and out of bacterial cells are maintained by sodium chloride. Dextrose Monohydrate/Anhydrous fuels bacterial metabolism. Certain bacteria may use soluble starch as a carbon source. Adding sodium pyruvate as a carbon source helps stressed organisms recuperate, which may stimulate bacterial growth. Agar solidifies the medium to make agar plates, which isolates colonies. Our forthcoming investigations will depend on these media to alter bacterial colonies and study microbial responses. This strong media methodology underpins our microbiological research. For stressed E. coli, we optimise medium composition using the Box-Behnken Design (BBD) to maximise efficiency, repeatability, and environmental responsibility[30, 21, 32].

Table 3. Composition of selective media with range of concentrations

S.No.	Composition	Concentration (mg/ml)
1	Pancreatic Digest of Casein	3.75 -11.25
2	Papaic Digest of Soybean	1.25- 3.75
3	Sodium Chloride	2.5 – 4.5
4	Dextrose Monohydrate/Anhydrous	0.5-1.5
5	Starch, soluble	0.5
6	Sodium pyruvate	1.0
7	Agar	15.0
8	Purified water	1000 ml
	pH	7.3 ± 0.2

2.6.Design of Experiments

The selective growth medium was optimised using Design of Experiments (DoE) and statistical modelling with Design-Expert® software (Version 13.0.5.0 by Sata-Ease). Table 4 shows the

selective growth medium compositions used in the experiment. Four independent variables were examined to determine how CFUs and percent recovery were affected. The four-factor, three-level Box-Behnken design yielded 29 experimental treatments. Note that Starch, Sodium pyruvate, and Agar concentrations were constant throughout tests. Each experiment is detailed in Table 4. Table 4 shows the dependent variables (CFUs, Y1, and percent recovery, Y2) from the studies that followed this strategy.

2.7.Characterisation of Culture Growth

2.7.1. Measurement of Colony Forming Units (CFUs)

CFUs measured viable microbial cells in the cultivated sample. To test this, dilutions from the initial culture were made to lower cell concentration to a level where colonies could be seen. To promote microbial development, each dilution was carefully distributed or poured onto agar plates. These plates were carefully nurtured to produce desired bacteria. Each visible colony represented a CFU and was counted after incubation. The CFUs were determined and expressed as CFU/ml.

2.7.2. Percent Recovery

Percent recovery was used to assess recovery efficiency, focusing on viable cell recovery after a given treatment or stress. Before treatment or stress, the number of viable cells in the original culture was counted. To treat or stress the culture, heat, chemicals, or other stressors were used. Viable cells in the culture were counted after treatment. Recovery percentage was obtained using formula 1:

$$Percent\ recovery = \frac{Final\ count}{\text{Inoculum count}}\ X\ 100$$

2.7.3. Microscopic Examination

To photograph cell morphology, we used an inverted microscope (Nikon Inc., model: TI-E) with a 100x phase-contrast objective lens (CFI PlanApo LambdaDM100 x 1.4 NA). Our solid-state light source (Lumencor Inc., model: Spectra X) and multi-band dichroic mirror (Chroma Technology Corp., model: 69002bs) helped with imaging.

Table 4. Box Behnken design with observed responses

Run	Factors				Responses					
					Y1-CFU			Y2- Percent recovery		
	A- Pancreatic Digest of Casein (mg/ml)	B - Papaic Digest of Soybean (mg/ml)	C - Sodium chloride (mg/ml)	D - Dextrose Monohydrate (mg/ml)	Actual	Predicted	Residual	Actual	Predicted	Residual
1	7.5	1.25	2.5	1	42.50	42.46	0.0445	48.33	49.01	-0.6722
2	3.75	2.5	3.5	1.5	40.50	40.35	0.1540	55.67	56.72	-1.05
3	7.5	3.75	4.5	1	21.83	20.87	0.9612	29.67	29.03	0.6334
4	7.5	2.5	2.5	1.5	32.83	32.74	0.0958	51.67	51.46	0.2055
5	7.5	3.75	3.5	1.5	29.67	29.22	0.4500	46.33	45.01	1.32
6	7.5	1.25	3.5	0.5	45.67	45.66	0.0055	50.50	50.23	0.2653
7	3.75	2.5	4.5	1	29.17	29.67	-0.5014	27.17	28.45	-1.28
8	7.5	3.75	2.5	1	39.00	38.48	0.5167	43.83	44.45	-0.6166

9	7.5	2.5	2.5	0.5	59.67	59.54	0.1236	53.67	52.13	1.54
10	7.5	2.5	3.5	1	42.83	43.11	-0.2765	43.67	42.56	1.11
11	11.25	2.5	3.5	0.5	40.17	39.37	0.7929	46.50	46.36	0.1403
12	11.25	2.5	2.5	1	33.33	33.83	-0.5014	32.50	32.84	-0.3389
13	7.5	2.5	3.5	1	43.50	43.11	0.3902	44.00	42.56	1.44
14	7.5	2.5	4.5	1.5	29.67	29.46	0.2069	37.83	36.04	1.79
15	11.25	3.75	3.5	1	29.17	28.73	0.4361	32.83	34.77	-1.93
16	11.25	1.25	3.5	1	32.67	32.70	-0.0361	40.33	39.32	1.01
17	3.75	1.25	3.5	1	47.50	46.15	1.35	48.17	50.35	-2.18
18	7.5	2.5	3.5	1	42.17	43.11	-0.9432	44.17	42.56	1.61
19	11.25	2.5	3.5	1.5	33.83	33.40	0.4318	36.67	37.86	-1.19
20	3.75	2.5	3.5	0.5	59.83	59.32	0.5152	49.83	49.55	0.2792
21	3.75	2.5	2.5	1	58.17	58.45	-0.2792	60.50	60.62	-0.1166
22	7.5	2.5	3.5	1	43.33	43.11	0.2235	43.50	42.56	0.9432
23	11.25	2.5	4.5	1	26.67	27.39	-0.7236	32.67	34.17	-1.51
24	7.5	2.5	3.5	1	43.17	43.11	0.0568	43.33	42.56	0.7765
25	7.5	2.5	4.5	0.5	27.83	27.60	0.2347	36.50	36.71	-0.2112
26	3.75	3.75	3.5	1	41.33	42.18	-0.8417	46.33	45.79	0.5404
27	7.5	1.25	3.5	1.5	32.00	33.19	-1.19	48.50	49.57	-1.07
28	7.5	1.25	4.5	1	24.67	24.84	-0.1777	34.17	33.59	0.5778
29	7.5	3.75	3.5	0.5	40.17	41.69	-1.52	43.67	45.68	-2.01

2.8.Data Collection and Analysis

Linear, quadratic, and cubic models can characterise independent-dependent relationships in four-component BBD studies. A crucial threshold with a p-value of less than 0.005 was set to determine the importance of these models, retaining only the most relevant terms. A thorough review, including numerous statistical parameters, determined the best fitting model. The model's p-value had to be less than 0.005 for validity, while the lack of fit p-value had to be more than 0.005 for adequacy. The coefficient of variation, multiple correlation coefficient (R^2), and adjusted R^2 were also assessed. This rigorous review was made possible by Stat-Ease Design Expert® (Version 13.0.5.0). Quadratic models were used for each response parameter to clarify them. This method used equation 2's multiple linear regression analysis.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1 X_2 + \beta_4 X_1^2 + \beta_5 X_2^2 + \beta_6 X_1 X_2^2 + \beta_7 X_1^2 X_2$$

The equations use Y as the measured response level, β0 as the intercept, and β1 to β7 as regression coefficients. X1 and X2 represent the major effects, X1X2 represents their interaction, and X12 and X22 represent the independent variables' quadratic terms, over the designed sample space. Data was tailored to this quadratic model using backward elimination to optimise the model. These regression-derived quadratic models were used to create three-dimensional graphics. Depending on X, these graphs showed Y as a curved surface. Contour plots helped show how independent variables affected response parameters[22].

2.9.Optimization and Confirmation Experiments

A numerical optimization strategy, the desirability approach, was used to find the ideal synthesis variable values and produce the desired result. Constraining the dependent and

independent variables allowed this strategy to optimize the protocol. Five further confirmation studies with different constituent molar concentrations followed. These confirmation tests confirmed the statistical experimental methodologies' efficacy and dependability.

3. Results and Discussion

Stress causes complex cellular changes that affect metabolic pathways, membrane integrity, and cell wall composition in microbiology and bacterial research. Stressed bacterial populations often have lower culturability due to collective alterations. Resuscitation of such populations is important in microbiology, biotechnology, and medicine. A popular model organism, *E. coli*, often experiences stresses that impair its physiological homeostasis. How stressed bacteria recover depends on the recovery medium, which bridges stress and full growth recovery. This medium supplies nutrients, growth ingredients, and environmental conditions for resurrection. To improve recovery efficiency, culturability restoration, and growth, the medium's composition must be optimised.

Optimisation involves optimising nutrition availability, growth factor concentrations, pH buffering, osmotic balance, and antibiotic selection. It produces an appropriate recuperation environment, adjusting to stress bacteria's needs and supporting their survival. It's essential for simulating these bacteria's native environment and evaluating stress responses. Optimisation allows for the optimal balance between encouraging target microorganism growth and suppressing undesirable contamination, resulting in pure cultures and accurate results. This enables for repeatable tests and consistent research findings across investigations. Optimisation maximises goal microorganism yield, research efficiency, cost-effectiveness, and environmental responsibility, making it essential in microbiological research.

Pancreatic Digest of Casein (A), Papaic Digest of Soybean (B), Sodium Chloride (C), and Dextrose Monohydrate (D) were the most effective variables for resuscitating challenged bacteria in the initial screening phase. The Box-Behnken Design (BBD) response surface methodology required twenty-nine experiments due to four components at three levels each. The experimental runs' independent and dependent variables and responses are listed in Table 4.This complex experimental design produced various answers according to component combinations. Polynomial equations encapsulating the variables were created by multiple linear regression analysis. Design Expert software was used to analyse the dataset, including ANOVA, regression coefficients, and 3D response surface plots. This thorough analysis allowed for a complete examination of the factors and their effects on the replies.

Equation 3 shows the polynomial equation for Y1-CFUs as a simultaneous function of A, B, C, and D.

$$CFUs\ (Y1) = 43.11 - 6.72A - 1.99B - 8.81C - 6.24D + 5.58AC + 3.25AD - 5.67B^2 - 5.78C^2$$

The polynomial equation quantifies how process variables A, B, C, and D and their interactions affect Y1. A, B, C, and D coefficients show how these variables affect Y1. Multifactor and higher-order coefficients represent interaction and quadratic relationships, respectively. A positive coefficient indicates synergy, while a negative value indicates opposition. This analysis' quadratic model showed that temperature and time levels affected practical yield

without interaction. Table 4 shows that theoretical (predicted) values and observed values coincide well. From -1.05 to 1.44, residuals show slight variations between actual and anticipated values. For CFUs, the model seems accurate.

An ANOVA was performed to determine the impact of these factors on the response. Table 5 shows that factors significantly affect response. The Model F-value, 510.94, emphasises its importance. A Model F-Value of this magnitude is just 0.01% likely due to random noise. This model's 26.19 Predicted Residual Sum of Squares (PRESS) indicates a strong predictor. P-values below 0.0050 indicate significance for model terms A, B, C, D, AC, AD, CD, B^2, and C^2. The Lack of Fit F-value of 2.47 indicates that it is not significant compared to the pure error, and there is a 19.81% possibility that it is attributable to random variability. A non-significant lack of fit means the model fits the data well.

Table 5. ANOVA of the quadratic model for the response CFUs (Y1)

Source of variations	Sum of squares	Degree of freedom	Mean squares	F-value	p-value Prob > F	R^2
Model	2756.70	9	306.30	510.94	< 0.0001	0.9959
A	542.26	1	542.26	904.55	< 0.0001	
B	47.34	1	47.34	78.96	< 0.0001	
C	930.45	1	930.45	1552.10	< 0.0001	
D	466.67	1	466.67	778.46	< 0.0001	
AC	124.69	1	124.69	208.00	< 0.0001	
AD	42.25	1	42.25	70.48	< 0.0001	
CD	205.44	1	205.44	342.70	< 0.0001	
B^2	221.96	1	221.96	370.26	< 0.0001	
C^2	230.19	1	230.19	383.99	< 0.0001	
Residual	11.39	19	0.5995			
Lack of Fit	10.28	15	0.6853	2.47	0.1981	
Pure Error	1.11	4	0.2778			
Cor Total	2768.09	28				

Figure 1 illustrates the main, interaction, and quadratic effects of A, B, C, and D on CFUs (Y1). The figure illustrates that C, A, and D have the greatest impact on Y1. B moderately affects Y1.Response surface plots assessed CFUs' relationship to independent factors. Figure 2A shows how A and C affect CFUs (Y1) while maintaining B and D constant. The interaction between A and D on CFUs (Y1) is shown in Figure 2B, with B and C constant. Figure 2C shows C and D's interaction with CFUs (Y1) while keeping A and B constant.

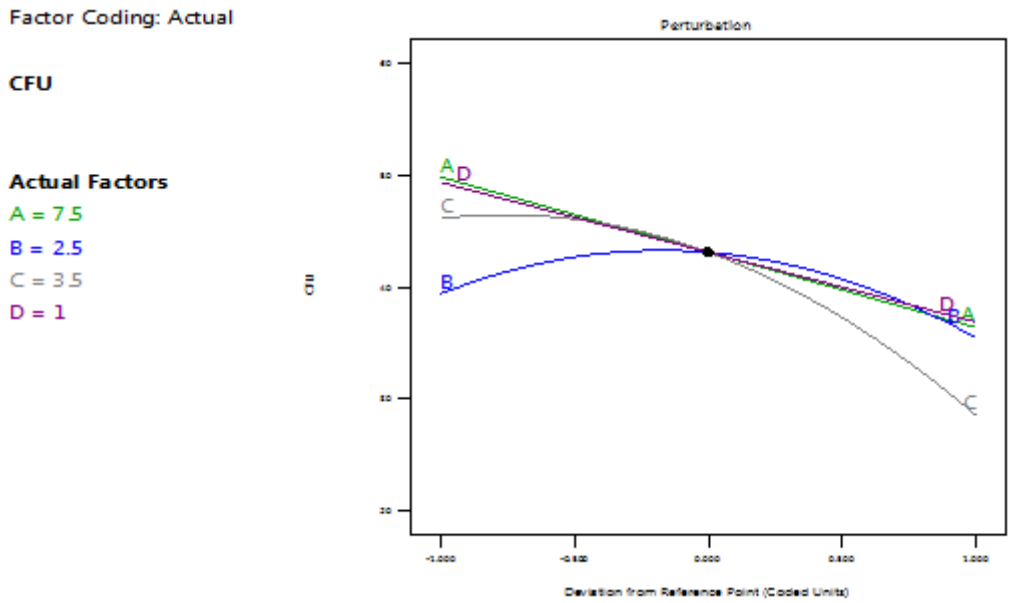

Figure 1. Perturbation plot showing the effect of A, B, C and D on CFUs

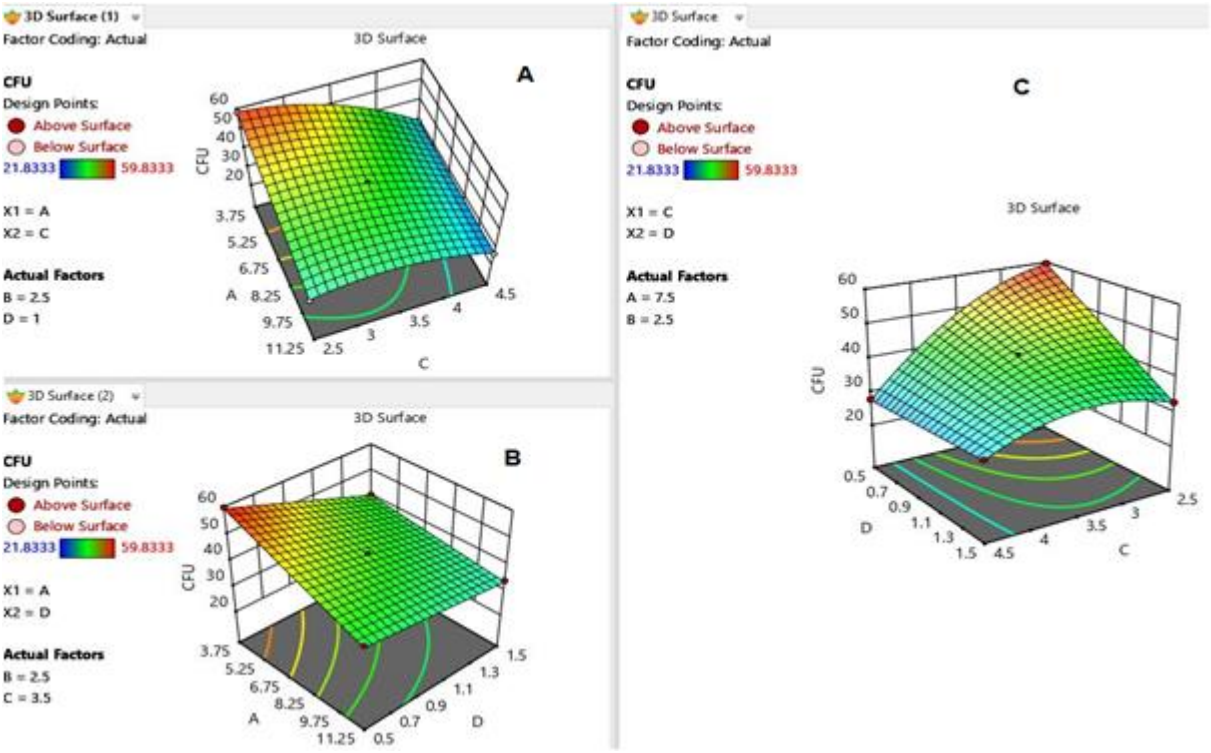

Figure 2: Response surface plots showing the interactive effect of CFUs (A) influence of AC at fixed levels of B and D (B) influence of AD at fixed levels of B and C (C) influence of CD at fixed levels of A and B

The polynomial equation below describes % recovery as a simultaneous function of A, B, C, and D.

$$Percent\ recovery\ (Y2) = 42.56 - 5.51A - 2.28B - 7.71C - 0.33D + 8.38AC - 3.92AD - 3.54C^2 + 5.07D^2$$

Table 4 shows recovery rates from 27.17 to 60.5. In the quadratic model, factors A, B, C, and D had a significant, antagonistic effect on mean particle size. A had a favourable interaction with C and a negative interaction with D. Particle size factorial equation had a 0.9791 correlation coefficient. Table 4 showed remarkable concordance between theoretical and observed values. Residuals vary from -2.18 to 1.79, indicating some differences from predictions. The model predicts % recovery more inconsistently than CFUs.

The analysis of variance (ANOVA) revealed a significant effect of the factors on the response ($p < 0.005$), as shown in Table 6. The Model F-value of 117.20 is significant because noise has a 0.01% chance of causing it. Compared to the sum of squares, the model's Predicted Residual Sum of Squares (PRESS) score (83.20) is low, indicating its predictability. In this analysis, model terms with p-values below 0.0500 were deemed significant, including A, B, C, AC, AD, C^2, and D^2. Model terms over 0.1000 were non-significant. Model reduction may improve model performance if there are many inconsequential model terms (excluding hierarchy terms). Lack of Fit was significant with a 19.69 F-value. Noise has a 0.53% chance of causing this large Lack of Fit F-value. Poor model fit is indicated by a substantial Lack of Fit.

Table 6. ANOVA of the quadratic model for the response percent recovery (Y2)

Source of variations	Sum of squares	Degree of freedom	Mean squares	F-value	p-value Prob>F	R^2
Model	1786.60	8	223.32	117.20	< 0.0001	0.9975
A	364.84	1	364.84	191.46	< 0.0001	
B	62.26	1	62.26	32.67	< 0.0001	
C	713.02	1	713.02	374.19	< 0.0001	
D	1.33	1	1.33	0.6997	0.4128	
AC	280.56	1	280.56	147.24	< 0.0001	
AD	61.36	1	61.36	32.20	< 0.0001	
C^2	86.37	1	86.37	45.32	< 0.0001	
D^2	177.19	1	177.19	92.99	< 0.0001	
Residual	38.11	20	1.91			
Lack of Fit	37.63	16	2.35	19.69	0.0053	
Pure Error	0.4778	4	0.1194			
Cor Total	1824.71	28				

Figure 3 summarises the main effects of factors A, B, C, and D on percent recovery (Y2). The Figure shows that variables C and A have the greatest impact on Y2, with C being the most important and A following closely. D has a small effect on Y2, while B has a large effect. Figure 4 shows response surface plots we used to analyse dependent-independent variable interactions. In particular, Figure 4A shows A and C's Y2 interaction with B and D held constant. Figure 4B shows A and D interacting on Y2 while keeping B and C constant.

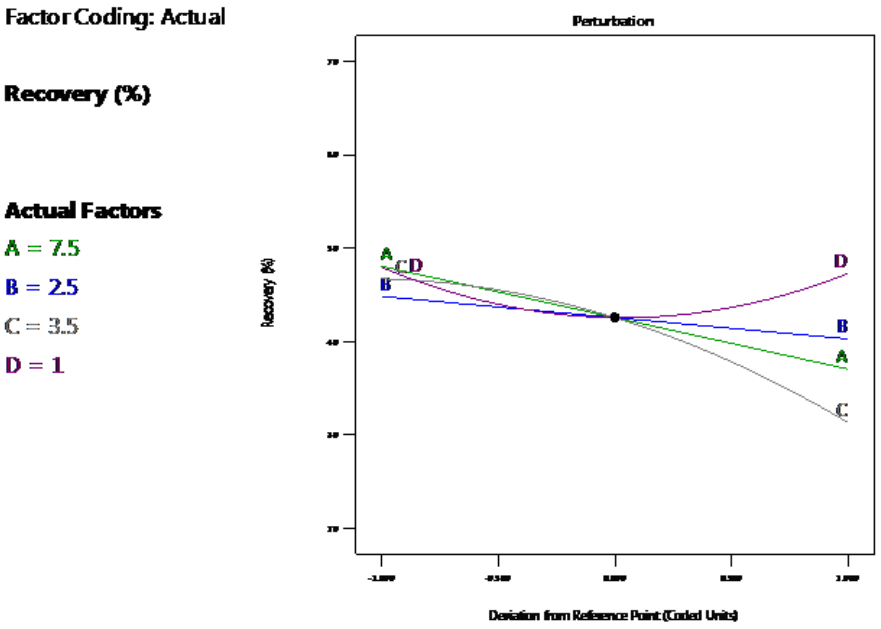

Figure 3: Perturbation plot showing the effect of A, B, C and D on percent recovery (Y2)

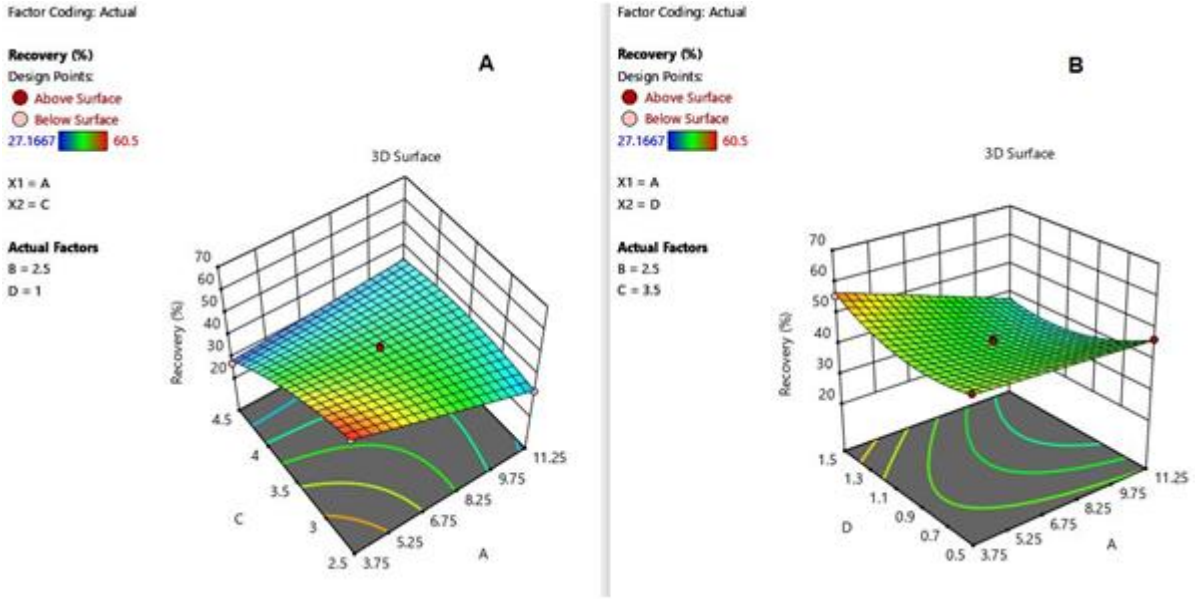

Figure 4: Response surface plots showing the interactive effect of percent recovery (A) influence of AC at fixed levels of B and D (B) influence of AD at fixed levels of B and C

3.1. Optimization and Confirmation Experiments

The media composition for nanosponges was optimised and confirmed using a systematic manner to maximise CFUs, % recovery, and desirable responses. Numerical optimisation, particularly attractiveness, was used first. CFUs and percent recovery can be optimised simultaneously using this method. The major goal was to find the best independent variable (factor) values for best results. To guide optimisation, limitations were set. Constraints guided optimisation with particular targets. Our goals were to maximise CFUs (Y1) and % recovery (Y2). The goal was to determine the elements that raised CFUs and recovery rates simultaneously. Results from the optimisation method included optimised independent variable

levels. These optimised settings should yield the highest CFUs and % recovery. To verify the optimisation technique, six confirmation trials were run. These studies employed the same optimised parameters and recorded CFUs and percent recovery figures. Optimization-predicted values were compared to observed values. The proximity of the predicted and observed values for Y1 and Y2 implies that the optimisation technique accurately predicted media composition operational parameters (Table 7).

Table 7.Optimized values obtained by the constraints applies on Y1 and Y2

Independent variable	Nominal values	Predicted values		Observed values		
		Y1	Y2	Batch	Y1	Y2
A	3.90	61.08	60.52	1	61.17 ± 1.60	59.33 ± 2.06
				2	62.33 ± 1.63	60.67 ± 2.73
B	1.90			3	60.83 ± 2.31	61.50 ± 2.88
				4	60.17 ± 2.48	59.33 ± 3.32
C	2.50			5	59.50 ± 2.07	61.50 ± 3.08
D	0.90			6	59.33 ± 1.36	61.33 ± 1.97

Microscopy was critical to our investigation, revealing how recovery medium compositions affected bacterial cell shape. We used modern equipment and procedures to precisely and clearly visualise and capture bacterial cell morphology for our research. The Nikon Inc. TI-E inverted microscope formed the foundation of our microscopic examination. Scientists admire its microscope's optics and precision. Its 100x phase-contrast objective lens, the CFI PlanApo LambdaDM100 x 1.4 NA, provides excellent magnification and clarity. Bacterial cells are tiny and require powerful lenses to explore. We used Lumencor Inc.'s Spectra X solid-state light source for optimum lighting and contrast during our observations. In microscopy, a good light source affects image quality and clarity. The Spectra X's durability and homogeneous lighting allowed us to take detailed photos of bacterial cells in different situations. We also used a Chroma Technology Corp. 69002bs multi-band dichroic mirror. Our examination relied on this optical component, which selectively reflects and transmits different wavelengths of light to enable phase contrast imaging of transparent biological specimens like bacterial cells. A dichroic mirror was vital for improving contrast and detail in our photos. Figure 5 shows our microscopic findings. Under diverse circumstances and recovery medium compositions, these photos showed bacterial cell morphology. Our study is more complete since this data supplements the quantitative data from CFUs and % recovery analysis. Our microscopy allowed us to see how recovery medium composition affected bacterial cell shape. Our findings are strengthened by this qualitative data and quantitative results, providing a more complete grasp of the topic.

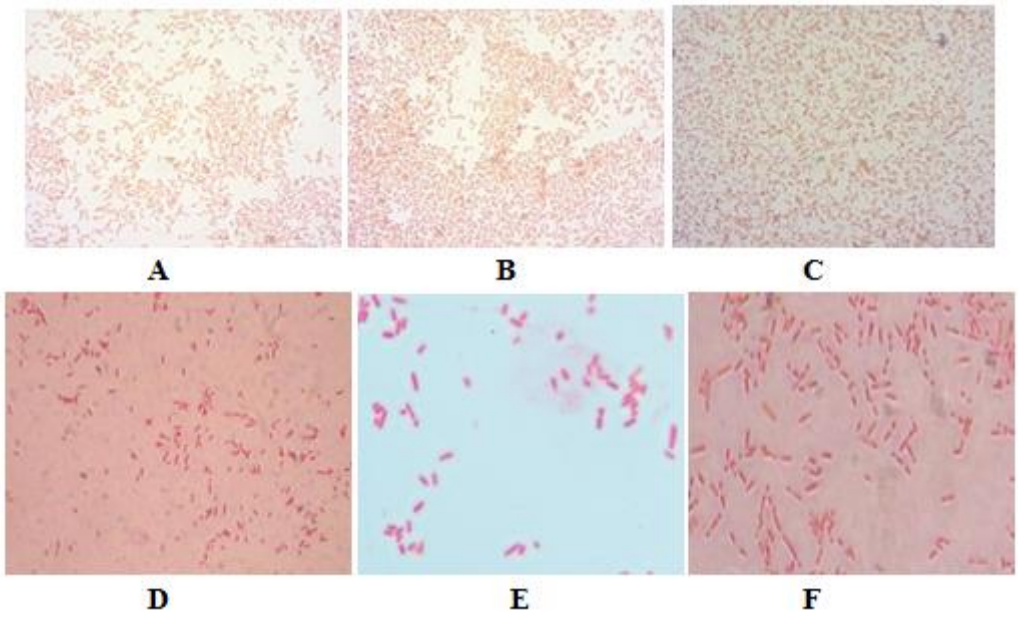

Figure 5: Microscopic images revealing the morphology of bacterial cells

4. Conclusion

Our microbiology research has focused on resuscitating challenged bacterial populations. Stress causes complex biological changes that affect bacterial culturability. *E. coli* is a model organism in microbiology, biotechnology, and medical research, thus recovery is crucial. Our research focused on recovery medium composition optimisation. Pancreatic Digest of Casein (A), Papaic Digest of Soybean (B), Sodium Chloride (C), and Dextrose Monohydrate (D) were fine-tuned. We used a Box-Behnken Design with 29 experiments, polynomial equations, ANOVA, and 3D response surface graphs. We predicted CFUs (Y1) and % recovery (Y2) with our models, and ANOVA verified their relevance. The desirability technique helped us find optimal levels for these elements during optimisation. The subsequent confirmation studies proved the optimisation procedure's reliability. Our method for selecting the best media composition for bacterial recovery was confirmed by the agreement between anticipated and observed values. This optimisation process is crucial to microbiology and other scientific fields, as shown by our work.

5. Conflicts of Interest

No conflict of interest.

6. Author Contributions

JKB conducted the investigation, collected data, and wrote the manuscript following statistical analysis. GPR helped develop the topic, design, supervise, correct, and approve the text.

7. References

[1]. Tuttle AR, Trahan ND, Son MS. Growth and maintenance of *Escherichia coli* laboratory strains. Curr Protoc. 2021;1(1):e20.

[2]. Dawan J, Ahn J. Bacterial stress responses as potential targets in overcoming antibiotic resistance. Microorganisms. 2022;10(7):1385.
[3]. Blount ZD. The unexhausted potential of E. coli. Elife. 2015;4:e05826.
[4]. Vila J, Sáez-López E, Johnson JR, Römling U, Dobrindt U, Cantón R, Giske CG, Naas T, Carattoli A, Martínez-Medina M, Bosch J. Escherichia coli: an old friend with new tidings. FEMS Microbiol Rev. 2016;40(4):437-63.
[5]. Uruén C, Chopo-Escuin G, Tommassen J, Mainar-Jaime RC, Arenas J. Biofilms as promoters of bacterial antibiotic resistance and tolerance. Antibiotics. 2020;10(1):3.
[6]. Jassim HM, Lafi SA, Majeed YH. Escherichia coli Biomarker Types in Colorectal Cancer Patients. Int J Drug Deliv Tech. 2022;12(2):622-628.
[7]. Raoof MAJ, Fayidh MA. Investigation of Biofilm Formation Efficiency in ESβLs of Pathogenic Escherichia coli Isolates. Int J Drug Deliv Tech. 2022;12(2):695-700.
[8]. Horcajo P, de Pedro MA, Cava F. Peptidoglycan plasticity in bacteria: stress-induced peptidoglycan editing by noncanonical D-amino acids. Microb Drug Resist. 2012;18(3):306-13.
[9]. Zhu M, Tse MW, Weller J, Chen J, Blainey PC. The future of antibiotics begins with discovering new combinations. Ann N Y Acad Sci. 2021;1496(1):82-96.
[10]. Booth IR. Regulation of cytoplasmic pH in bacteria. Microbiol Rev. 1985;49(4):359-78.
[11]. Lagier JC, Edouard S, Pagnier I, Mediannikov O, Drancourt M, Raoult D. Current and past strategies for bacterial culture in clinical microbiology. Clini Microbiol Rev. 2015;28(1):208-36.
[12]. Li L, Mendis N, Trigui H, Oliver JD, Faucher SP. The importance of the viable but non-culturable state in human bacterial pathogens. Front microbiol. 2014;5:258.
[13]. Oliver JD. The viable but nonculturable state in bacteria. JbMicrobiol. 2005;43(1):93-100.
[14]. Thorfinnsdottir LB, Bø GH, Booth JA, Bruheim P. Survival of *Escherichia coli* after high-antibiotic stress is dependent on both the pregrown physiological state and incubation conditions. Front microbiol. 2023;14:1149978.
[15]. Rouiller Y, Périlleux A, Collet N, Jordan M, Stettler M, Broly H. A high-throughput media design approach for high performance mammalian fed-batch cultures. InMAbs. 2013;5(3):501-511.
[16]. Ju Y, Son KH, Jin C, Hwang BS, Park DJ, Kim CJ. Statistical optimization of culture medium for improved production of antimicrobial compound by Streptomyces rimosus AG-P1441. Food Sci Biotechnol. 2018;27:581-90.
[17]. Chen J, Lan X, Jia R, Hu L, Wang Y. Response Surface Methodology (RSM) Mediated Optimization of Medium Components for Mycelial Growth and Metabolites Production of Streptomyces alfalfae XN-04. Microorganisms. 2022;10(9):1854.
[18]. Wang CN, Dang TT, Nguyen NA. A computational model for determining levels of factors in inventory management using response surface methodology. Mathematics. 2020;8(8):1210.

[19]. Ramyasree A, Umadevi S. An efficient RP-HPLC-PDA Method for Estimating Dolutegravir and Lamivudine in Combined Pharmaceutical Formulations using a Box-Behnken Design Approach. Int J Pharm Qual Assur. 2023;14(3):507-513.

[20]. Mamatha P, Bhikshapathi DVRN. Preparation and In-vitro Evaluation of Pemigatinib Nanosponges Tablets by Box-Behnken Design. Int J Pharm Qual Assur. 2023;14(3):791-800.

[21]. Collins LM, Dziak JJ, Li R. Design of experiments with multiple independent variables: a resource management perspective on complete and reduced factorial designs. Psychol Methods. 2009;14(3):202.

[22]. Singireddy A, Pedireddi SR, Subramanian S. Optimization of reaction parameters for synthesis of Cyclodextrin nanosponges in controlled nanoscopic size dimensions. J Polym Res. 2019;26:1-2.

[23]. Tekindal MA, Bayrak H, Özkaya B, Yasemin GE. Box-behnken experimental design in factorial experiments: the importance of bread for nutrition and health running head. Turk J Field Crops. 2012;17(2):115-23.

[24]. Elfghi FM. A hybrid statistical approach for modeling and optimization of RON: A comparative study and combined application of response surface methodology (RSM) and artificial neural network (ANN) based on design of experiment (DOE). Chem Eng Res Des. 2016;113:264-72.

[25]. Anandam S, Selvamuthukumar S. Optimization of microwave-assisted synthesis of cyclodextrin nanosponges using response surface methodology. J Porous Mater. 2014;21:1015-23.

[26]. Son MS, Taylor RK. Growth and maintenance of *Escherichia coli* laboratory strains. Curr Protoc Microbiol. 2012;27(1):5A-4.

[27]. Booth A, Aga DS, Wester AL. Retrospective analysis of the global antibiotic residues that exceed the predicted no effect concentration for antimicrobial resistance in various environmental matrices. Environ Int. 2020;141:105796.

[28]. Sharma N, Choudhary Jain A, Anamika A, Sharma P. Evaluation of microbiological pattern, antimicrobial sensitivity among patients of chronic otitis media. Asian J Pharm Clin Res. 2023;16(5):118-23.

[29]. Rebbapragada D, Kalyanaraman R. Statistical optimization of medium components by plackett burman design and response surface methodology for enhanced antioxidant activity by xylaria feejeensis hmjau22039. Int J Pharm Pharm Sci. 2016;8(11):159-64.

[30]. Hema JN, Shobha, Shruthi SD. Isolation and characterization of cellulose-degrading bacteria from decomposing plant matter. Int J Pharm Pharm Sci. 2023;15(4):22-27.

[31]. Shaji J, Shah A. Optimization of tenoxicam loaded niosomes using quadratic design. Int J Curr Pharm Res. 2016;8(1):62-67.

[32]. Konda M, Sampathi S. Qbd approach for the development of capsaicin-loaded stearic acid-grafted chitosan polymeric micelles. Int J Appl Pharm. 2013;15(4):131–142.