RESEARCH PAPER

Formulation and Evaluation of Oral Drop for the Treatment of Gastrointestinal Disorder in Infants

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ABSTRACT

Background: Probiotics have been found to be helpful in treating conditions like diarrhea and eczema in infants. The lactic acid bacteria (LAB), 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.

Results: In this study, the central composite design method was used to successfully design and optimise an oral probiotic drop of *Bacillus coagulans* for newborns. The pepsin enzyme was discovered to be compatible with the *Bacillus coagulans* spores. The emulsifier (xanthum gum) concentration and magnetic stirrer speed both had an impact on the responses (independent variables), droplet size, polydispersity index, and zeta potential. The researchers came to the conclusion that the ideal formulation of *Bacillus coagulans* oral probiotic drop should be made with an emulsifier (xanthum gum) of 0.1 gm, with a magnetic stirrer speed 500 rpm, and droplet size, polydispersity index, and zeta potential measured at 243.1 nm, 0.138, and -28.28 mV, respectively. The optimized formulation shows about 50 % of pepsin activity, viability of probiotic in simulated gastric fluid of 0.99×10^7 CFU, total viable count of formulation containing pepsin enzyme is 4.02×10^7 CFU, and total viable count of formulation without pepsin enzyme is 1.85×10^7 CFU. Finally, stability tests performed at room temperature highlighted a bacterial viability of 4.0×10^7 CFU and 4.02×10^7 CFU after 1 and 3 months, respectively.

Conclusions: The optimized formulation were demonstrated for the viability of the oral probiotic drop containing *Bacillus coagulans* in simulated gastric condition, pepsin activity, total viable count and 3 month stability study was performed.

Keywords: Bacillus coagulans, Dill oil,Anise oil,Probiotic, Oral Drop, Pepsin activity, Total viable count.

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INTRODUCTION

Postnatal adaptation of the GI tract is not always smooth. Even healthy term infants commonly have reflux, but more serious problems are likely in the very preterm infant. In addition, a number of serious congenital anomalies of the gastrointestinal tract present in the first few days of life. Postnatal adaptation of the gastrointestinal tract can be maximized by early feeding.[1] Necrotising Enterocolitis (NEC) an inflammatory disorder of the intestine, usually seen in premature infants. It involves coagulation necrosis, bacterial overgrowth, inflammation and in severe cases necrosis of the bowel wall. It is a major cause of death and morbidity in neonates.

- Prematurity (seen in 90%) is the most important risk factor. The incidence of NEC is inversely proportional to the gestational age at birth. Immaturities in intestinal barrier function, gastric acid production, motility, some digestive enzymes and an imbalance in the inflammatory response may contribute to NEC
- Abnormal intestinal microbiota, either from a lack of beneficial commensal microbes, a low diversity of bacteria or a preponderance of pathogenic bacteria

- Ischemia re-perfusion injury; many risk factors involve decreased mesenteric perfusion and intestinal ischemia e.g. intrauterine growth restriction, perinatal asphyxia, congenital heart disease and patent ductus arteriosus

The use of formula instead of breast milk increases the risk of NEC. Older studies suggested rapid advancement of enteral feedings was associated with an increased risk of NEC, however recent meta-analyses have not shown an effect of aggressive feeding or delayed initiation of enteral feeding on NEC.[2,3]Gastroesophageal reflux (GER) is the retrograde movement of gastric contents into the oesophagus and above due to decreased lower oesophageal sphincter tone. While GER can be physiologic in healthy thriving infants, it is more frequently associated with symptoms of disease in infants < 1500g. Acid reflux has the potential to cause injury of the oesophageal mucosa and oesophagitis.

Clinical features vary from simple regurgitation to vomiting of feeds to the association of GER with apnoea and bradycardia and aspiration.[4]

The most common gastrointestinal (GI) ailments seen by a pediatrician include abdominal pain, diarrhea, vomiting, constipation, failure to gain weight, and feeding problems.

Chronic, functional abdominal pain occurs in about 15% of all children

Prebiotics are nondigestible substances that stimulate the growth of health-promoting bacteria in the colon, such as bifidobacteria.They are live microbial feed supplements which improves the intestinal microbial balance,Intestinal microflora development in infants is described as rapid and immense variations in diversity, abundance and composition of microorganisms.These disparities are influenced by environmental, therapeutic and cultural factors such as diet, mode of child birth, familial settings, diseases and medications. The microflora performs different biological functions in the gastrointestinal tract that include production and availability of nutrient, stimulation of bowel motility and maintenance of intestinal mucosa. An abnormal bacterial colonization of the infant's gut is, therefore, a major concomitant factor in feeding intolerance.

MATERIALS AND METHODS:

Bacillus coagulans (powder) were gift sample from SK Biobiz Limited (Jaulke Dindori, Nashik, India) and Pepsin enzyme (IP 1:3000) were gift samples from Advanced Enzyme Technology Limited (Sinnar, Nasik, India).Xanthum gum was procured from Modern Science,Nashik, Dill oil, Anise oil was procured from Katyani manufacturers. Hi Media Laboratories Pvt. Limited (Mumbai, India) supplied the soyabean caesin 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:

Mixing method by magnetic stirrer

The liquids and glassware used in the procedures had been thoroughly sterilized. The probiotic drop was prepared by using dill oil,anise oil, Xanthum gum, 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 water,oil and emulsifier, at different emulsifier concentrations and probe sonication times. A magnetic stirrer spinning for 20 minutes with a speed of 500 rpm was used for mixing them. Then, probiotic and pepsin was 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 to maintain aseptic condition.

Experimental design for optimization:

The effect of factors emulsifier concentration (0.01 – 0.1 gm) and magnetic stirrer speed (500- 1500 rpm) on the dependent variables such as MPS (R1), PDI (R2), ZP (R3), 2-factor face-centered 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 is determined using mathematical model. The significant terms ($P < 0.005$, analysis of variance- ANOVA), coefficient of variance, least significant lack of fit, the multiple correlation coefficient, and adjusted multiple correlation coefficient 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.

Both graphical and numerical method were used to find out the design space and optimization of emulsion base. The interaction effect between independent variables on the dependent variables was assessed by utilizing the response surface and 3D contour plots of the fitted polynomial regression equation. Furthermore, the predicted versus observed value curve was used for the quantitative comparison between theoretically predicted and experimentally obtained values of CQAs in order to revalidate the chosen face-centered CCD design. The prediction error is calculated from the following equation,

$$\text{Predicted error} = \frac{\text{Observed experimental value} - \text{Predicted value}}{\text{Predicted value}}$$

The design matrix was obtained from a face-centered CCD in order to optimize the effect of independent variables along with their working ranges and obtained values of dependent variables to make emulsion base.

The polynomial regression equation is used to examine the factor response connection for the response function Y_i used the generalized response surface model. In equation, the independent variable such as the concentration of emulsifier

and probe sonication time are denoted as X_1 andX_2 respectively. While the term a_0 indicates intercept (a constant), the term
a_1, a_{11}, a_{12} represents regression coefficients of the linear, quadratic, and interactive term respectively.

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

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 optimisation were done.

Preformulation Studies:

Pre-formulation studies are designed to ensure the development of a stable, safe and therapeutically effective dosage form. Pre-formulation testing is designed to assess the influence of physicochemical properties of drug and excipients on formulation properties of dosage form, method of manufacturer, pharmacokinetic and biopharmaceutical properties of the resulting product. A thorough understanding of physicochemical properties may ultimately confirm that no significant barriers are present for the formulation development.

Characterization of Bacterial spores of *B. coagulans*:

1. Organoleptic Characteristics:

The *Bacillus coagulans* powder was subjected for physical examination, such as Color, Odor, texture etc.

2. Total viable count determination :

1) Weigh accurately 1 gm of *Bacillus coagulans* powder in sterile container.
2) Add all the 1 gram in the 99 ml of the sterile saline and shake well. Then pour the prepared sterile soyabean casein digest agar media into the sterile petri plate.
3) Depending upon the estimated count of microorganism in sample 10 fold dilution made by using sterile saline solution, and those dilutions give number of colonies between 30 to 300 were used for plating. Then placed the petri plate in incubator at 40°C for 48 hrs.
4) Count and determine the total number of colonies of sample by multiplying with the dilution factor.

3. Identification of *Bacillus coagulans*:

Weigh accurately 1 gm of *Bacillus coagulans* powder and placed in the 99 ml of the sterile saline and shake well. Then pour the sterile soyabean casein digest agar media into the sterile petri plate. Then by using sterile nichrome wire loop strike the suspension of bacillus coagulans on the media containing petri plate and placed the petri plate in incubator at 40°C for 48 hrs.

4. Probiotic-Excipient compatibility studies:

The knowledge of the interaction of probiotic and excipients is essential in the initial formulation of a product. Probiotic excipient compatibility studies are important in the formulation development process, as the knowledge gained from excipient compatibility studies is used to select the dosage form components, to study stability profile of the probiotic and to identify degradation products.

Probiotic powder and other excipients were weighed accurately. The probiotic was mixed separately witheachexcipientasproportionmentionedinTableNo.8.1.

The probiotic excipient compatibility studywascarriedoutbytakingprobiotic-excipientin1:1%W/Wratioforsamples. The mixtures were then transferred to previously clean and dried vials, vials were sealed using rubber closure and aluminium crimp. The samples were kept at room temperature for a period of 7 days. The samples were then observed visually for change in color, odour and appearance, also the samples were analysed for total viable count at day 7. [6]

5. Assay of Pepsin activity:

Activity of Pepsin enzyme were performed by using the biuret protein assay. The Biuret method is based on the complexation of Cu^{2+} to functional groups in the protein's peptide bond. In the copper ion based protein assays, protein solutions are mixed with an alkaline solution of copper salt, (cupric ions, Cu^{2+}). The protein assay is based on the interaction of cupric ions with protein in an alkaline solution and is commonly referred to as the Biuret assay. The interaction of cupric ions (Cu^{2+}) with protein results in a purple color that can be read at 540 nm. The amount of color produced is proportional to protein concentration.

Glassware and Equipment's: Cuvettes, Spectrophotometer, Test tubes, Test tubes stand.

Chemicals and Reagents:

i. Biuret reagent: Dissolve1.5 gm of CuSO4 and 4.5 gm of Sodium Potassium Tartrate in 250 ml 0.2 N NaOH solution. Add 2.5 gm of KI and make up the volume to 500 ml with 0.2 N NaOH.

ii. 0.2 N NaOH: Dissolve 0.8 gm NaOH in 100 ml distilled water.

iii. Albumin stock (standard) solution (20 mg/ml): Dissolve 400 mg of Albumin in 20 ml 0.5 w/v % NaCl.

iv. Distilled water.

Procedure:

i. A stock solution of Albumin protein was prepared (20mg/ml) in volumetric flask.

ii. 150 ml of Biuret reagent was prepared.

iii. In the test tube, dilution of proteins of concentration 2, 4, 6 mg/ml was made with one unknown dilution by appropriate amount of Albumin stock and water as mentioned in the Table 8.11.

iv. 3 ml of Biuret reagent is added to all the test tube. Calibration were performed by taking absorbance in triplicate.

v. In another test tube, test sample

vi. Incubate all the test tubes for 15 minutes at room temperature.

vii. Then taken the absorbance at 540 nm.[7,8,9]

Then protein concentration calculate by using formula,

$$\mathbf{C_u = A_u - A_b / A_s - A_b \times C_s}$$

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

6. Total Viable Count:

1) Add the 1 ml of sample in the 99 ml of the sterile saline and shake well. Then pour the prepared sterile soyabean casein digest agar media into the sterile petri plate.
2) Depending upon the estimated count of microorganism in sample 10 fold dilution made by using sterile saline solution, and those dilutions give number of colonies between 30 to 300 were used for plating. Then placed the petri plate in incubator at 40°C for 48 hrs.
3) Count and determine the total number of colonies of sample by multiplying with the dilution factor.

7. Determination of viability of probiotic in simulated gastric fluid:

1) In this study viability of probiotic drop in simulated gastric fluid were determined by in-vitro technique. By using paddle type Dissolution apparatus this study is performed.
2) Preparation of simulated gastric fluid: Weigh accurately 0.2 gram of NaCl, 0.32 gram of pepsin, 0.7 ml of the hydrochloric acid and make up the volume upto 100 ml by using distilled water. And then adjust the pH at 1.2 by using 0.1 N HCl.
3) Then placed the 90 ml of simulated gastric fluid in the dissolution flask and add 10 ml formulation in it. Maintained the dissolution medium temperature at 37 ± 0.5 °C and set paddle revolution at 50 RPM for 3 hr.
4) After 3 hr remove the 1 ml of sample and placed it in the 99 ml of the sterile saline and shake well. Then pour the prepared sterile soyabean casein digest agar media into the sterile petri plate.
5) Depending upon the estimated count of microorganism in sample 10 fold dilution made by using sterile saline solution, and those dilutions give number of colonies between 30 to 300 were used for plating. Then placed the petri plate in incubator at 40°C for 48 hrs.
6) Count and determine the total number of colonies of sample by multiplying with the dilution factor.[10,11,12]

7) Stability Study:

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

RESULTS :

PREFORMULATION STUDY OF BACILLUS COAGULANS:

1. Organoleptic properties of *Bacillus coagulans*

Table No.1. Organoleptic properties of *Bacillus coagulans*

Identification test	Observed Result	Reported standard
Color	Light brown color	Light brown to brown color
Odor	Characteristic odor	Mild characteristic odor
Appearance	Amorphous	Amorphous

2. Total viable count determination:

Table No.2. Total viable count determination

Sample	Observed
Bacillus coagulans	21×10^9 CFU

Identification of *Bacillus coagulans*:

Identification of bacterial sample were done by strike plate method

Fig No.1. Strike plate method of *Bacillus coagulans*

3. Probiotic-Excipient Compatibility Study

Table No.3. Probiotic-excipient compatibility test

Sr.No	Sample	0 Day		7 Days	
		Color	Odor	Color	Odor
1	1	Light brown	Characteristics	Light brown	Characteristics
2	2	Whitish brown	Characteristics	Whitish brown	Characteristics

4. Total viable count Assay

Table No.4. Total viable count Assay

Sr. No.	Sample	0 Day	7 Day
1	1	21×10^9 CFU	21×10^9 CFU
2	2	44×10^9 CFU	44 10^9 CFU

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

Table No.5.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)	Magnetic stirrer speed (rpm)	MPS	PDI	ZP
1	1	Factorial	0.01	500	396	0.145	-15
3	2	Factorial	0.01	1500	354	0.286	-19
2	3	Factorial	0.1	500	256	0.215	-22
4	4	Factorial	0.1	1500	228	0.284	-28
6	5	Axial	0.055	292.8	226	0.231	-23
5	6	Axial	0.11864	1000	254	0.281	-29
7	7	Axial	0	1000	450	0.192	-19
8	8	Axial	0.055	1707.11	221	0.241	-22
12	9	Center	0.055	1000	362	0.286	-19
10	10	Center	0.055	1000	316	0.254	-23
13	11	Center	0.055	1000	271	0.218	-22
11	12	Center	0.055	1000	358	0.247	-28
9	13	Center	0.055	1000	246	0.187	-18

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

$PS=310.60-55.40A+9.87B-7.50AB+19.64.\ A^2\ -44.61\ B^2$

$PDI=0.2312+0.0245A+0.0283$

$Zeta\ Potential= -22.00-0.2500A+0.0303B+6.00AB$

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, response surface approach can be used to optimize a formulation and look at how many components interact to produce the desired results.

Table No.6. ANOVA for quadratic model generated from face-centered central composite design effects along with three different dependent variable to determine the best fitted model equation:

Response 1: MPS

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	43986.92	5	8797.38	4.62	<0.0349	significant
A-Emulsifier	24551.71	1	24551.71	12.90	<0.0088	
B-magnetic stirrer	778.72	1	778.72	0.4091	0.5428	
AB	225.00	1	225.00	0.1182	0.7411	
A^2	2682.65	1	2682.65	1.41	<0.2739	
B^2	13845.39	1	13845.39	7.27	0.0308	
Residual	13323.39	7	1903.34			
Lack of Fit	2664.19	3	888.06	0.3333	0.8033	not significant
Pure Error	10659.20	4	2664.80			
Cor Total	57310.31	12				

Response 2: PDI

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.0112	2	0.0056	5.82	0.0210	significant
A-Emulsifier	0.0048	1	0.0048	4.99	0.0495	
B-magnetic stirrer	0.0064	1	0.0064	6.65	0.0274	
Residual	0.0096	10	0.0010			
Lack of Fit	0.0068	6	0.0011	1.60	0.3379	not significant
Pure Error	0.0028	4	0.0007			
Cor Total	0.0208	12				

Response 3: ZP

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	144.51	3	48.17	5.90	0.0165	significant
A-Emulsifier	0.5000	1	0.5000	0.0612	0.8101	
B-magnetic stirrer	0.0074	1	0.0074	0.0009	0.9767	
AB	144.00	1	144.00	17.63	0.0023	
Residual	73.49	9	8.17			
Lack of Fit	10.69	5	2.14	0.1362	0.9747	not significant
Pure Error	62.80	4	15.70			
Cor Total	218.00	12				

6. Analysis of 3D response surface plot:

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

From Figure 9, the DS of emulsion decreases with the increase in the concentration of emulsifier (at 0.01 gm concentration the DS is around 396 nm, whereas at 0.1 gm its decreases to 256 nm) whereas magnetic stirrer speed does not produced any effect on droplet size

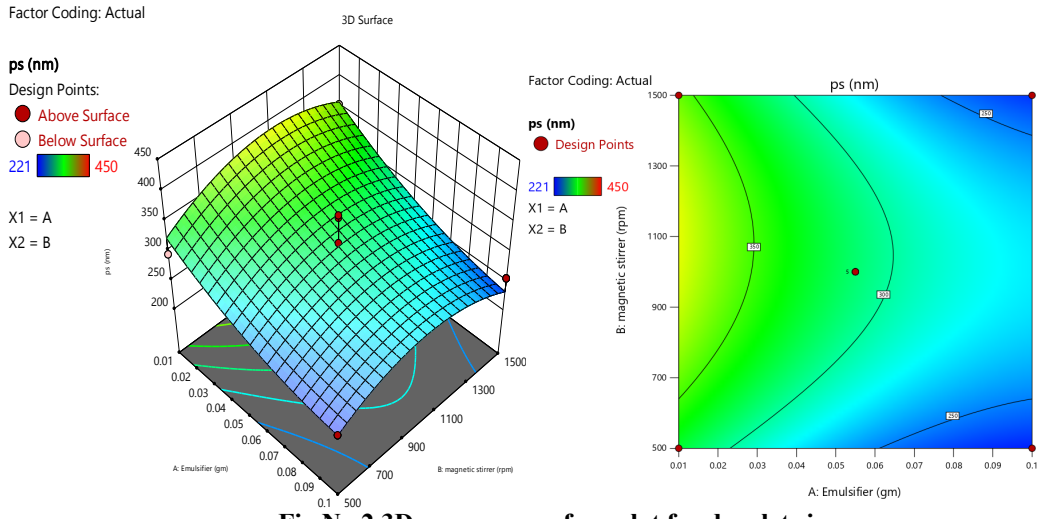

Fig No.2.3D response surface plot for droplet size

From Figure 10, the polydispersity index value lies between 0 (for a perfectly uniform sample with respect to the particle size) to 1 (for a highly polydisperse sample with multiple particle size population), wherein PDI < 1 represents a nearly homogeneous monodispersed formulation. The PDI of emulsion base increases with the increase magnetic stirrer speed (at 500 rpm of magnetic stirrer speed the PDI is around 0.145, whereas at 1500 rpm it increases to 0.286). Whereas the emulsifier not produces the any significant effect.

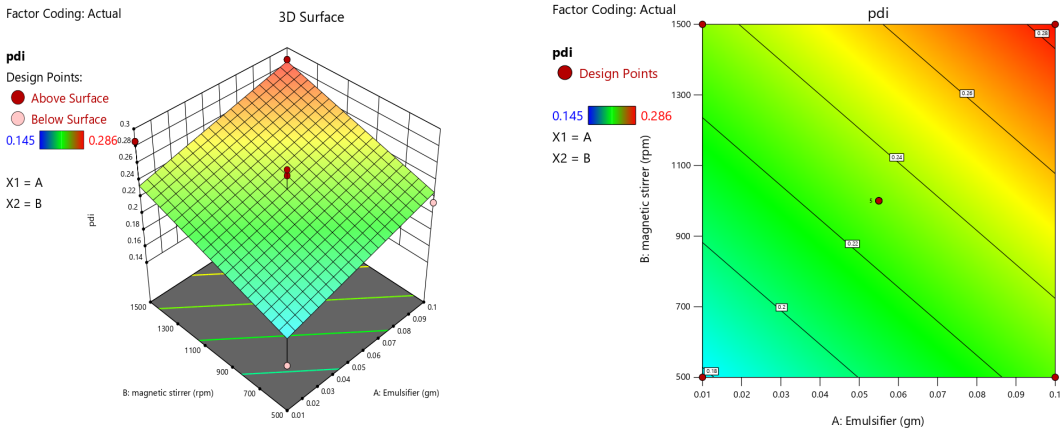

Fig.No.3. 3D response surface plot for polydispersity index

From Figure 11, the zeta potential of emulsion was increased with increment in the concentration of emulsifier (at 0.01 gm concentration the ZP is around -15 mV, whereas at 0.1 gm concentration, the ZP is around -19 mV). The possible explanation of this observation is the emulsifier is producing the surface charge which might influence the physical and chemical stability of emulsion and it is believed to ensure that the stability of the emulsion by electrostatic repulsion as per the DLVO theory

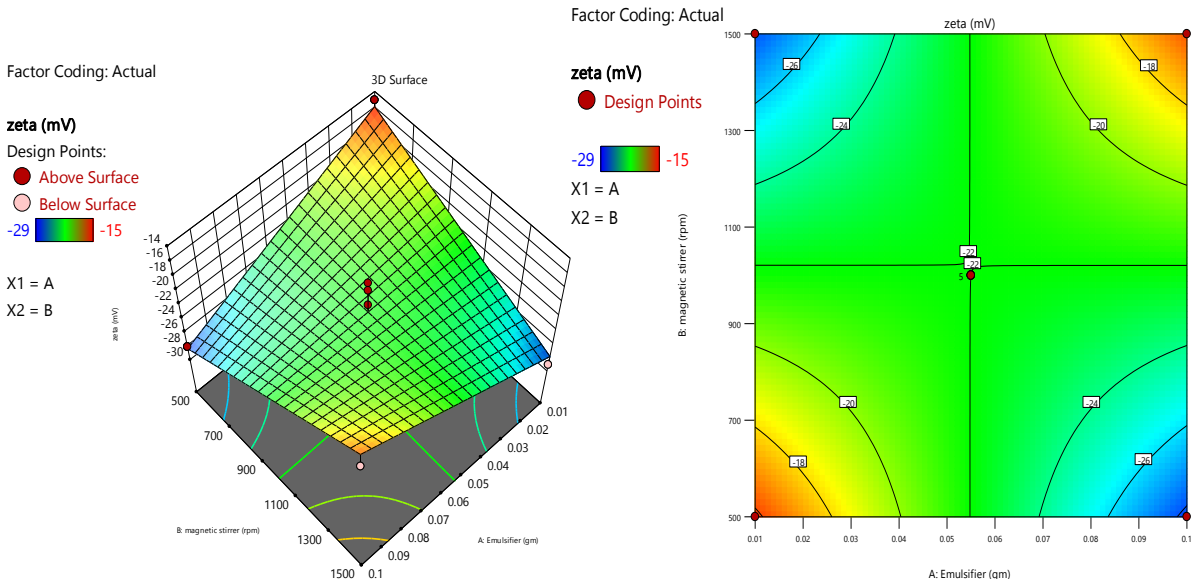

Figure.4. 3D response surface plot for zeta potential

Optimization of responses:

Design-Expert® software was used in order to determine the working region (design space) by graphical optimization as shown in overlay plot Figure 17. In the overlay plot, the working region is observed in yellow colour whereas the region which does not come under the working region is observed in grey colour. The design space in the overlay plot represents the desired amount of independent variables and the chosen dependent variables (MPS, PDI, ZP) values of emulsion base. The optimized formula was selected within the design space in which the MPS, ZP and PDI is minimum. The independent variables values given in the optimized formula for emulsifier is 0.1 gm, magnetic stirrer speed is 500 rpm,whereas, the response values given for the optimized formula within the design space are 227.86nm, 0.227, and -28 mV for MPS, PDI, ZP respectively. Table 29 displayed the prediction error values for CQAs calculated after preparing formulation using optimized formula

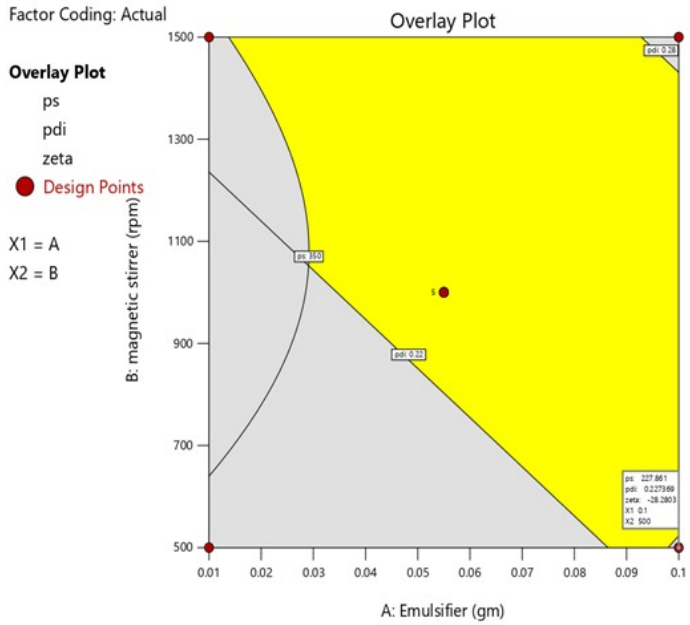

Fig.No.5.Overlay plot obtained from face-centered central composite design

Table No.7.Comparative data of predicted and observed experimental values of responses

Dependent variables	Predicted value	Observed experimental value*	Prediction error
Mean particle size (nm)	227.86	243.1	0.06
Polydispersity index	0.227	0.138	-0.86
Zeta potential (mV)	-28.28	-28.32	-2.00

7. Assay of Pepsin activity:

Calibration curve data for the albumin solution are shown in table no.6.

Table No.8. Assay of Pepsin activity

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

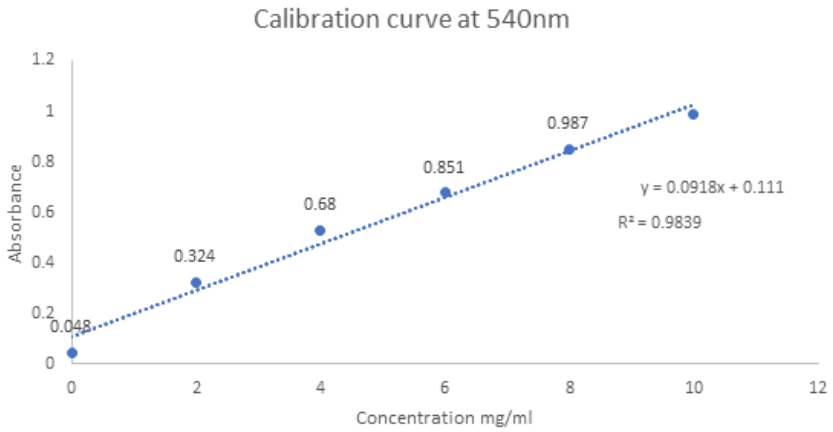

Fig.No.6.Calibration Curve of Pepsin Enzyme Activity

Table No.9. Protein concentration remained after the action of pepsin enzyme present in formulation in various dilutions

Sr. No	Conc (mg/ml)	Volume of formulation (ml)	Volume of protein (ml)	Volume of Biuret (ml)	Volume of Distilled water (ml)	Absorbance			Mean
1	2	2	1	3	4	0.200	0.205	0.203	0.203
2	4	2	2	3	3	0.303	0.305	0.308	0.305
3	6	2	3	3	2	0.420	0.420	0.419	0.420

Protein conc (mg/ml)	Avg Protein conc (mg/ml)	% Protein conc
1.12	2.24	56
2.13		53.25
3.49		58.16

From the obtained results we found that Pepsin enzyme present in the formulation shows proteolytic activity. Pepsin enzyme present in the formulation able to digest 50.5 % of the albumin protein present in the sample. And also found that the Bacterial spores present in the formulation not affect the pepsin action on the protein.

8. Total viable count of Formulation:

The probiotic drop were tested for total viable count of probiotic *Bacillus coagulans* using pour plate technique. This pour plate test was performed by making serial dilutions of probiotic drop containing Probiotics *Bacillus coagulans* by using saline solution.

Table No.10. Total viable count of Formulation

Sample	Observed
Formulation with pepsin enzyme	4.02×10^7 CFU
Formulation without pepsin enzyme	1.85×10^7 CFU

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

9. Determination of viability of probiotic in simulated gastric fluid:

Probiotic survival at low pH is very crucial for resisting initial stress in the stomach. Table no. 8.15 illustrates the survivability of *Bacillus coagulans* strain under simulated conditions of low pH.

Table No.11. Total Viable Count of Determination of viability of probiotic in simulated gastric fluid

Sample	Time under acidic condition (hr)	Observed
Bacillus coagulans probiotic	3	0.99×10^7 CFU

From the above data of the probiotic survival in the simulated gastric fluid or condition, probiotic shows 25 % of the survivability in the simulated gastric condition. Which means survived probiotic entered in the intestine region through the duodenum and able to form the colonies in the intestine and shows its action.

10. Stability Study:

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 optimized batch of probiotic drop is stable and reproducible.

Table No.12. Stability study of the optimized batch

Sr. No.	Parameter	Day 0	Day 30	Day 90
1.	pH	5.56	5.57	5.56
2.	Viscosity	0.952 cp	0.955 cp	0.9553
3.	Total viable count	3.8×10^7 CFU	4.0×10^7 CFU	4.02×10^7 CFU
4.	Physical appearance	No physical change	No physical change	No physical change

DISCUSSION

The oral probiotic drop of *Bacillus coagulans* for infants was prepared using the fennel oil, pepsin enzyme, lecithin and distilled water. Using design expert software, the central composite design optimized the formulation. The formulation was optimized by CCD design and carried forward to evaluate pepsin activity and viability in simulated gastric fluid. The factor emulsifier concentration and probe sonication time are independent variable consider in CCD design for optimization and their influence on the responses droplet size, polydispersity index and zeta potential was determined. The emulsifier was observed to have reduced the droplet size. The minimum polydispersity index produced through mixing method results in homogenizing the formulation by avoiding the aggregation of oil droplets that are scattered [13,14].

As emulsifier concentration and magnetic stirrer speed increased, the droplet size, reduced. Viscosity and pH were also tested with total viable count of probiotic bacteria since they play a critical role in determining the efficacy of the formulation, in addition to characterizing formulation based on their physical stability [22]. According to the findings, the ideal formulation of oral probiotic drop of *Bacillus coagulans* should be prepared using emulsifier (xanthum gum) is 0.1 gm and with 500 rpm and droplet size, polydispersity index and zeta potential were found to be 243.1 nm, 0.138 and -28.32 mV, respectively. The optimized formulation shows about 50 % of pepsin activity, viability of probiotic in simulated gastric condition of 0.99×10^7 CFU, total viable count of formulation containing pepsin enzyme is 4.02×10^7 CFU, and total viable count of formulation without pepsin enzyme is 1.85×10^7 CFU. Further, room temperature stability testing revealed a bacterial survivability of 4.0×10^7 CFU and 4.02×10^7 CFU after 1 and 3 months, respectively.

CONCLUSION

In this investigation, fennel oil and pepsin enzyme containing oral probiotic drop of *Bacillus coagulans* for infants were successfully designed and optimized using central composite design. The *Bacillus coagulans* spores were found to be compatible with the pepsin enzyme. The responses (independent variable) droplet size, polydispersity index and zeta potential were influenced by the emulsifier (xanthum gum) concentration and magnetic stirrer speed. According to the findings, the ideal formulation of oral probiotic drop of *Bacillus coagulans* should be prepared using emulsifier (xanthum gum) is 0.1 gm and with 500 rpm magnetic stirrer speed.. The optimized formulation were demonstrated for the viability of the oral probiotic drop containing *Bacillus coagulans* in simulated gastric condition, pepsin activity, total viable count and 3 month stability study was performed. The technique for the preparation of oral probiotic drop of *Bacillus coagulans* was found simple, easily controllable and economical.

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Abbreviations:
CFU: Colony forming unit, ZP: Zeta potential, PDI: Polydispersity index, DS: Droplet size, nm: Nanometer, LAB: Lactic acid bacteria, IP: Indian Pharmacopoeia

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