

Research

Synergistic fermentation of vitamin B2 (riboflavin) bio-enriched soy milk: optimization and techno-functional characterization of next generation functional vegan foods

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Abstract

Vitamin B2 (riboflavin) is essential for cellular growth, energy production, and redox potential. Certain lactic acid bacteria (LAB) can synthesize B2 in low levels in fermented products, however it is mostly retained inside the cell. This study aimed to develop B2-enriched soymilk by fermenting with B2-producing probiotic *Lactiplantibacillus plantarum* strains and traditional starter culture *Lactobacillus acidophilus* NCIM2902. Using the central composite design approach, processing parameters were optimized for enhanced B2 content and probiotic count. Six independent variables were assessed: temperature (A: 35–45 °C), pH (B: 4–6), time (C: 3–18 h), and inoculum size for strains *L. plantarum* MTCC 25432 (D: 1–2%), *L. plantarum* MTCC 25433 (E: 1–2%), and *L. acidophilus* NCIM 2902 (F: 1–2%). The second-order model effectively predicted responses, identifying optimal fermentation conditions for developing vitamin B2-enriched soymilk: temperature (A) 36 °C, pH (B) 5.5, fermentation time (C): 11 h, inoculum size for *L. plantarum* MTCC 25432 (D): 2%, MTCC 25433 (E): 2%, and *L. acidophilus* NCIM (F): 0.43%. These conditions resulted in a threefold increase in B2 concentration (481 µg/L) while maintaining a probiotic count of 9 logs CFU/mL. Additionally, techno-functional characterization, including rheology and texture profile analysis, showed that enhanced protease activity of co-cultured LAB improved protein hydrolysis (6259 nm), positively impacting the water holding capacity (WHC) and overall acceptability of the fermented soymilk. This optimized fermentation process represents a novel approach to developing nutritionally enhanced dairy-free soy products with high riboflavin content, utilizing the synergistic benefits of co-fermentation by two riboflavin-producing *L. plantarum* strains and traditional starter culture of *L. acidophilus*. This advancement is particularly significant for lactose-intolerant and vegan consumers who may lack sufficient dietary sources of Vitamin B2.

Keywords Riboflavin · Lactic-acid bacteria · Probiotics · Optimization · Response surface methodology

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1 Introduction

B-group vitamins are vital for maintaining the body's homeostasis, playing key roles in metabolic processes such as energy production and red blood cell formation. Among these, Vitamin B2, or riboflavin, is particularly crucial. It serves as a precursor to the essential coenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), which are integral to the body's core metabolic functions [1]. Additionally, riboflavin plays a role in the metabolism of folate, vitamin B12, vitamin B6, and other vitamins. For this reason, in some genotypes linked to cardiovascular diseases (CVD), pregnancy complications, and cognitive impairment, plasma homocysteine is determined by plasma riboflavin [2]. Furthermore, it supports the safeguarding of the nervous system, skin, eyes, and mucous membranes. Humans are susceptible to developing riboflavin deficiency during times of dietary restriction or physiological and pathological stresses [3]. Consuming riboflavin may help mitigate several health issues, including growth retardation, anemia, skin lesions, renal damage, and degenerative changes in the nervous system.

This water-soluble vitamin boasts a unique physiological function, making it indispensable in various fields, including medicine, feed and food additives, and other industries. In the realm of industrial riboflavin (B2) production, biotechnological processes have gradually superseded chemical synthesis due to a myriad of advantages [4]. By harnessing the power of microorganisms, the synthesis of B2 has become more efficient, sustainable, and environmentally friendly, thereby aligning with contemporary principles of green manufacturing of functional foods [5]. Recent studies have led to a resurgence of interest in B2, particularly its role in cellular biochemistry. There has been a notable expansion in our understanding of the mechanisms and regulation of intestinal absorption of B2 and its implications for health [6].

Many dairy-based fermented foods are known for their probiotic potential, largely due to their connection with lactic acid fermentation [7]. However, these products are unsuitable for individuals with lactose intolerance [8]. As a favorable alternative, soymilk has been identified as an effective medium for developing probiotic-rich functional foods [9]. Soy-based products are recognized not only for their capacity to carry probiotics but also for their health-promoting benefits, including lowering lipid levels, preventing atherosclerosis, and providing antioxidant and anti-allergenic properties [10]. The fermentation-induced acidification is a key factor in food processing, as it inhibits the growth of undesirable microorganisms while enhancing the desirable sensory qualities of the final product, such as aroma, flavor, and texture [11]. This property makes soymilk suitable for supporting probiotic growth, promoting acidification, and ensuring probiotic survival [9]. The high protein content of soy-based products has been shown to enhance the growth of probiotic strains such as *Lactobacillus acidophilus*, *Streptococcus thermophilus*, and *Lactobacillus casei*. Notably, in 2003, Shimakama first reported probiotic-fermented soy beverages with both good sensory acceptance and significant health benefits [12].

Soybean (*Glycine max*) remains a leading non-dairy option, particularly in Asia, due to its high content of proteins, phytosterols, phenolic acids, and polyunsaturated fatty acids, which are ideal for those who are lactose intolerant [9]. The health benefits of soy foods are largely attributed to soy proteins and isoflavones, which are phytochemicals offering various health advantages [13] depending on their bioavailability in the host organism based on molecular structures [14]. A suitable method for enhancing palatability, fortifying, and optimising physicochemical and sensory properties of soymilk-based foods is by in vitro fermentation processes. The fermented soymilk with probiotic strains is a food product that has gained popularity as a viable way to develop a nutrient-rich probiotic product of non-dairy origin [15]. The protein gel formation in soymilk is a crucial step in developing non-dairy fermented products. However, there has been limited research on the rheological properties and textural profiles of these fermented soy products [9]. Microorganisms like *Candida flaveri*, lactic acid bacteria (LAB), *Bacillus subtilis*, *Eremothecium ashbyii*, and *Ashbya gossypii* are known to produce substantial quantities of riboflavin/B2, though most of it is retained inside the cell [16]. The advanced methodologies in bioprocessing offer the potential to optimize and enhance the production of the vitamins, making *B. subtilis* a promising candidate for efficient B2 production using biotechnological and metabolic engineering approaches [5]. Recent advancements have been made in the in situ bacterial overproduction of B vitamins, such as vitamin B2. Nowadays, fermentation-based methods are increasingly replacing chemical synthesis of B2 due to economic and environmental considerations [17].

A study by Narayan et al. [9] investigated the development of riboflavin-enriched fermented soy curds using *Lactobacillus plantarum* strains MTCC 25432 and MTCC 25433, both individually and in combination. This approach focused

on the technological and functional properties of the products, showing that the use of *L. plantarum* accelerated acidification, allowing the product to reach a pH of 4.7 more quickly. The co-cultured strains significantly increased the hardness and cohesiveness of the fermented soymilk, with higher values for G' (6.25×10^2 Pa), G'' (2.30×10^3 Pa), and G^* (8.00×10^2 Pa), indicating a firmer gel. Additionally, the riboflavin content was notably higher in the combination product (342.11 $\mu\text{g/L}$) compared to individual strains, and the final product achieved a probiotic count exceeding 9 log CFU/mL, meeting probiotic functional food criteria [9].

To further develop a B2-enriched probiotic soymilk beverage with enhanced B2 titer, co-fermentation with a cocktail of cultures *L. plantarum* MTCC 25433, *L. plantarum* MTCC 25432, and *Lactobacillus acidophilus* NCIM 2902, along with a bio-process optimization approach, has been employed in this study. To enhance the productivity and efficiency of the riboflavin fermentation process, it is crucial not only to utilize a productive strain but also to carefully design appropriate fermentation parameters [18]. The medium composition and physical parameters of the fermentation process play a significant role in determining the final product's concentration, yield, and volumetric production, as well as the overall cost and ease of product separation [19]. Therefore, thoughtful consideration and optimization of the fermentation parameters are essential to achieve optimal results in riboflavin production [18].

Traditional methodologies such as the “one-factor-at-a-time” approach have been used to optimize riboflavin production by altering one variable while keeping others constant. However, this method overlooks the overall interactions between physicochemical factors and is time-consuming [20]. In order to address the constraints of single-factor optimization, statistical experimental designs such as Plackett–Burman and response surface methodology (RSM) are utilized to simultaneously optimize all factors that have a significant effect [18]. RSM uses factorial design and regression analysis to identify critical factors, construct models to investigate their interactions and choose the best settings for these variables to obtain the intended output [18]. Despite the notable achievement of a 1.77 g/L increase in riboflavin production by a UV-mutant of *E. ashbyii* reported by Venugopal and Chandra in 2000, a comprehensive examination using statistical methods to optimize riboflavin synthesis by bacterial strains has not been conducted yet [21].

Further, there is currently a dearth of knowledge regarding how the LAB affects the texture of soy yogurt, particularly in terms of hardness and water holding capacity (WHC).

The majority of research focussed on the effect of EPS production by LAB on the texture of fermented soymilk gel [19]. Pang et al. [22] reported that LAB with high EPS production [23] enhance the lubricating properties and texture of soymilk gel making it a better product choice. However, low EPS production in the soymilk gel fermented by LAB may also result in low hardness and high WHC. This could be because of LAB's varying capacities to hydrolyse soybean proteins [15]. However, not much research has been done on how proteolysis affects the texture of fermented soymilk gel. This study examines the connection between LAB's capacity for protein hydrolysis and the hardness and WHC of fermented soymilk gel [9]. Furthermore, a novel comparison was made between the control variables, hardness, WHC, and other texture parameters of soymilk gel fermented by different LAB at the same pH [24].

The present study evaluates the impact of various physical parameters and culture combinations of lactic acid bacteria (LAB) including time, temperature, pH, and different inoculum concentrations of *L. plantarum* MTCC 25432, *L. plantarum* MTCC 25433, and *Lactobacillus acidophilus* NCIM 2902 on riboflavin (B2) production during fermentation. Using Response Surface Methodology (RSM) with a Central Composite Design (CCD) model, we aim to determine the optimal conditions for maximizing riboflavin production and probiotic count. The response variables examined include riboflavin content and viable probiotic count, while the optimized product is characterized by its texture profile, water holding capacity (WHC), and sensory evaluation. Variations in gel hardness and WHC of the fermented soymilk were analysed, and the zeta potential, intermolecular forces, and particle size of the soymilk gel were assessed to understand the physical and chemical properties of soymilk gel. The results provide technical parameters and a theoretical foundation for enhancing riboflavin content in fermented soymilk, ensuring reliable production. This study additionally presents a novel approach by employing a synergistic combination of lactic acid bacteria strains, specifically two riboflavin-producing *Lactobacillus plantarum* strains alongside the traditional starter culture *Lactobacillus acidophilus* for developing fermented soymilk. This innovative formulation aims to enhance the riboflavin content while simultaneously improving the probiotic profile of soy beverages. By leveraging the metabolic capabilities of these strains, we anticipate a significant increase in both nutritional and functional properties, offering potential health benefits that surpass those of conventional fermentation methods.

2 Materials and methods

2.1 Microbial strains

The probiotic strain utilised in this investigation, *Lactiplantibacillus plantarum* MTCC 25432 and 25,433, were isolated from human feces sample at National Institute of Food Technology Entrepreneurship and Management-Kundli (NIFTEM-K), India and kept under safe deposit regulations at the Microbial Type Culture Collection (MTCC), Chandigarh, India. The starter culture *Lactobacillus acidophilus* NCIM 2902 was purchased from National Collection of Industrial Microorganisms, Pune, India.

Bhushan et al. [25] conducted an evaluation of the probiotic properties of *Lactiplantibacillus plantarum* strains MTCC 25432 and MTCC 25433 through in vitro assessments and multivariate principal component analysis (PCA) to identify the most promising strain. The study also examined the safety, antioxidant activity, and exopolysaccharide production of the strains. The results indicated that *L. plantarum* MTCC 25432 and MTCC 25433 exhibited the strong probiotic potential. Both MTCC 25432 and MTCC 25433 demonstrated tolerance to simulated gastrointestinal conditions, and their overnight cell-free supernatants (CFSS, pH 4.0–4.3) inhibited the growth of several pathogens, including *Escherichia coli* AF10, *Salmonella* Typhi MTCC98, *Bacillus cereus* NCDC250, and *Pseudomonas aeruginosa* NCDC105. Neither strain degraded mucin, and both adhered to human colorectal adenocarcinoma Caco-2 cells (22–25%) and showed aggregation with indicator bacteria (30–50%). Additionally, the strains were non-haemolytic and susceptible to most antibiotics tested. The PCA confirmed these in vitro findings, supporting the selection of *L. plantarum* MTCC 25432 and MTCC 25433 as promising probiotic candidates [25].

2.2 Chemical and reagents

The chemicals required for are purchased from Hi-Media, India. The MRS (deMan, Rogosa and Sharpe) broth and agar were purchased from Hi-Media, India. The reagents for HPLC were purchased from Sigma-Aldrich, India.

2.3 Procurement of soybean

Soybean (*Glycine max*) seeds were collected from Azadpur Mandi, New Delhi, India (28° 43' 0" North, 77° 11' 0" East) between November and December 2023 accordance with the national and local guidelines. The soybean was harvested from agriculture farm Indore, India (22° 4'37"N 75° 52'7"E) in November 2023. The size of the soybeans ranged between 5 and 11 mm in diameter and 120–180 mg of weight of each soybean seed with spherical shape and yellow color.

3 Experimental methods

3.1 Bacterial strains and growth conditions

The starter culture *L. acidophilus* NCIM 2902, as well as indigenous vitamin B₂-producing strains *L. plantarum* MTCC 25432 (BBC32B) and *L. plantarum* MTCC 25433 (BBC33), were isolated using the method described by Bhushan et al. [25] in the microbiology laboratory, National Institute of Food Technology Entrepreneurship and Management-Kundli (NIFTEM-K), India. All strains were sub-cultured three times in MRS broth (Hi-Media laboratories, India) to activate them biologically and incubated at 37 °C for 24 h. Afterward, the soymilk was fermented using these activated strains.

3.2 Soymilk preparation and fermentation conditions

The procured soybeans (*Glycine max*) were used to preparation according to the procedure of described by Božanić et al. [26] with some modifications. Soybean grains were soaked in distilled water in a 1:3 (w/v) ratio for 12 h at room temperature (28 °C). Subsequently, the water was drained from the soybeans, and the testa, or outer covering of the rehydrated soybeans, was manually peeled off. The peeled soybeans were then placed in a food grade mixer grinder (Sujata Home Appliance Private Limited, Mumbai-India) and ground for 10 min with 400 mL distilled water. Subsequently, the slurry

Table 1 Levels of independent variables in coded form as per the experimental design

Variables	Temperature (°C) (A)	pH (B)	Time (hours) (C)	Inoculation concentration of <i>L. plantarum</i> (%) (D)	Inoculation concentration of <i>L. plantarum</i> (%) (E)	Inoculation concentration of <i>L. acidophilus</i> (%) (F)
	Coded levels	Actual levels				
−2	26.60	3.15	0.044	0.43	0.43	0.43
−1	30	4	4	1	1	1
0	36	5.5	11	2	2	2
+1	42	7	18	3	3	3
+2	45.39	7.84	21.93	3.56	3.56	3.56

underwent filtration using a double-layered muslin cloth at room temperature (28 °C). After this, the soymilk's final volume was adjusted to 1000 mL by incorporating distilled water. The obtained soymilk was autoclaved for 15 min, at 15 psi, and 121 °C for sterilization. The fermentation of the soymilk was done using overnight grown cultures *L. plantarum* (MTCC 25432), *L. plantarum* (MTCC 25433) with starter culture *L. acidophilus* (NCIM 2902) at different inoculum concentrations as per optimization design (1×10^8 to 2.1×10^9 CFU/mL) inoculated in 100 mL autoclaved soymilk. The fermentation and incubation conditions were selected according to the output based on the CCD by RSM (Table 1).

The selection of the level range for each variable (temperature, pH, time, and inoculum concentration) in the fermentation process is typically based on optimizing conditions that favor the growth and activity of probiotics, as well as the production of desired metabolites like riboflavin.

3.3 Experimental design

3.3.1 Central composite design (CCD)

RSM was used to assess the impact of six independent factors, including temperature (A: 35–45 °C), pH (B: 4–6), time (C: 3–18 h), inoculation concentrations for strains *L. plantarum* MTCC 25432 (D: 1–2%), *L. plantarum* MTCC 25433 (E: 1–2%), and *L. acidophilus* NCIM 2902 (F: 1–2%) on two response variables namely, probiotic count (R1) and riboflavin content (R2). Based on the design matrix total 52 runs were conducted (Table 2). Each variable was examined at five different coded levels (−2, −1, 0, 1, and 2), with zero as the central coded value for all variables. A 2^{5-1} fractional factorial design with six central points, ten axial points, and one variable set to an extreme level (± 2) made up the design matrix for the CCD, while other variables were set to their central point values.

Experiments were randomized to reduce the effects of unexplained variability in the actual responses caused by outside influences. Eight repeats of the centre point were used to calculate the method's repeatability.

3.4 Estimation of response parameters

3.4.1 Evaluation of total probiotic count

The viability of cells in fermented soymilk was assessed using a standard procedure [27], which included serial dilution and plating of colony-forming units (CFUs). To assess the efficiency of probiotics in fermented soymilk, a sample was diluted tenfold using a 0.85% saline solution. Subsequently, 1 mL of samples from various dilutions was distributed onto melted MRS agar plates using the pour-plating technique. The plates were placed in a static incubator and incubated at 37 °C for 24 h. The number of colonies was determined and expressed as the logarithm of colony-forming units per millilitre (log CFU/mL).

3.4.2 Extraction and quantification of riboflavin

The riboflavin content of the fermented soymilk was extracted according the methodology followed by Del Valle et al. [28] with minor modifications. About, 10 mL of fermented soymilk was immersed with an equivalent of 1% acetic acid. The mixture was then subjected to autoclaving (Labtech-LAC 5060S) at a temperature of 121 °C for 15 min. Subsequently,

Table 2 Central composite design with experimental results for fermented probiotic soymilk using response surface methodology

Runs	Independent variables						Responses	
	A	B	C	D	E	F	Total probiotic count (log CFU/mL)	Riboflavin concentration (µg/L)
1	30	7	4	1	3	1	2.7	129
2	36	5.5	11	2	2	2	8.1	394
3	30	4	18	3	3	3	3.2	87
4	36	3.15	11	2	2	2	4.7	155
5	36	5.5	21.95	2	2	2	4	179
6	42	7	4	3	3	1	4	231
7	42	4	4	3	3	3	7	299
8	30	4	18	1	1	3	7	340
9	30	7	18	3	1	3	4	108
10	30	4	4	1	3	3	5	286
11	42	4	18	3	3	1	4	127
12	36	5.5	11	2	2	2	9	401
13	36	5.5	11	0.43	2	2	9	455.83
14	36	5.5	11	2	2	0.43	9	481
15	42	7	18	3	1	1	7	384
16	36	5.5	11	2	0.43	2	9	460
17	36	5.5	11	2	3.57	2	9	419
18	30	7	18	1	3	3	4	158
19	45.39	5.5	11	2	2	2	2	78
20	26.61	5.5	11	2	2	2	2	91
21	42	7	4	1	1	1	3	109
22	42	4	4	1	1	3	4	143
23	42	7	18	3	3	3	3	159
24	36	5.5	11	2	2	2	8.5	443
25	30	4	18	1	3	1	7	369
26	36	5.5	0.04	2	2	2	2	55
27	42	4	4	1	3	1	4	137
28	36	5.5	11	2	2	2	8.5	449
29	36	7.85	11	2	2	2	4.5	117
30	42	4	4	3	1	1	4	128
31	36	5.5	11	2	2	2	9	457
32	42	7	4	1	3	3	5	231
33	30	4	4	3	1	3	5	234
34	42	7	18	1	3	1	7	352
35	30	4	4	3	3	1	5	214
36	30	7	18	3	3	1	4	148
37	30	4	18	3	1	1	7	371
38	42	7	4	3	1	3	5	261
39	30	7	4	3	3	3	7	319
40	30	7	4	1	1	3	4	183
41	36	5.5	11	2	2	2	9.5	478
42	42	4	18	1	3	3	4	147
43	42	4	18	1	1	1	8	389
44	42	4	18	3	1	3	4	129
45	42	7	18	1	1	3	7	382
46	30	4	4	1	1	1	2	94
47	36	5.5	11	2	2	2	9	463
48	30	7	18	1	1	1	8	401

Table 2 (continued)

Runs	Independent variables						Responses	
	A	B	C	D	E	F	Total probiotic count (log CFU/mL)	Riboflavin concentration (µg/L)
49	36	5.5	11	3.57	2	2	9.6	407
50	36	5.5	11	2	2	2	8	394
51	36	5.5	11	2	2	3.57	9.6	480
52	30	7	4	3	1	1	3.27	154

where; A: Temperature (°C); B: pH; C: Time (h); D: Inoculum concentration of *L. plantarum* MTCC 25432 (%); Inoculum concentration of *L. plantarum* MTCC 25433 (%); Inoculum concentration of *L. acidophilus* NCIM 2902 (%)

the mixture was centrifuged (Sigma 2-6E Centrifuge) at 10,000 × g for 10 min. The supernatant was filtered using 0.22 µm filters (Merck, Germany) and stored under freezing temperature (− 20 °C) for further analysis.

3.4.3 HPLC analysis of riboflavin

To quantify riboflavin, the supernatant from fermented soymilk was analyzed chromatographically using high-performance liquid chromatography (HPLC) (Model 2707, Waters India Pvt. Ltd., Kolkata, India) with a reverse-phase C18 column (Kromasil, SIGMA, St. Louis, MO, USA, 5µ 100A, 250 × 4.6 mm), and a fluorescence detector operating at 440 nm for excitation and 520 nm for emission. As a control, pure riboflavin was used. A freshly prepared mobile phase of methanol/water (35:65v/v) was used to analyze the sample by isocratic elution at a flow rate of 1 mL min^{−1}.

3.5 Data analysis and optimization of parameters

The analysis of variance (ANOVA) and model generation, including assessments of lack-of-fit, coefficient of variation, coefficient of determination, and 3-D plotting for statistical analysis, were conducted using Design Expert version 13 software (State-Ease Inc., Minneapolis, MN, USA). The role of each variable, their interactions, and statistical analysis to determine anticipated yields can be derived by applying the quadratic Eq. (1):

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \quad (1)$$

In this equation, Y denotes the expected response. The term β_0 signifies the offset term, while β_i indicates the linear effect. The squared effect is represented by β_{ii} , and the interaction effect is denoted by β_{ij} . Additionally, X_i corresponds to the dimensionless coded value of x_i .

Based on the desirability function, optimization was carried out by applying certain constraints to the factors and responses. The software predicted values for the responses, which were statistically compared to the experimental value of the developed optimized fermented probiotic soymilk using a two-tailed, one-sample *t*-test using the difference between specific pairs of means was analysed using independent sample *t*-test at a 95% confidence level ($p < 0.05$) using IBM SPSS software version 22 (IBM Cooperation, New York, USA).

4 Techno-functional characterization of fermented soymilk

4.1 Rheological characterization

Rheological measurements of the fermented soymilk were conducted to assess its viscoelastic and gelation properties, following a modified standard method by Donkor et al. [29]. The rheological properties of fermented soy milk samples were analyzed utilizing an Anton Paar Rheometer (MCR 302, Graz, Austria) configured with a parallel plate setup, ensuring a 2.5 mm gap at a temperature of 20 °C. A small portion of the sample was positioned at the centre of the inset plate. A frequency of 1 Hz was first applied to establish the viscoelastic range (0.01–100 Pa). This was succeeded by a frequency sweep test conducted at 0.1–10 Hz with a maximum strain of 0.06. The rheological measurement results were processed

using the instrument software, which calculated the dynamic moduli (G' , G'' , G^*), and viscosity (η) from the frequency sweep test.

4.2 Texture profile analysis

The texture of fermented soymilk samples was evaluated using a TA-XT2i texture analyzer (Anton Paar Particle size Analyser), fitted with a 25 kg load cell at 22 °C. The resulting profile curves were used to determine the hardness, cohesiveness, gumminess, adhesiveness, and springiness of the fermented soymilk samples [30].

4.3 Water holding capacity (WHC)

WHC was determined as per protocols reported by Zeng et al. [31] with minor modifications. The samples of fermented soymilk were centrifuged at 2900 rpm for 10 min, followed by calculation using the formula (2):

$$WHC\% = \frac{W_1}{W} \times 100 \quad (2)$$

where, W_1 = weight of the sample after centrifugation in which water has been removed (g); W = weight of the sample before centrifugation (g).

4.4 Particle size and zeta potential analysis of fermented soymilk

Using a laser particle size analyzer, the zeta potential and particle size of the fermented soymilk were determined by Particle size analyzer (Anton Paar by Diamitron Technologies Private Limited). Zeta potential measurement was taken at pH 4.7 (equal to fermented soymilk pH). Before the operation, the fermented soymilk was diluted with demineralized water, and the pH was set to 4.7 using lactic acid [32]. Unfermented soymilk was used as a control. For accurate particle size analysis, samples often need to be diluted to ensure that the concentration of particles is appropriate for the measurement equipment. The ideal dilution factor depends on the sample's original concentration, in this study a dilution of 1:10 was used. For zeta potential, dilution is often required to maintain a low ionic strength, preventing particle agglomeration or flocculation. In present study the dilutions of 1:100 was used. Both particle size and zeta potential are measured over multiple runs to improve accuracy. The experiment was conducted 3–5 runs for each measurement. The unfermented soymilk was used as a control. Zeta potential values greater than +30 mV or less than -30 mV indicate strong electrostatic repulsion, leading to high stability.

4.5 Sensory evaluation

The sensory assessment of the fermented probiotic soymilk was conducted on a 9-point hedonic scale by a panel of semi-trained evaluators consisting of 30 participants aged 22 to 45. The scale is understood in the following manner: 9 denotes 'extremely like,' 8 indicates 'very much like,' 7 represents 'moderately like,' 6 signifies 'slightly like,' 5 stands for 'neutral,' 4 conveys 'slightly dislike,' 3 implies 'moderately dislike,' 2 expresses 'very much dislike,' and 1 reflects 'extremely dislike.' The average scores were derived from the aggregated responses to explain the results. Protocol number. 12/7 L/NECHR/23 granted approval for the sensory evaluation research of the developed fortified soymilk by the "NIFTEM Ethical Committee for Human Research" of the National Institute of Food Technology Entrepreneurship and Management in Kundli, Sonapat (Haryana), India. All individuals involved in the study have given their verbal or written permission to take part.

5 Statistical analysis

The average value and standard deviation (SD) of the triplicate data (Mean $\pm$ SD) was reported as results. The differences between the groups were analysed by one-way analysis of variance (ANOVA) considering a 5% of significance level, and an independent sample *t*-test at 95% confidence level ($p < 0.05$) was conducted using IBM SPSS software version 22 (IBM Cooperation, New York, USA).

6 Results and discussion

Fermentation is a crucial step in the creation of many bio-based products, including food ingredients, fuels, fine chemicals, agrichemicals, personal care items, and pharmaceuticals. In order to maximise titre, productivity, yield, and qualities that are vital to the process, fermentation optimisation entails intensifying microbial cell factories and strains, culture media, bioreactor design, and operating parameter conditions. Finding the ideal values for process variables, such as temperature, pH, dissolved oxygen, nutrient composition, critical substrate delivery, and mixing characteristics, in order to maximise desired fermentation and commercial performance is known as fermentation optimisation. There are several ways to carry out fermentation optimisation, including design of experiment (DOE) and classical experimental design. There are numerous goals unique to each product in fermentation optimisation. For example, increasing the ratio of product yield to substrate consumption, or fermentation efficiency, can shorten the process's duration. Optimizing fermentation can enhance product quality by determining the ideal parameter ranges that facilitate the production of a fully functional and highly active product. Enhancing the fermentation process contributes to increased robustness and consistency. The influence of fermentation parameters on riboflavin content and probiotic count was evaluated utilizing Central Composite Design (CCD) through Response Surface Methodology (RSM). The response surface modeling incorporated six independent parameters: temperature, pH, time, and concentration, across three strains: *L. plantarum* MTCC 25432, *L. plantarum* MTCC 25433, and *L. acidophilus* NCIM 2902. Riboflavin and probiotic count served as response variables, resulting in a total of 52 experimental runs, as detailed in Table 2.

Table 3 Regression coefficients of different responses and analysis of variance for fermented probiotic soymilk

Model	Total probiotic count (log CFU/mL)	Riboflavin concentration (µg/L)
A	0.05	− 0.20
B	− 0.07	4.21
C	0.58**	29.62**
D	− 0.12	− 15.54
E	− 1.17	− 13.04
F	− 0.02	− 7.39
AB	0.19	31.41**
CD	− 0.82**	− 48.53**
CE	− 0.79**	− 46.78**
CF	− 0.93**	− 55.97**
A ²	− 2.27**	− 110.16**
B ²	− 1.20**	− 89.13**
C ²	− 1.86**	− 96.89**
D ²	0.71**	31.47*
E ²	0.59**	34.77*
F ²	0.71**	51.51*
ANOVA		
Type	Quadratic	Quadratic
F-value	18.03	13.15
p-value	< 0.0001	< 0.0001
Lack of fit	Ns	Ns
C.V. %	13.22	18.92
R ²	0.95	0.94

The *p*-values indicated the statistical significance of the regression coefficients

A: temperature (°C), B: pH, C: time (hours), D: inoculation concentration of *L. plantarum* MTCC 25432 (%), E: inoculation concentration of *L. plantarum* MTCC 25433 (%), F: inoculation concentration of *L. acidophilus* NCIM 2902 (%); A, B, C, D, E and F are linear, A², B², C², D², E², F² are quadratic and AB, CD, CE, and CF are the interactive coefficient of the responses; *significant at 5% level (*p* < 0.05); **significant at 1% level (*p* < 0.01); ns non-significant

6.1 The influence of process parameters on the probiotic count in fermented soymilk

As Table 2 shows that probiotic count in fermented soymilk varied from 2 log CFU/mL to 9 log CFU/mL. The maximum probiotic count was achieved when the soymilk was fermented at a temperature of 36 °C, pH 5.5, incubation time of 11 h with 2% inoculum concentration of *L. plantarum* MTCC 25432, 2% *L. plantarum* MTCC 25433, and 0.43% *L. acidophilus* NCIM 2902. The influence of independent parameters on the responses is presented in Table 3. According to the regression model, the model for total probiotic count was quadratic and significant. The linear effect of fermentation time, the quadratic effect of fermentation parameters i.e., temperature, pH, time, and inoculum concentration of different LAB strains and the interactive effect of time and different combinations of LAB strains had significant effects on total probiotic count ($p < 0.05$).

The interaction of the six independent parameters on the probiotic count of fermented soymilk was determined through 3D surface plots to better understanding the interaction of physical factors and to identify the ideal ranges of concentration for the variables needed to get the highest probiotic count and riboflavin content. The response surface and 3D plots for changes in probiotic counts and riboflavin content and as a function of three factors are shown in Fig. 1a–f, while the levels of other three factors remain constant. As indicated in the response surface models, the probiotic count of fermented soymilk was substantially affected by the incubation time and temperature (Fig. 1a–c). Probiotic count decreased with increase (36 °C–45.5 °C) or decrease (26.5 °C) in temperature at a constant incubation time and pH. The lowest value of probiotic count was observed at 26.5 °C is 2.16 log CFU/mL. Previous studies reported that fermentation by different probiotic strains synergistically increased the riboflavin content and resulted in higher probiotic count at a temperature of 36 °C. The temperature at which fermentation occurs is a crucial

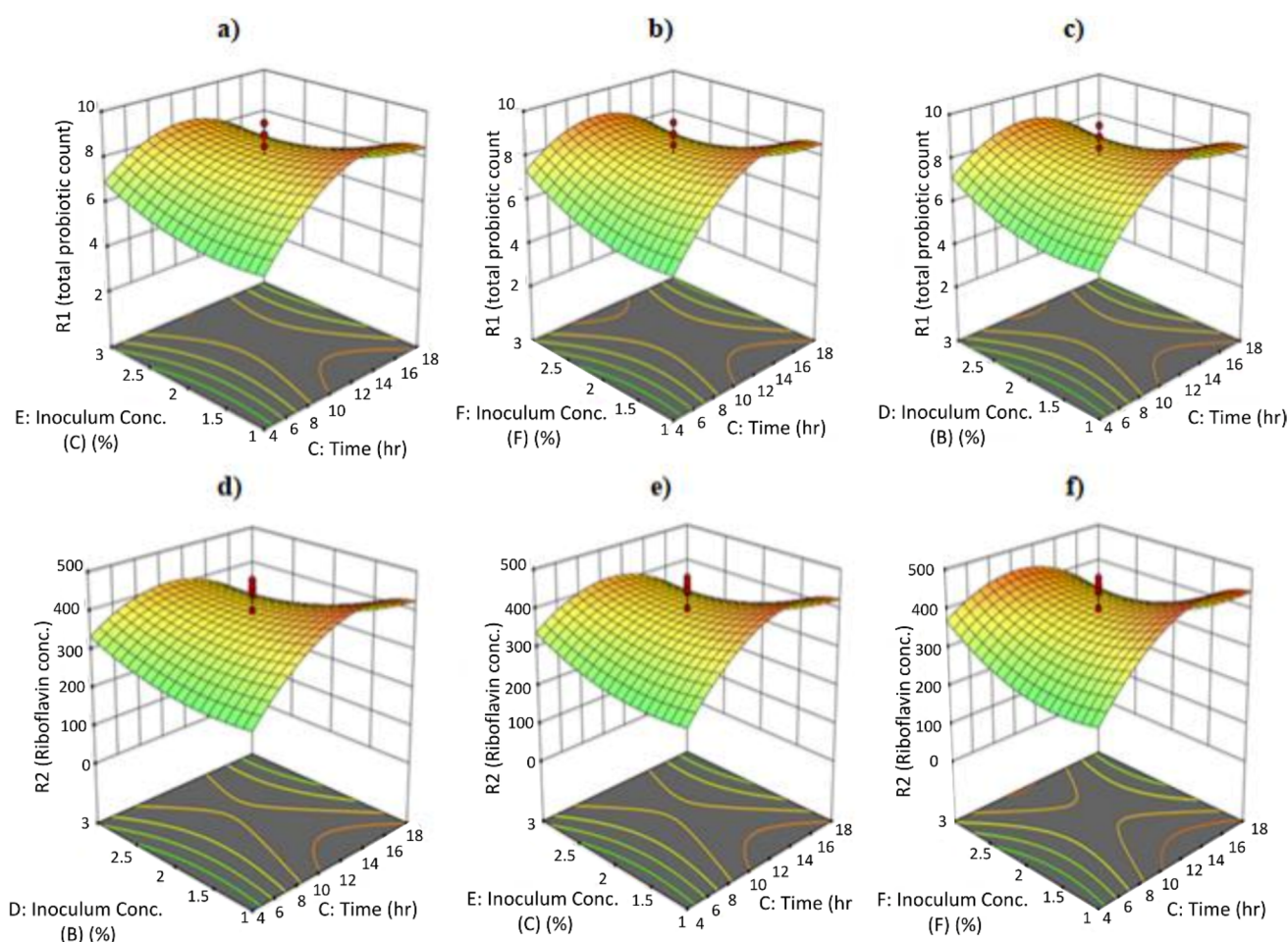


Fig. 1 a–c and d–f: Response surfaces showing the interaction effect of variables on two responses: total probiotic count (R1) and riboflavin content (R2) of fermented probiotic soymilk, respectively

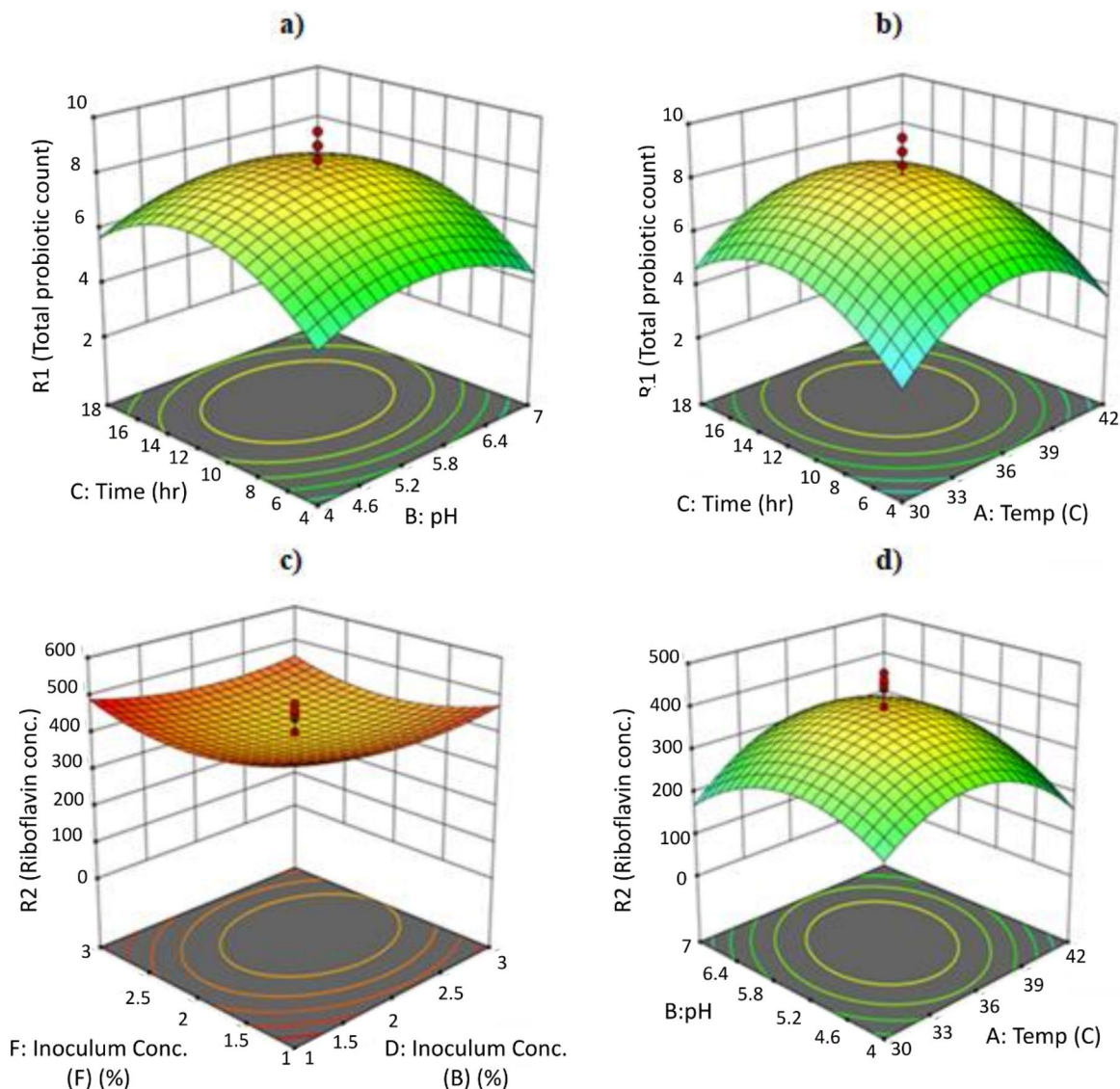


Fig. 2 a–b and c–d: Response surface and contour plots of fermented soymilk [*L. plantarum* MTCC 25432, MTCC 25433, and *L. acidophilus* NCIM 2902] showing the significant interaction between both responses: R1, R2 and non-significant interaction between inoculation concentration of B, C, and F respectively

factor that influences both the rate of bacterial growth and the effectiveness of substrate conversion. Hence, it can be considered their optimum temperature [9]. In addition, comparable data were reported by Singh et al. [9] who fermented soymilk with *L. plantarum* to enrich with vitamin B₂. Furthermore, from the interaction between incubation time and inoculum concentrations of *Lactobacilli*, a log increase in probiotic count from 3 log CFU/mL at 7 h to 9 log CFU/mL at 11 h was reported. At lower incubation time, the probiotic count was low i.e., 2 log CFU/mL at 30 min of incubation. The interactive effect among time, temperature, and pH on the probiotic count in fermented soymilk is represented in (Fig. 2a, b). Hence, by optimising the process parameters for soymilk fermentation, riboflavin production is increased maintaining the probiotic count for the beverage [20]. The result is in line with the previous findings of Thakur [15], they fermented the milk with *L. plantarum* at similar conditions. Due to the excellent probiotic qualities, *L. plantarum* is commonly used as a probiotic in food industry [33]. A group of researchers [3] isolated *L. plantarum* obtained from dahi and kinema which resulted in improved probiotic production. Another study of Bhushan et al. [34] indicated the role of temperature on the fermentation performance of probiotic strain of LAB where *L. plantarum* ZFM4 could adapt well to the temperature of dairy and fermented food.

6.2 The influence of process parameters on riboflavin content in fermented soymilk

The results demonstrated that the concentration of riboflavin was significantly affected by the linear effect of time, the quadratic effect of all fermentation variables such as temperature, pH, time, and inoculum concentration of different strains, interactive effect of time and inoculum concentration of different strains as well as temperature and pH (Table 2). All independent parameters, viz. temperature, time, pH, and inoculum concentration, presented a positive interactive effect, i.e., statistically significant ($p < 0.05$) on the production of riboflavin in the fermented soymilk (Fig. 1d–f). Furthermore, at the interaction level (Fig. 2c, d), the incubation time, temperature, and pH presented a statistically significant ($p < 0.05$) positive influence on the riboflavin content of fermented soymilk. The combination effect of the LAB strains resulted in a sixfold higher riboflavin content, i.e., 481 $\mu\text{g/L}$ at optimum fermentation parameters, thereby optimizing the riboflavin-producing capacity of these strains. Both riboflavin-producing strains, *L. plantarum* MTCC 25432 and MTCC 25433, possess *rib* genes required for riboflavin biosynthesis [29]. It can be concluded that the strains of *L. plantarum* in optimized conditions lead to maintaining a high probiotic count in fermented soymilk (9 log CFU/mL) and riboflavin content (481 $\mu\text{g/L}$) which are higher than those obtained by the former research conducted on the riboflavin-production by these strains (342.11 $\mu\text{g/L}$) [20] individually. Thompson et al. [30], reported statistically significant increase in riboflavin content on fermentation with *L. plantarum* strains of unfermented cauliflower, white beans and their 50:50 mixture. Similarly, Mohedano et al. [35] concluded potential of *L. plantarum* as a probiotic strain increasing the riboflavin content. In addition, Kumar et al. [36], reported enhanced riboflavin content in yogurt from cow milk using *L. plantarum* strains (MTCC 25432, 25,433, and 25,434).

HPLC was used to quantify the amount of riboflavin produced following bio-fortification with a different probiotic strain of soymilk. HPLC was used to quantify the amount of riboflavin produced following bio-fortification with a different probiotic strain of soymilk. The total riboflavin present in the samples was estimated using the peak values obtained from the chromatogram obtained after HPLC analysis. The soymilk fermented with a highest concentration combinations of *L. plantarum* and *L. acidophilus* showed the highest recorded riboflavin production (481 $\mu\text{g/L}$) strains MTCC 25433 (2%), MTCC 25432 (2%), and NCIM 2902 (0.43%).

The convex form of the response surfaces observed in our investigation suggests clearly defined optimum circumstances. Shapes of the 3D plots revealed the type and degree of interactions. The 3D plot in Fig. 2a elliptical shape illustrated the significant interaction between temperature, time, and pH; while the plot in Fig. 2d circular shape illustrated the nonsignificant interaction between other physical factors (other plots of non-significant interactions are omitted) for response R1 (probiotic count). Similar pattern was observed for response R2 (riboflavin content) as shown in Fig. 2a–d respectively. The graphical and numerical optimization was done keeping the significant values of independent variables.

6.3 Statistical analysis and model fitting

The probiotic and riboflavin content responses were fitted on the mathematical multiple regression model using second order polynomial equations. Analysis of the data revealed the high statistical significance of the respective mathematical model. All CCD optimized variables have confidence levels above 95%. Nonlinear regression with SAS software correlated prediction and experimental data as a second-order polynomial model (Eq. 2). The data fit the following second-order polynomial equation, which links the response and screening variables empirically (non-significant terms were removed from equation). The equation was remodified omitting all the non-significant variables and placing significant variables as shown in Eqs. (3) and (4) for responses R1 and R2.

$$R1 = +0.5789C - 0.8241CCD - 0.7884CE - 0.9322CF - 2.27A^2 - 1.20B^2 - 1.86C^2 + 0.7139D^2 + 0.5914E^2 + 0.7139F^2 \quad (3)$$

$$R2 = +31.41AB - 48.53CD - 46.78CE - 55.97CF - 110.16A^2 - 89.13B^2 - 96.89C^2 + 31.47D^2 + 34.77E^2 + 51.51F^2 \quad (4)$$

where, R1 and R2 are the response of probiotic counts (log CFU mL⁻¹) and riboflavin concentration ($\mu\text{g/L}$ or PPB).

The CCD's anticipated responses based on Eqs. (3) and (4) and suitability of this polynomial equation was further examined using analysis of variance (ANOVA). According to the ANOVA results, the model F value of R1 and R2 is 18.03 and 13.15, respectively, which is statistically significant ($p < 0.05$), and the possibility that a large model F value will occur by chance is extremely unlikely 0.01%. Furthermore, a lack of fit values of R1-2.76 and R2-2.86 indicated the difference between the fit and the pure error is not statistically significant (lack of fit $p > 0.05$). As a measure of the model's quality

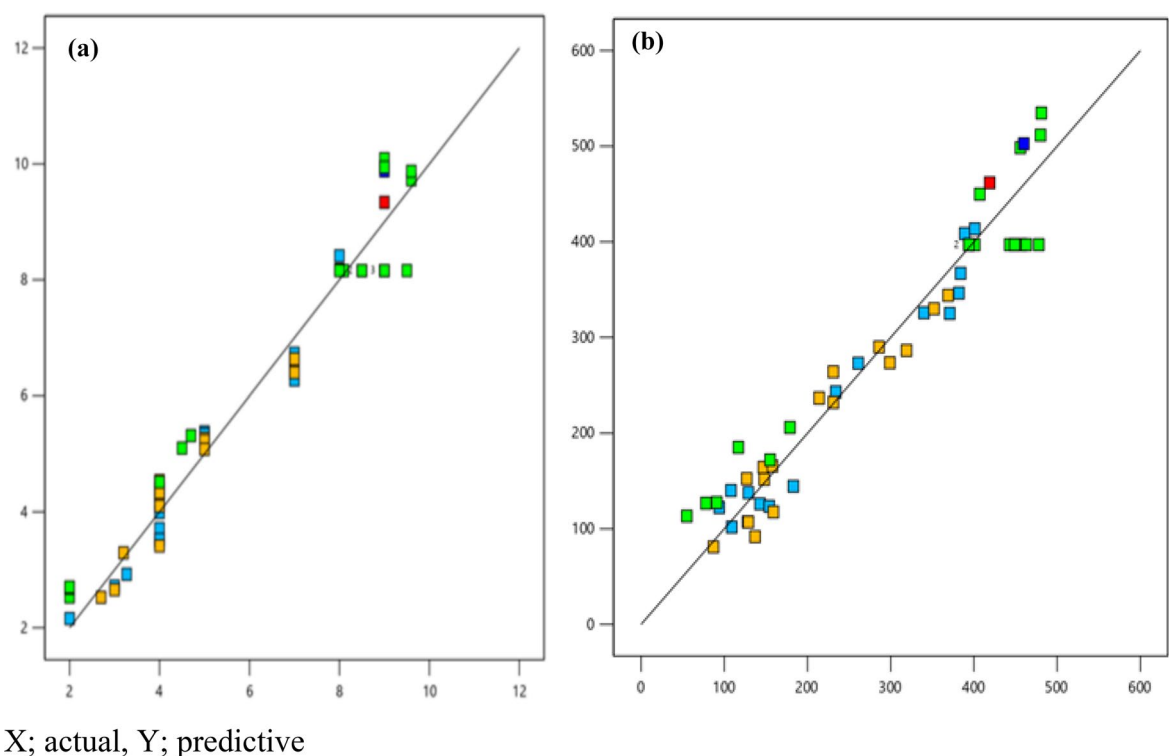


Fig. 3 Represent predictive and experimental values for both responses; **a** total probiotic count (R1) and **b** riboflavin content (R2) of fermented soymilk

of fit, the R^2 value showed that the model explained 95.3% and 93.67% of the total variation for response R1 and R2, respectively. The adjusted R^2 value of R1 is 90.02% and for R2, 86.55% was observed. All the RSM model's projected values are situated close to the actual values, as shown by the plot of predicted values versus experimental values in Fig. 3.

6.4 Process optimization and validation

This study aimed to optimize the fermentation process of soymilk utilizing LAB strains that produce riboflavin. The objective was to identify the optimum conditions for selected independent variables. By performing mathematical regression analysis, the solution with the highest desirability (100%) was selected for riboflavin fortification of probiotic soymilk through in-situ fermentation by LAB strains, as presented in Table 3. The optimized values for riboflavin content (481 $\mu\text{g/L}$) and probiotic count (9 log CFU/mL) were achieved by controlling the variables. The optimized values for the variable were determined as follows: incubation temperature (36 $^{\circ}\text{C}$), pH (5.5), incubation time (11 h), and the inoculum concentrations of LAB strains *L. plantarum* MTCC 25432 (2%), *L. plantarum* MTCC 25433 (2%), and *L. acidophilus* NCIM 2902 (0.43%).

The predicted and experimental data of the process parameters exhibited statistically significant ($p < 0.05$) differences and low error percentages. The analysis showed that optimizing the fermentation of soymilk with riboflavin-producing strains efficiently increases riboflavin content while maintaining the probiotic count in the fermented soymilk. Moreover, this approach also reduces the incubation time of fermentation experiments to 11 h. Optimization criteria were set according to the selection of individual variables.

Table 4 Predicted and measured values for responses of the optimized fermented probiotic soymilk

Responses	Predicted values	Measured values
Total probiotic count (log CFU/mL)	9.6 ^a	9 ^b
Riboflavin concentration ($\mu\text{g/L}$)	480.61 ^a	481 ^b

The appropriateness of the established models elucidating the influence of the variables on the responses were confirmed with the optimum settings predicted by design expert software. The predicted optimized values of RSM variables were verified by conducting experiments. The actual values of the responses were compared with the predicted values using one sample *t*-test (Table 4).

6.5 Physiochemical and functional properties of optimized fermented soymilk

6.5.1 Rheological characterization

Shear stress and shear rate refer to the magnitude of force applied perpendicularly or tangentially to the cross-sectional area of the product. The fermented soymilk is characterized by its apparent viscosity (η), consistency index, storage modulus (G'), loss modulus (G''), and loss tangent (or G''/G') [24]. The viscoelastic properties of fermented soymilk were shown by frequency sweep experiments. The frequency sweep test of the prepared fermented soymilk showed significantly higher G' 3.26×10^2 ; G'' 2.14×10^2 ; and G^* 5.34×10^2 values as compared to the control soymilk G' 2.1×10^2 ; G'' 1.49×10^2 ; and G^* 4.9×10^2 which described the firmness and solid characteristic of fermented soymilk (Table 5). Similarly, the angular frequency of the fermented sample was also observed to be significantly higher than the control. The cultures' combinations used for the fermentation of probiotic soymilk demonstrated a fine gel-like activity; as a result, G' was observed to be higher than G'' . In this case, it can be hypothesized that the primary soy protein subunits were degraded to tiny peptides with the rise of the G' values [33]. Li et al. [37] was described a similar rheological behavior in set-type soy-based yogurt.

In our study, the small deformation analysis of each distinct fermented soymilk was designed to analyse the rheological perspective of the fermentation process of soymilk as depicted in Fig. 4.

Table 5 Comparative analysis of unfermented and optimized fermented soymilk

Parameter	Control/unfermented soymilk	Optimized Fermented soymilk
Rheological analysis		
Storage modulus G' (Pa)	2.1×10^{2a}	3.26×10^{2b}
Loss modulus G'' (Pa)	1.49×10^{2a}	3.26×10^{2b}
Complex modulus G^* (Pa)	4.9×10^{2a}	3.26×10^{2b}
Texture profile analysis		
Hardness	74.81 ± 1.02^a	81.20 ± 1.14^b
Adhesiveness	-56.81 ± 0.09^a	-29.37 ± 1.06^b
Springiness	0.027 ± 1.81^a	0.035 ± 1.08^b
Cohesiveness	0.19 ± 1.27^a	0.37 ± 1.61^b
Gumminess	1.26 ± 1.73^a	2.19 ± 1.07^b
Chewiness	24.07 ± 1.92^a	28.16 ± 1.08^b
Water holding capacity (%)	39.29 ± 0.24^a	41.3 ± 0.27^b
Particle size distribution		
$D_{[3,4]}$	8621^a	6259^b
D_{10}	2118^a	1156^b
D_{50}	2856^a	1429^b
D_{90}	3667^a	1707^b
Span	0.54^a	0.39^b
Sensory analysis		
Body and Texture	7.12 ± 1.08^a	8.82 ± 1.16^b
Flavor	5.32 ± 0.51^a	8.76 ± 0.14^b
Color	6.06 ± 0.12^a	7.12 ± 0.44^b
Overall acceptability	6.86 ± 1.07^a	8.06 ± 1.56^b

Values with different superscripts within a row are significantly different at 5% level ($p < 0.05$); Span = $(D_{90} - D_{10})/D_{50}$

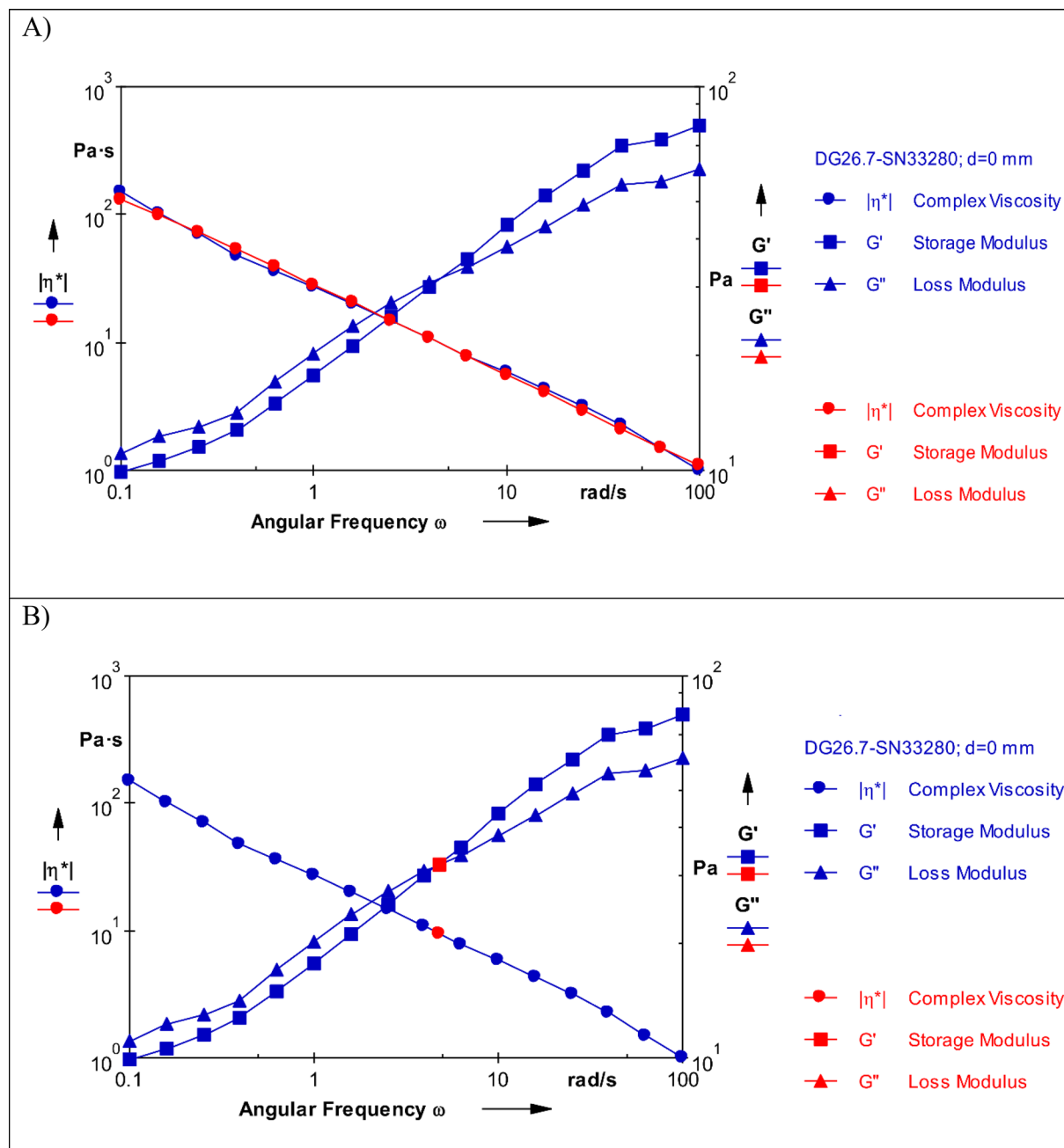


Fig. 4 Rheological analysis of fermented probiotic soymilk sample by using frequency sweep test A: Control B) fermented soymilk

6.5.2 Texture profile analysis (TPA)

According to Narayan et al. [9] the Eta (η) values were maximal for *Lactobacillus rhamnosus* GG (23,902 Pa), succeeded by *Lactiplantibacillus plantarum* MTCC 25432 (2710.5 Pa), MTCC 25433 (1963.8 Pa), and the combined strains MTCC 25432 and MTCC 25433 (967.34 Pa). The G' and G'' values for soy curd fermented with *L. plantarum* MTCC 25432 had significant increases compared to those for curds fermented with alternative cultures. The stress sweep test, similar to the frequency sweep, exhibited variability among samples. The combined strains MTCC 25432 and MTCC 25433 exhibited high shear rate (2.56), shear stress (10), viscosity (3.91), speed (2.45), and torque (245) in comparison to other probiotic strains, including *L. rhamnosus* GG, *L. plantarum* MTCC 25432, and MTCC 25433, which demonstrated nearly identical stress sweep parameters [9].

In this study, it was found that the fermented soymilk had significantly ($p < 0.05$) higher values for hardness (81.20 ± 1.14), adhesiveness (29.37 ± 1.06), springiness (0.035 ± 1.08), gumminess (2.19 ± 1.07), and chewiness (28.16 ± 1.08), while cohesiveness (0.37 ± 1.61) was non-significant as compared to the control sample (Table 5). The fermented probiotic soymilk had a hardness that ranged from 81.20 to 82.41 g. It might depend on whether a single culture or combinations of cultures are used to replicate the action of jaw compression. As previously reported, the fermented soymilk became harder when it had many large particles ($\sim 20 \mu\text{m}$) [35]. Here, it can be concluded that the hardness of the fermented soymilk is related to the hydrolysis of the proteins during the fermentation [36]. Overall, the fermented soymilk had a substantially better textural profile than the control, which may have been influenced by the strains used, the rate of protein hydrolysis, EPS formation etc. Kumar et al. [20] conducted a study that involved the analysis and comparison of yogurt-based fermented milk samples, focusing on riboflavin content, antimicrobial activity, and various physicochemical and functional properties. The findings indicated that yogurt made with *Lactobacillus plantarum* MTCC 25432 exhibited a notably elevated riboflavin concentration of 2.49 mg/L, in contrast to MTCC 25433 at 2.33 mg/L, MTCC 25434 at 2.14 mg/L, and the control group at 1.70 mg/L. The incorporation of probiotic strains facilitated the maintenance of pH and titratable acidity levels within the specified range of 4.1–4.4 and 1.0–1.05% (lactic acid/100 mL), aligning with Indian yogurt standards. The incorporation of riboflavin-producing probiotics improved the yogurt's rheological characteristics, texture, and antimicrobial properties. Additionally, all yogurt-based fermented milk samples received acceptable ratings according to sensory evaluation scores. In summary, the integration of riboflavin-producing *L. plantarum* strains with conventional yogurt cultures presents a viable method for enhancing riboflavin levels in yogurt-based fermented milk, thereby contributing to the fulfilment of daily riboflavin needs for individuals [36].

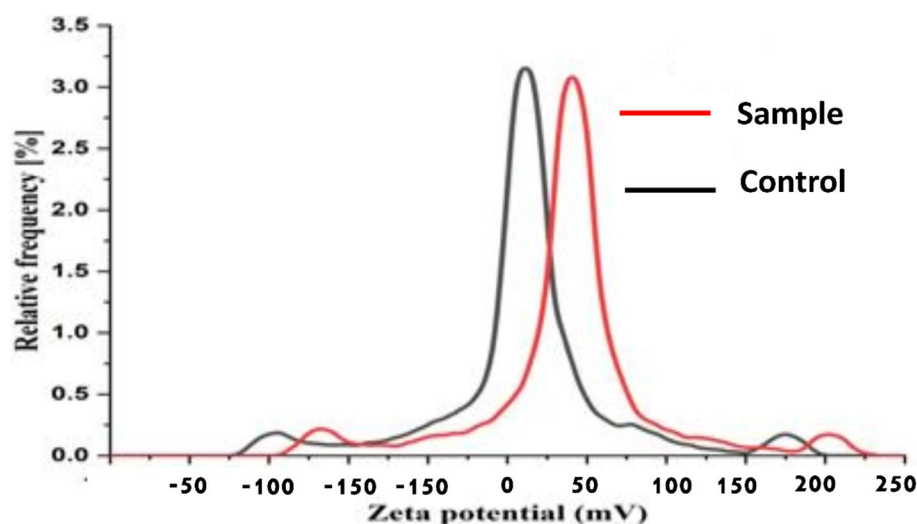
6.5.3 Water holding capacity

The water-holding capacity (WHC) of the fermented soymilk showed a higher value for WHC than the non-fermented control soymilk (Table 5). An increase in WHC could be attributed to increased protein particle hydration or accumulation of solids in the pellet due to protein hydrolysis during fermentation. It was observed that the high WHC group had a lower ratio of hydrophilic to hydrophobic subunits. This could be as a result of the hydrolysis of hydrophobic subunits exposing more hydrophobic groups, which negatively impacted the fermented soymilk water content [38]. Previous studies have reported that protein structure and conformation are altered by the hydrolysis of soymilk protein, exposing additional charges [38, 39]. According to the findings, hydrolysis of hydrophobic subunits might lead to strong electrostatic attractions between fermented soymilk particles, impacting the system's stability and resulting in fermented soymilk with a high WHC [40, 41]. When compared to the control, the impact of LAB on fermented soymilk varied. Variations in soymilk texture upon fermentation were caused by changes in the zeta potential, intermolecular forces, and particle size distribution caused by the hydrolysis of various protein subunits. In fermented soymilk, hydrogen bonds and hydrophobic forces are the primary intermolecular forces [42]. This study demonstrates that the protease properties of LAB can be used to screen LAB used to ferment low hardness and high WHC soymilk.

6.5.4 Particle size and zeta potential analysis

The volume percentage of the fermented soymilk particles was used to determine the size and zeta potential. The volume-weighted average particle diameter, often known as D [12], quantifies the mean diameter of the particles. The particle sizes denoted by the numbers $d(0.1)$, $d(0.5)$, and $d(0.9)$ correspond to 10%, 50%, and 90%, respectively (Table 5), of the cumulative particle size distribution. The findings demonstrated that the control soymilk particles were not uniform and were primarily scattered above the maximum threshold of 75,089.16 nm. The broadly and unevenly dispersed soymilk particles were comparable to earlier research [43]. The peak from the particle size distribution of unfermented soymilk represented the protein-containing particles at 100 nm [44], fat balls were represented by the peak at 1000 nm [38], and large particle fibers were represented by the peak at around 10,000 nm [43]. After LAB fermentation, the particle size of the soymilk was observed at 8260 nm. As a result of LAB fermentation, which caused the pH to drop to a level close to the protein's isoelectric point, protein acid precipitation may have occurred, which may have contributed to the particle aggregation [45]. Based on particle size, the particles with small average diameter and uniform overall distribution can be used to form thick gel network with high WHC which would aid in product formulation in the food industry as well as improved consumer acceptance [45].

Fig. 5 Zeta potential (mV) of control and sample (Fermented soymilk)



In systems where the zeta potential is high, whether positive or negative, the particles are more likely to repel each other, which enhances stability and prevents aggregation. Conversely, when the zeta potential is low, the electrostatic repulsion is weaker, increasing the likelihood of particle aggregation and leading to instability.

A zeta potential greater than +30 mV or less than −30 mV typically indicates strong stability, whereas values near zero imply that the system is likely to experience flocculation or coagulation due to inadequate particle repulsion. In this study, the control exhibited a zeta potential of 20 mV, while the sample showed approximately 50 mV, suggesting that the sample is more stable than the control as shown in Fig. 5.

6.5.5 Sensory evaluation of optimized fermented soymilk

Sensory evaluation and consumer preferences play a crucial role in product acceptance. The fermented probiotic soymilk developed in this study received an overall acceptability score of 8.06, indicating it was “liked very much.” Sensory evaluation scores for body and texture, color, flavor, and overall acceptability showed a statistically significant difference ($p > 0.05$) when compared with control samples (Table 5). The increased acceptability of the fermented probiotic soymilk may be attributed to the production of riboflavin, exopolysaccharides (EPS), and the hydrolysis of proteins, which can positively influence consumer purchasing decisions. The fermentation process produces compounds such as lactic acid, acetaldehyde, and diacetyl, which decrease the natural beany flavour of soybean caused by presence of volatile compounds n-hexanal and pentanal), which may affect the consumer acceptability.

The present results are consistent with another study performed by Ahsan et al. [46], they fermented soy milk using *Lactobacillus acidophilus* (ATCC® 4356™) and *Lactobacillus casei* (ATCC® 393™). Similarly, the sensory properties of fermented soymilk showed significant improvement when fermented with five different probiotic bacterial strains by [47]. It is evident from the current study, the capability of certain strains of LAB to synthesize and improve riboflavin content and act as probiotic fermented food acting as an alternative method for fortification. Further understanding of the nutritional and bioactive compounds of the formulated fermented soymilk will further drive to provide consumers seeking health benefits with functional food product.

7 Conclusion

The fermentation parameters for the growth of riboflavin-producing probiotic strains of *L. plantarum* in soymilk were optimized using response surface methodology and techno-functional characterization. CCD has been demonstrated to be a practical approach to optimizing physical parameters to enhance riboflavin production and probiotic viability. The statistical experimental design effectively integrated these optimizations into a feasible practice for industrial application. The study identified the optimal conditions for maximizing riboflavin production (481 µg/L) and probiotic viability (9 log CFU/mL), specifically at a temperature of 36 °C, pH of 5.5, fermentation time of 11 h, inoculation concentrations of *L. plantarum* MTCC 25432 and *L. plantarum* MTCC 25433 at 2% each, and *L. acidophilus* NCIM 2902 at 0.43%, suggesting

that this combination of probiotic cultures can be effectively utilized in industrial processes to achieve desired outcomes. Fermenting soymilk is better suited to the combination fermentation of *Lactobacillus acidophilus* and *Lactiplantibacillus plantarum*. Researchers studied the enhancement of flavouring compounds and nutritional components like riboflavin in soymilk beverages by utilising the synergistic effect of mixed bacterial fermentation. The research findings provide theoretical underpinnings and process parameters for the industrial production of soy-based fermented beverages, as well as expanding concepts for the deep processing of soymilk products.

The study highlighted significant textural modifications in the fermented soymilk, particularly in terms of hardness, adhesiveness, and springiness. The combination of *Lactiplantibacillus plantarum* MTCC 25432 and MTCC 25433 led to increased hardness, resulting in a firmer product. Additionally, the soy curds displayed improved springiness, indicating better elasticity. Adhesiveness, however, was reduced, contributing to a smoother and less sticky texture. These changes reflect enhanced textural quality, making the fermented soy curds more appealing in terms of both sensory and functional properties.

In addition, this study demonstrates that protease properties of LAB may be used to hydrolyze proteins and enhance the WHC of fermented probiotic soymilk. The fermented probiotic soymilk was also observed to have a substantially better textural profile than the control. This improvement increases the overall acceptability of soy-based fermented products, making them more suitable for lactose-intolerant consumers. The optimised fermented beverage has a soft taste, a moderate sour and sweet flavour, and the distinct flavour of a LAB-fermented beverage, according to the comprehensive data. Future research should investigate the LAB protease activity and protein alterations in the fermented soymilk. Further its functional properties should be measured to test the anti-oxidant properties of the developed soymilk.

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Data availability The authors confirmed that, the data supporting the finding of the present study are available within the article.

Declarations

Ethics approval and consent to participate The research was carried out following the guidelines of the ethics committee. The protocol was approved by NIFTEM ethical committee in accordance with the Protocol no. 12/7 L/NECHR/23.

Consent for publication The Institutional Ethics Committee (No. 12/7 L/NECHR/23) approved the study protocol. The participants have provided their informed consent to engage in the study.

Competing interests The authors declare no competing interests.

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