ORIGINAL ARTICLE

Design, Formulation, and Evaluation of an Oral Drop of Probiotic for Infants

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Abstract

Background: Probiotics are helpful in treating conditions like diarrhea and eczema in infants. The lactic acid bacteria, from diverse sources, are of great importance as probiotics. The current study deals with the formulation development and optimization of oral probiotic drop of *Bacillus coagulans* for infants by the central composite design (CCD). **Materials and Methods:** Oral probiotic drops of *B. coagulans* for infants were manufactured with the help of fennel oil, pepsin enzyme, lecithin, and distilled water. The formula was optimized through CCD design and taken further for pepsin activity and viability assessment in SGF. **Results:** In this study, the CCD method was used to successfully design and optimize an oral probiotic drop of *B. coagulans* for newborns. The pepsin enzyme was discovered to be compatible with the *B. coagulans* spores. The emulsifier (lecithin) concentration and probe sonication time both had an impact on the responses (independent variables), droplet size (DS), polydispersity index (PDI), and zeta potential (ZP). The researchers concluded that the ideal formulation of *B. coagulans* oral probiotic drop should be made with an emulsifier (lecithin) of 7 mg, with a probe sonication time of 10 min, and DS, PDI, and ZP measured at 161.24 nm, 0.219, and −33 mV, respectively. The optimized formulation shows about 50% of pepsin activity, viability of probiotic in simulated gastric fluid of 1×10^7 colony-forming unit (CFU), total viable count of formulation containing pepsin enzyme is 4.1×10^7 CFU, and total viable count of formulation without pepsin enzyme is 2.02×10^7 CFU. Finally, stability tests performed at room temperature highlighted a bacterial viability of 4.1×10^7 CFU and 4.04×10^7 CFU after 1 and 3 months, respectively. **Conclusion:** The optimized formulation was demonstrated for the viability of the oral probiotic drop containing *B. coagulans* in simulated gastric conditions, pepsin activity, total viable count, and a 3-month stability study was performed.

Key words: *Bacillus coagulans*, oral drop, pepsin activity, probiotic, total viable count

INTRODUCTION

Healthy newborns benefit from probiotics because they help strengthen their immune systems. *Bifidobacterium*, *Lactobacillus*, *Streptococcus*, and *Saccharomyces boulardii* are a few examples of probiotic microbes. These bacteria are found in probiotic supplements, which help prevent infections by taking up residence in the digestive tract and preventing the growth of other hazardous microbes there. Nevertheless, probiotics should not be given to infants who have major medical issues. Prebiotics, a non-digestible component of probiotic supplements, encourage the growth of gut flora. Prebiotics are typically obtained by infants through their mother's breast milk. Up to the age of 6 months, breast milk is the recommended source of prebiotics for infants.[1]

It has been discovered that probiotics are effective in treating ailments, including diarrhea and infantile eczema (atopic dermatitis). In addition, they might aid in controlling allergies, avoiding urinary tract infections, and easing newborn colic

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symptoms. Probiotics in the form of live microorganisms are increasingly being used to promote health in both humans and animals. Today, a wide variety of fermented foods and drinks are offered, making up around one-third of all human diets globally. Probiotics have also been studied for their potential to improve immune function and dental health. They are also a crucial component of the food processing industry and the agricultural sector. When probiotic and prebiotic substances are applied together, the health advantages of probiotics can be increased.[2,3]

The process of ultrasonic emulsification, which uses high-energy ultrasound to apply a significant shear stress and reduce droplet size (DS), has proven to be quite effective. The most alluring high-energy way to create a stable emulsion is ultrasonication. Due to its capacity to produce tiny DSs with the least amount of energy use, ultrasonic homogenization has proven to be both economical and effective. The fundamental idea behind ultrasonication is the interaction of waves produced by an acoustic field with a frequency in the 20–100 kHz range with a liquid.[4-6]

However, it is discovered that no effort has been made to create probiotic *Bacillus coagulans*-loaded drop with the fennel oil (essential oil) using lecithin as an emulsifier.

First, this study's goals were to improve the circumstances of emulsification for the probiotic strain *B. coagulans* using the ultrasonication method to obtain a probiotic drop and finally to assess the probiotic cells' ability to survive following technological manufacturing, under simulated gastrointestinal environment, as well as during storage at room temperature for 3 months.

MATERIALS AND METHODS

B. coagulans (powder) was a gift sample from SK Biobiz Limited (JaulkeDindori, Nashik, India), and Pepsin enzyme (Indian pharmacopoeia 1:3000) was a gift sample from Advanced Enzyme Technology Limited (Sinnar, Nasik, India). Lecithin and Fennel oil was procured from Katyani manufacturers. Hi Media Laboratories Pvt. Limited (Mumbai, India) supplied the soybean casein digest media and other analytical grade laboratory chemicals. *B. coagulans* identification tests (description, microscopic examination), total viable *B. coagulans* cell count, and internal compliance testing were carried out in accordance with the manufacturer's instructions. In all of the trials carried out for this investigation, distilled water was used.

Formulation of probiotic drop

The liquids and glassware used in the procedures had been thoroughly sterilized. The probiotic drop was prepared using fennel oil, lecithin, pepsin, *B. coagulans,* and distilled water. In this study, the preparation of the emulsions was a two-part procedure, with the first phase being the creation of emulsions by combining the three essential ingredients, namely oil, water, and emulsifier, at different emulsifier concentrations and probe sonication times. A magnetic stirrer spinning for 10 min at a speed of 500 rpm was used for mixing them. Then, these emulsions were subjected to an ultrasonicator, a Probe Sonicator.[7] Then, probiotics and pepsin were added to the optimized formulation and mixed well. On a bench of a clean air work station with horizontal laminar flow, the entire procedure was done aseptically.

Experimental design for optimization

The effect of factors emulsifier concentration (1–7 mg) and probe sonication time (2–10 min) on the dependent variables such as DS (R1), polydispersity index (PDI) (R2), Zeta potential (ZP) (R3), and s2-factor face-centered central composite design (CCD) was used for optimization. A design-expert® (version 13.0.3.0, Stat-Ease Inc., Minneapolis, MN, USA) software was used to run a total of 13 experiments, including 5 repetitions of central points along with 4 factorial and axial points, respectively. The values of responses obtained after preparing the experimental runs were fed into the Design Expert software, and the connection between dependent variables and independent variables was determined using a mathematical model. The significant terms ($P < 0.005$, analysis of variance), coefficient of variance, least significant lack of fit, the multiple correlation coefficient, and adjusted multiple correlation coefficient were used for the selection of a suitable polynomial model provided by Design-Expert® software. The repeatability of the method was determined by repeating the center points for 5 times. The polynomial regression equation is used to examine the factor response connection for the response function Y_i, which uses the generalized response surface model. In the equation, the independent variables, such as the concentration of emulsifier and probe sonication time, are denoted as X_1 and X_2, respectively. While the term a_0 indicates intercept (a constant), the terms a_1, a_{11}, a_{12} represent regression coefficients of the linear, quadratic, and interactive terms, respectively.

$$Y_i = a_0 + a_1X_1 + a_2X_2 + a_{12}X_1X_2 + a_{11}X_1^2 + a_{22}X_2^2$$

To determine the impact of independent factors on the measured responses, the response surface plots and contour plots were examined. Overlay plot analysis and numerical optimization were done.

Preformulation studies

A detailed understanding of the properties of bacterial spores is essential to minimize the formulation problems in the later stages of formulation development. Preformulation is the term for the initial stage of gathering physicochemical data on bacterial spores, which examines the appropriateness of excipients

before formulation. Preformulation serves as the link between the discovery of novel medicinal entities and formulations. To create the best possible medication delivery system, preformulation entails applying biopharmaceutical principles to the physicochemical characteristics of the medicine.

Characterization of bacterial spores of *B. coagulans*

Organoleptic characters

The *B. coagulans* powder was subjected to physical examination, such as color, odor, and appearance.

Total viable count determination

Weigh accurately 1 g of *B. coagulans* powder in a sterile container. Add all the 1 g in the 99 mL of the sterile saline and shake well. Then, pour the prepared sterile soybean casein digest agar media into the sterile petri plate. Depending on the estimated count of microorganisms in the sample, a 10-fold dilution was made using sterile saline solution, and those dilutions that gave numbers of colonies between 30 and 300 were used for plating. Then, the petri plate was placed in the incubator at 40°C for 48 h. Count and determine the total number of colonies in the sample by multiplying by the dilution factor.

Identification of B. coagulans

Weigh accurately 1 g of *B. coagulans* powder and place it in 99 mL of sterile saline and shake well. Then, pour the sterile soybean casein digest agar media into the sterile petri plate. Then, using a sterile nichrome wire loop, strike the suspension of *B. coagulans* on the media in a petri plate and place the petri plate in an incubator at 40°C for 48 h.

Probiotic excipient compatibility studies

In the initial manufacturing process of a product, understanding how probiotics and excipients interact is crucial. Probiotic excipient compatibility investigations are crucial to the formulation development procedure since the information they provide is utilized to choose the dosage form's components, analyze the probiotic's stability profile, and pinpoint degradation products. The probiotic excipient compatibility study was carried out by taking probiotic-excipient in 1:1% W/W ratio for samples. The mixtures were then transferred to previously clean and dried vials, vials were sealed using a rubber closure and aluminum crimp. The samples were kept at room temperature for 7 days. The samples were then observed visually for a change in color, odor, and appearance, and the samples were analyzed for total viable count at day 7.[8]

Thermodynamic stability study

The optimized formulations of the probiotic drop were evaluated for the following stability testing methods.

Centrifugation

The stability of probiotic drops in terms of phase separation was evaluated by high-speed centrifugation (force-free separation). Optimized formulations were centrifuged for 30 min at 3,000 rpm using Remi C-854/8. And phase separation was analyzed visually. Formulations that did not undergo phase separation were selected for the next stability tests.

Thermal stability of probiotic drop

Stability of optimized formulations was detected by placing the solution in vials at three different temperatures, i.e., 4, 25, and 45°C, in a temperature-controlled oven for 48 h. Samples were taken periodically for analysis to look for any physical changes, such as coalescence.

Freeze-thaw method

The optimized batch of formulation was filled into the prewashed and dried glass vials and sealed with an aluminum cap. The formulation was frozen for 18 h. After 18 h, the formulations were removed and kept at room temperature (25°C) for 6 h. The thermodynamically stable formulation returned to its original form within 2–3 min, and such 2–3 cycles were repeated.

Assay of pepsin activity

The biuret protein assay was utilized to measure the activity of the pepsin enzyme. The cupric ions (Cu^{2+}) complexation to functional groups in the protein's peptide bond is the foundation of the Biuret technique. Protein solutions are combined with an alkaline solution of copper salt (Cu^{2+}) in the copper ion-based protein tests. The protein assay, also known as the Biuret assay, is based on the interaction of Cu^{2+} with protein in an alkaline solution. When Cu^{2+} interact with proteins, a purple hue is produced that may be seen at 540 nm. Protein content directly relates to color production. A stock

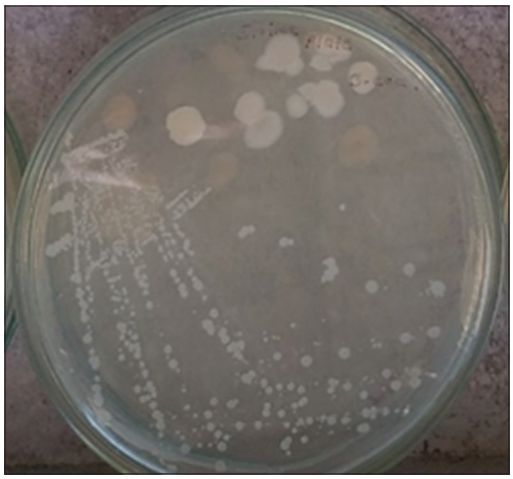

Figure 1: Observed result of the strike plate method of *Bacillus coagulans*

solution of albumin protein was prepared (20 mg/mL) in a volumetric flask. Biuret reagent was prepared by dissolving 1.5 g of copper(II) sulfate and 4.5 g of sodium potassium tartrate in 250 mL 0.2 N sodium hydroxide (NaOH) solution. Add 2.5 g of potassium iodide and make up the volume to 500 mL with 0.2 N NaOH. In the test tube, dilution of albumin protein of concentrations 0, 2, 4, 6, 8 10 mg/mL were made by an appropriate amount of albumin stock and water. 3 mL of Biuret reagent is added to all the test tubes. Calibration was performed by taking absorbance in triplicate. In another set of test tubes, three different dilutions of 2, 4, 6 mg/mL protein solutions were prepared, and 2 mL formulation with 3 mL of biuret reagent in each tube. Incubate all the test tubes for 15 min at room temperature, and then take the absorbance at 540 nm.[9-12]

Then the protein concentration is calculated using the formula,

$$C_u = A_u – A_b / A_s – A_b \times C_s$$

Where,

- C_u = unknown concentration of protein
- C_s = stock or standard concentration of protein
- A_s = absorbance of standard sample
- A_u = absorbance of unknown sample
- A_b = absorbance of blank sample

Total viable count

Add 1 mL of the sample to the 99 mL of sterile saline and shake well. Then, pour the prepared sterile soybean casein digest agar media into the sterile petri plate. Depending on the estimated count of microorganisms in the sample a 10-fold dilution was made using sterile saline solution, and those dilutions that gave numbers of colonies between 30 and 300 were used for plating. Then, the petri plate was placed in the incubator at 40°C for 48 h. Count and determine the total number of colonies in the sample by multiplying by the dilution factor.

Probiotic viability testing in simulated gastric fluid (SGF)

In this study, the viability of probiotic drops in SGF was determined by *in vitro* technique. Using paddle type Dissolution apparatus this study is performed. Preparation of SGF: 0.2 g of sodium chloride, 0.32 g of pepsin, 0.7 mL of hydrochloric acid (HCl) were accurately measured and make up the volume of 100 mL using distilled water. And then adjust the pH to 1.2 by using 0.1 n HCl. Then placed the 90 mL of SGF in the dissolution flask and added 10 mL of the formulation to it. Maintained the dissolution medium temperature at 37 ± 0.5°C and set paddle revolution at 50 rpm for 3 h. After 3 h remove 1 mL of the sample and place it in 99 mL of sterile saline and shake well. Then pour the prepared sterile soybean casein digest agar media into the sterile petri plate. Depending on the estimated count of microorganisms in the sample, a 10-fold dilution was made using sterile saline solution, and those dilutions that gave numbers of colonies between 30 and 300 were used for plating. Then, the petri plate was placed in the incubator at 40°C for 48 h. Count and determine the total number of colonies in the sample by multiplying by the dilution factor.[13,14]

Stability study

Stability study of optimized formulation was performed by keeping the sample at room temperature for 1 month to analyze the physical stability of the formulation over a period of time. The optimized batch of probiotic drop was filled in a prewashed, dried transparent glass vial and sealed with an aluminum cap and stored at room temperature for 3 months. Total viable count, viscosity, pH, and physical appearance were determined at 0, 1, and 3 months.

RESULTS

Preformulation study of *B. coagulans*

Characterization of bacterial spores of B. coagulans

The *B. coagulans* powder was subjected to physical examination and was found to be an amorphous light brown powder with a characteristic odor.[15] Total viable count of probiotic powder was found to be 21×10^9 Colony Forming Unit (CFU), and probiotic *B. coagulans* strain identified by the strike plate method and grown colonies confirms that the probiotic powder contains *B. coagulans* strain [Figure 1].

Probiotic excipient compatibility studies

Compatibility study of the probiotic and excipient was performed, and results were found satisfactory on the basis of the analysis of sample color, odor, and total viable count. The excipient does not affect the probiotic strength. At day 0 and day 7, the strength was found to be 21×10^9 CFU of pure probiotic powder, and the strength of the sample containing probiotic powder with the pepsin enzyme at day 0 and day 7 was found to be 44×10^9 CFU. From the above data of the compatibility study of probiotics with pepsin, probiotics show good viability/stability in the presence of the pepsin enzyme. That means the pepsin enzyme is compatible with the probiotic.

Coded equations for DS, PDI, and ZP are given below:

$$DS = 231.81 - 91.28\,A + 0.1464\,B - 1.5\,AB + 21.50\,A^2 - 2.20\,B^2$$

$$PDI = 0.2658 + 0.0011\,A - 0.0660\,B + 0.0013\,AB - 0.0022\,A^2 + 0.0240\,B^2$$

$$ZP = -27.60 - 5.91\,A - 0.7286\,B - 1.25\,AB + 1.25\,A^2 + 0.0631\,B^2$$

It should be noticed that each of the three mathematical models mentioned above had a coefficient with statistical significance ($P < 0.05$). Response surface methodology was used to clarify how factors affected the replies that were under investigation. According to reports, the response surface approach can be used to optimise a formulation and look at how many components interact to produce the desired results.[16-18]

Analysis of 3D response surface plot

The 3D response surface plots for the selected responses of emulsion base, such as DS, PDI, and ZP, are shown in the figures.

As shown in Figure 2, the DS of emulsion decreases with the increase in the concentration of emulsifier (at 1 mg surfactant concentration, the DS is around 340 nm, whereas at 7 mg, it decreases to 161 nm), whereas probe sonication time does not produce any effect on DS.

As shown in Figure 3, the PDI value lies between 0 (for a sample that is completely consistent in terms of particle size) and 1 (for a strongly polydisperse sample with a mixed population of particle sizes), wherein PDI <1 represents a nearly homogeneous monodispersed formulation. The PDI of emulsion base decreases with the increase in probe sonication time (at 2 min of probe sonication time, the PDI is around 0.352, whereas at 10 min its decreases to 0.22), whereas the emulsifier does not produce any significant effect.

As shown in Figure 4, the ZP of emulsion was decreased with increment in the concentration of emulsifier (at 1 mg concentration, the ZP is around −21 mV, whereas at 7 mg concentration, the ZP is around −31 mV). This observation may be explained by the fact that the emulsifier generates surface charges that may affect the emulsion's chemical and physical stability. According to the DLVO theory, these charges are thought to maintain the emulsion's stability through electrostatic repulsion.

Optimization of responses

Design-Expert® software was used in order to determine the working region (design space) by graphical optimization, as shown in the overlay plot in Figure 5. In the overlay plot, the

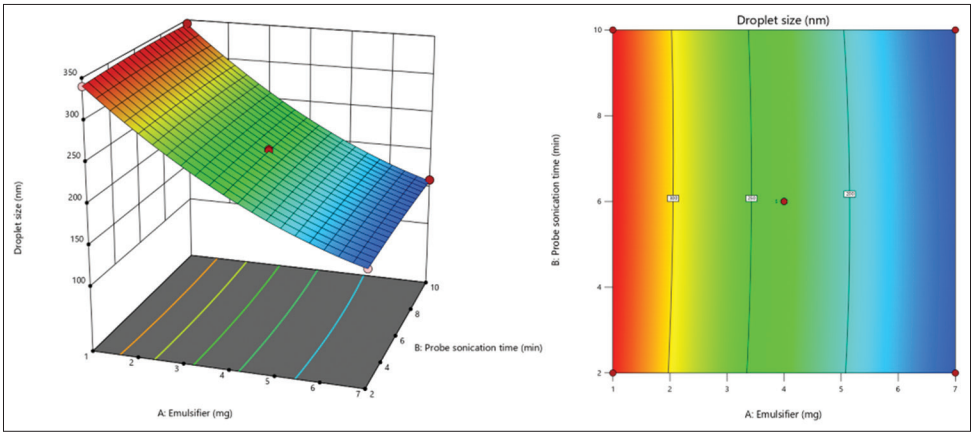

Figure 2: 3D response surface plot for droplet size

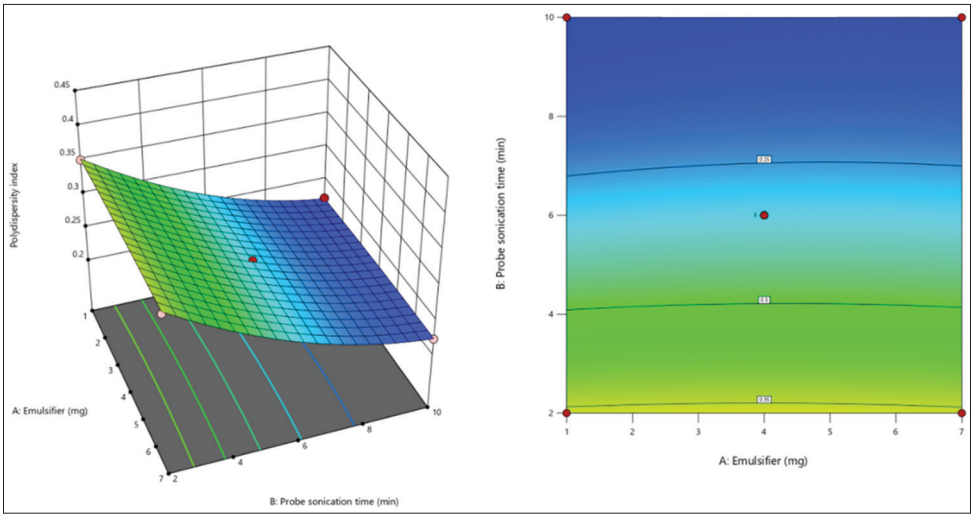

Figure 3: 3D response surface plot for polydispersity index

Table 1: Matrix of face-centered central composite design along with observed independent variable values for preparation of emulsion base

Std	Run	Space type	Independent variables		Dependent variables		
			Factor 1	Factor 2	Response 1	Response 2	Response 3
			Emulsifier concentration (mg)	Probe sonication time (min)	DS	PDI	ZP
1	1	Factorial	1	2	340	0.352	−21
3	2	Factorial	1	10	345	0.220	−20
2	3	Factorial	7	2	161	0.350	−31
4	4	Factorial	7	10	160	0.223	−35
6	5	Axial	8.24	6	146	0.261	−32
5	6	Axial	8.31	6	144	0.267	−34
7	7	Axial	4	0.34	228	0.410	−26
8	8	Axial	4	11.65	226	0.220	−28
12	9	Center	4	6	230	0.268	−27
10	10	Center	4	6	232	0.265	−28
13	11	Center	4	6	232	0.265	−27
11	12	Center	4	6	234	0.267	−28
9	13	Center	4	6	231	0.264	−28

DS: Droplet size, PDI: Polydispersity index, ZP: Zeta potential

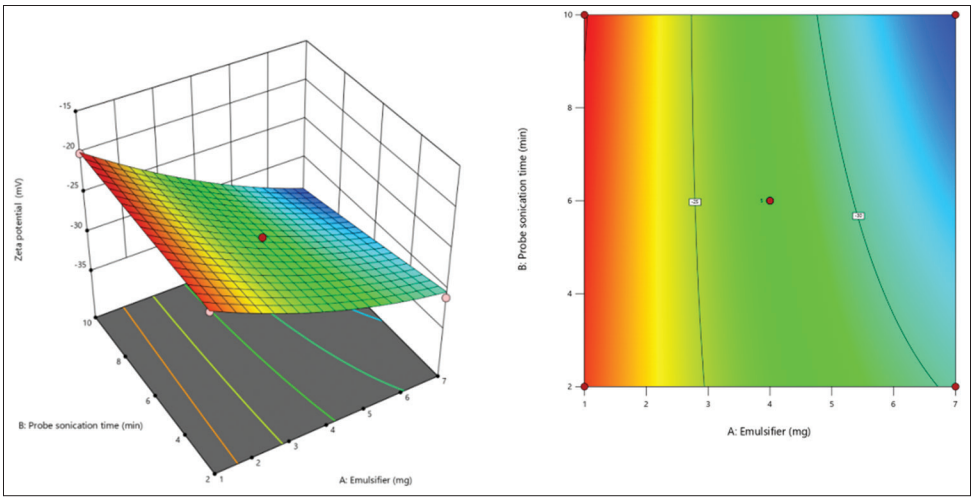

Figure 4: 3D response surface plot for zeta potential

working region is observed in yellow color, whereas the region that does not come under the working region is observed in gray color. The design space in the overlay plot represents the desired amount of independent variables and the chosen dependent variables (DS, PDI, ZP) values of the emulsion base. The optimized formula was selected within the design space in which the DS, ZP, and PDI are minimum [Table 1]. The independent variables values given in the optimized formula for emulsifier are 7 mg, probe sonication time is 10 min, whereas the response values given for the optimized formula within the design space are 158.47 nm, 0.223, and −34 mV for DS, PDI, ZP, respectively. Tables 2 and 3 displayed the prediction error values for Critical Quality Attributes (CQAs) calculated after preparing the formulation using the optimized formula.

Thermodynamic stability study

Prepared formulation found stable under the centrifugation and thermal stability study, which showed no physical change. And returned to the original form within 4–5 min after freeze-thaw cycle.

Assay of pepsin activity

Calibration curve data for the albumin solution are shown in Table 4.

From the obtained results, we found that the Pepsin enzyme present in the formulation shows proteolytic activity. The

Table 2: ANOVA for quadratic model generated from face-centered central composite design effects along with three different dependent variables to determine the best fitted model equation

Response 1: DS						
Source	**Sum of squares**	**Degrees of freedom**	**Mean square**	**F-value**	***P*-value**	**Significance**
Model	48961.25	5	9792.25	4017.60	<0.0001	Significant
A-Emulsifier concentration	41589.90	1	41589.90	17063.66	<0.0001	
B-Probe sonication time	0.1716	1	0.1716	0.0704	0.7984	
AB	9.00	1	9.00	3.69	0.0961	
A^2	2347.72	1	2347.72	963.23	<0.0001	
B^2	32.36	1	32.36	13.28	0.0082	
Residual	17.06	7	2.44			
Lack of fit	8.26	3	2.75	1.25	0.4024	Not significant
Pure error	8.80	4	2.20			
Cor total	48978.31	12				
Response 2: PDI						
Source	**Sum of Squares**	**Degrees of freedom**	**Mean square**	**F-value**	***P*-value**	**Significance**
Model	0.0389	5	0.0078	961.06	<0.0001	Significant
A-Emulsifier concentration	6.238E-06	1	6.238E-06	0.7702	0.4093	
B-Probe sonication time	0.0348	1	0.0348	4297.62	<0.0001	
AB	6.250E-06	1	6.250E-06	0.7717	0.4088	
A^2	0.0000	1	0.0000	3.16	0.1188	
B^2	0.0038	1	0.0038	473.17	<0.0001	
Residual	0.0001	7	8.099E-06			
Lack of fit	0.0000	3	0.0000	5.67	0.0635	Not significant
Pure error	0.0000	4	2.700E-06			
Cor total	0.0390	12				
Response 3: ZP						
Source	**Sum of squares**	**Degrees of freedom**	**Mean square**	**F-value**	***P*-value**	**Significance**
Model	223.59	5	44.72	58.64	<0.0001	Significant
A-Emulsifier concentration	174.62	1	174.62	229.00	<0.0001	
B-Probe sonication time	4.25	1	4.25	5.57	0.0504	
AB	6.25	1	6.25	8.20	0.0242	
A^2	7.99	1	7.99	10.48	0.0143	
B^2	0.0265	1	0.0265	0.0348	0.8573	
Residual	5.34	7	0.7625			
Lack of fit	4.14	3	181.14	4.60	0.0.873	Not significant
Pure error	1.20	4	0.3000			
Cor total	228.92	12				

ANOVA: Analysis of variance, DS: Droplet size, PDI: Polydispersity index, ZP: Zeta potential

Table 3: Comparative data of predicted and observed experimental values of responses

Dependent variables	Predicted value	Observed experimental value	Prediction error
DS (nm)	158.47	161.24±2.20	1.74
PDI	0.223	0.219±0.024	−1.79
ZP (mV)	−34	−33±1.37	−2.94

DS: Droplet size, PDI: Polydispersity index, ZP: Zeta potential

pepsin enzyme present in the formulation can digest about 50% of the albumin protein present in the sample.

Total viable count

The probiotic drops were tested for the total viable count of probiotic *B. coagulans* using the pour plate technique. This pour plate test was performed by making serial dilutions of a probiotic drop containing probiotics *B. coagulans,* using a saline solution [Tables 5 and 6].

Table 4: Absorbance values of different dilutions of standard solution

S. No.	Concentration (mg/mL)	Volume of protein (mL)	Volume of biuret (mL)	Volume of distilled water	Absorbance (540 nm)			Mean
1.	0	0	3	5	0.048	0.046	0.050	0.048
2.	2	1	3	6	0.324	0.326	0.323	0.324
3.	4	2	3	5	0.528	0.530	0.530	0.530
4.	6	3	3	4	0.680	0.681	0.679	0.680
5.	8	4	3	3	0.851	0.852	0.850	0.851
6.	10	5	3	2	0.986	0.987	0.987	0.987

Table 5: Protein concentration remained after the action of the pepsin enzyme present in the formulation in various dilutions

S. No.	Concentration (mg/mL)	Volume of protein (mL)	Volume of biuret (mL)	Volume of sample (mL)	Absorbance (540 nm)			Mean	Protein concentration (mg/mL)
1.	2	1	3	2	0.203	0.200	0.205	0.203	1.12
2.	4	2	3	2	0.303	0.305	0.308	0.305	2.13
3.	6	3	3	2	0.418	0.419	0.418	0.418	3.48

Table 6: Total viable count of prepared formulation

Sample	Observed result
Formulation with pepsin enzyme	4.1×10^7 CFU
Formulation without pepsin enzyme	2.02×10^7 CFU

CFU: Colony-forming unit

Table 7: Total viable count under influence of simulated gastric fluid

Sample	Time under acidic condition (hour)	Observed result
Bacillus coagulans probiotic	3	1×10^7 Colony forming unit

From the above data of the compatibility study of probiotics with pepsin and total viable count of formulation with pepsin, probiotic shows the good viability/stability in the presence of the pepsin enzyme.

Probiotic viability testing in SGF

It is essential for probiotic survival at low pH levels to withstand the initial stress in the stomach. The *B. coagulans* strain's capacity to survive in low pH environments is displayed in Table 7.

From the above data on the probiotic survival in the SGF or condition, the probiotic shows 25% of the survivability in the simulated gastric condition. This means the probiotic entered into the intestinal region through the duodenum and can form colonies in the intestine showing its action.

Stability study

A stability study was performed, and it was found that there was no significant change in physical form, viscosity, pH, and total viable count. It exclaimed that the optimized batch of probiotic drops is stable and reproducible.

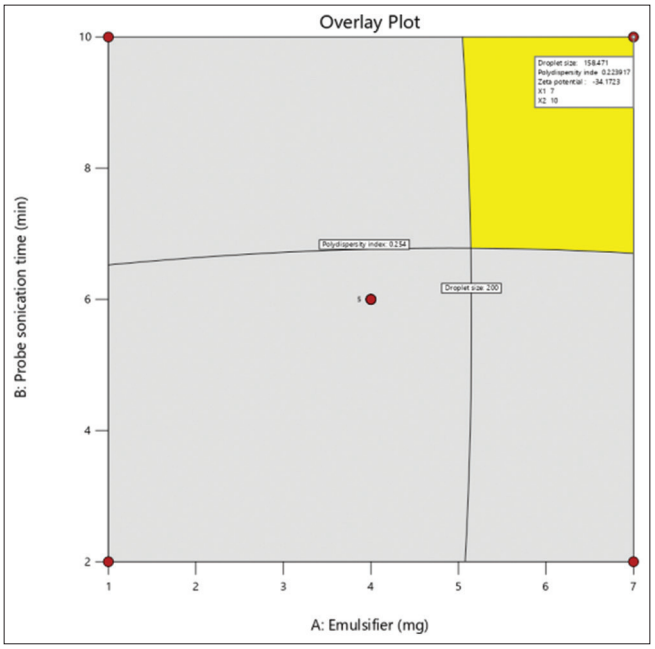

Figure 5: Overlay plot obtained from face-centered central composite design

DISCUSSION

The oral probiotic drop of *B. coagulans* for infants was prepared using fennel oil, pepsin enzyme, lecithin, and distilled water. Using design expert software, the CCD optimized the formulation. The formulation was optimized by CCD design and carried forward to evaluate pepsin activity and viability in SGF. The factor emulsifier concentration and

Table 8: Stability study of the optimized batch

S. No.	Parameter	At day 0 of stability study	At day 30 of stability study	At day 90 of stability study
1.	pH	6.53	6.54	6.54
2.	Viscosity	0.930 cP	0.9318 cP	0.935 cP
3.	Total viable count	4.1×10^7 CFU	4.1×10^7 CFU	4.04×10^7 CFU
4.	Physical appearance	No physical change	No physical change	No physical change

CFU: Colony-forming unit

probe sonication time are independent variables considered in CCD design for optimization, and their influence on the responses DS, PDI, and ZP was determined. The emulsifier was observed to have reduced the DS and ZP. The minimum PDI produced through probe ultrasonication results in homogenizing the formulation by avoiding the aggregation of oil droplets that are scattered.[19]

As emulsifier concentration and probe ultrasonication time increased, the DS, PDI, and ZP were significantly reduced. Viscosity and pH were also tested with the total viable count of probiotic bacteria, since they play a critical role in determining the efficacy of the formulation, in addition to characterizing the formulation based on its physical stability.[20] According to the findings, the ideal formulation of oral probiotic drop of *B. coagulans* should be prepared using emulsifier (lecithin) at 7 mg, with 10 min probe sonication time, and DS, PDI and ZP were found to be 161.24 nm, 0.219, and −33 mV, respectively.[21] The optimized formulation shows about 50% of pepsin activity, viability of probiotic in simulated gastric condition of 1×10^7 CFU, total viable count of formulation containing pepsin enzyme is 4.1×10^7 CFU, and total viable count of formulation without pepsin enzyme is 2.02×10^7 CFU. Further, room temperature stability testing revealed a bacterial survivability of 4.1×10^7 CFU and 4.04×10^7 CFU after 1 and 3 months, respectively [Table 8].

CONCLUSION

In this investigation, fennel oil and pepsin enzyme-containing oral probiotic drops of *B. coagulans* for infants were successfully designed and optimized using CCD. The *B. coagulans* spores were found to be compatible with the pepsin enzyme. The responses (independent variable) DS, PDI, and ZP were influenced by the emulsifier (lecithin) concentration and probe sonication time. According to the findings, the ideal formulation of an oral probiotic drop of *B. coagulans* should be prepared using emulsifier (lecithin) at 7 mg and with 10 min probe sonication time. The emulsifier was employed to stabilize the formulation, then centrifugation, thermal stability study, and freeze-thaw cycles were utilized to study it. The optimized formulation was demonstrated for the viability of the oral probiotic drop containing *B. coagulans* in simulated gastric conditions, pepsin activity, total viable count, and a 3-month stability study was performed. The technique for the preparation of oral probiotic drops of *B. coagulans* was found simple, easily controllable, and economical.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

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