The Research of Medical Science Review
ISSN: 3007-1208 & 3007-1216 Volume 4, Issue 3, 2026

METABOLOMIC PROFILING OF PROBIOTIC-FERMENTED PLANT-BASED BEVERAGES USING HPLC AND FTIR SPECTROSCOPY

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DOI: https://doi.org/10.5281/zenodo.18898276

Keywords
Probiotic fermentation, Metabolomic profiling, rice milk, date pulp, Saccharomyces boulardii, HPLC, FTIR spectroscopy.

Article History
Received: 07 January 2026
Accepted: 20 February 2026
Published: 07 March 2026

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Abstract

The increasing global demand for vegetarian and natural foods has driven the development of probiotic-fermented plant-based beverages. This study aimed to enhance the nutritional and functional properties of rice milk fortified with date pulp through controlled fermentation using Saccharomyces boulardii. *Response Surface Methodology (RSM) with Central Composite Design (CCD) was employed to optimize fermentation conditions including date pulp concentration (1-4 g), inoculum size (2-5 mL), and incubation time (24-96 hours). The optimal fermentation conditions (1 g date pulp, 5 mL inoculum, 96 hours) yielded a pH of 4.52, titratable acidity of 0.45%, and viable cell count of 7.98 log CFU/mL. ANOVA revealed significant model terms ($p < 0.05$) with strong interactions between variables, particularly AB and AC ($p < 0.05$). Proximate analysis demonstrated substantial nutritional enhancement following fermentation: crude protein increased from 8.5% to 12.8%, ash content rose from 3.8% to 4.2%, while carbohydrates decreased from 84.5% to 80.2%, indicating active microbial metabolism. HPLC analysis confirmed the production of bioactive metabolites including organic acids and phenolic compounds. FTIR spectroscopy revealed characteristic functional groups including broad O-H/N-H stretching at 3281 cm^{-1}, carbonyl (C=O) groups at 1636 cm^{-1}, and aliphatic C-H stretches at 2927 and 2856 cm^{-1}, confirming structural modifications of macronutrients and enzymatic activity during fermentation. This research validates rice milk supplemented with date pulp as an effective substrate for probiotic fermentation, yielding a functional beverage with enhanced nutritional profile, viable probiotic content, and diverse bioactive metabolites. Future studies may explore additional plant-based substrates and probiotic strains to further optimize product quality.*

1. Introduction

Metabolomics is the holistic research of small molecules in the biological systems, giving information about the biochemical processes, physiological reactions, and food performance (Wishart, 2016). Metabolomic profiling is becoming more popular in the context of functional and fermented foods in food science to assess bioactive compounds and their involvement in nutrition, sensory properties, and health. Probiotics which are *Saccharomyces boulardii* have been shown to have health benefits like improved digestion, immune system and metabolism, and are being applied in plant-based substrates to benefit lactose-intolerant, vegan, and health-aware consumers. Fermentation enhances the nutritional and functional qualities of plant-based beverages by converting bioactive metabolites, enhancing digestibility, and improving flavor, among other

advantages, and serves as an alternative to dairy that could be widely used to provide nutritional advantages (Cebi, Bekiroglu, Erarslan, & Rodriguez-Saona, 2023).

Rice milk was enriched with date pulp using *S. boulardii* and Response Surface Methodology (RSM) was used to optimize the fermentation conditions such as temperature, inoculum concentration, and incubation period. Detailed metabolomic profiling by High-Performance Liquid Chromatography (HPLC) and Fourier-Transform Infrared (FTIR) spectroscopy were conducted to identify the biochemical alterations, probiotic viability, and functional improvements by using plant-based beverages and proved the great potential of plant-based beverages as a carrier of probiotics (Okur, Yıldırım, Yousefvand, & Saris, 2025).

The objectives of this study were to analyze the metabolomic profile of the probiotic-fermented plant-based beverages using RSM and to ascertain HPLC, FTIR and proximate biochemical alterations.

Material and Methods

2.1 Microorganism

In this study the probiotic culture was *Saccharomyces boulardii* CNCM I-745. Lyophilized culture (Biflor, 250 mg) which contained viable cells was bought at Medi Life Pharmacy, Satyana Road, Faisalabad, Pakistan. To activate the powder under aseptic conditions the powder was rehydrated and inoculated into sterile medium.

2.2 Inoculum Preparation

A 10 mL of distilled water in which 1g sugar had been sterilized was inoculated aseptically with 250 mg Biflor sachet of *S. boulardii*. The mixture was left to incubate to rehydrate and activate yeast giving a slightly turbid solution, which was the sign of successful inoculum preparation. This was maintained at room temperature until it was used which is shown in fig 1. (Murhekar et al., 2021).

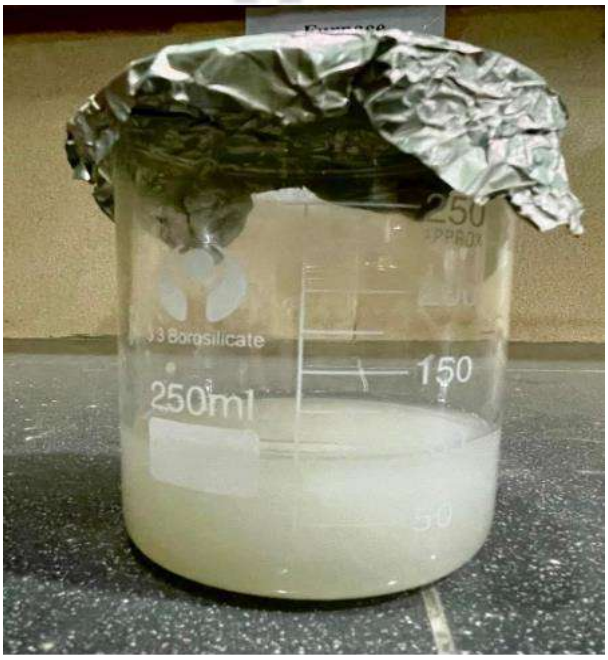

Figure 1. ***Inoculum of Saccharomyces boulardii***

2.3 Probiotic Fermentation

2.3.1 Rice Milk Preparation

White rice had been washed, soaked overnight in distilled water, ground and filtered through a muslin cloth to separate the milk and the rice pulp. 2 L of rice milk was prepared (Zhao et al., 2020).

2.3.2 Date Pulp Preparation

High quality dates were fully washed, deseed, soaked, ground and filtered to get fine pulp (Mohammed Al-Farsi, 2022).

2.3.3 Probiotic Inoculation

The inoculum of *S. boulardii* was prepared then added to the sterilized mixture of rice milk and date pulp (Afshin et al., 2019). Optimization of fermentation conditions was done through Response Surface Methodology (RSM) to achieve the maximum growth and metabolic activities of probiotics. The independent variables were date pulp concentration, inoculum size and the time of incubation. Experimental runs were based on a Central Composite Design (CCD) and run in duplicates. RSM software was used to statistically analyze the data to identify the best fermentation conditions (Mehta et al., 2020).

2.4 RSM Optimization of Probiotic Production

Different series of reactions were designed by using Response Surface Methodology (RSM) which are given in the table 2.1.

Table 2.1: ***Design Matrix and Experimental Runs Based on RSM***

Run	Factor 1 A: Date pulp (g)	Factor 2 B: Innoculum Size (mL)	Factor 3 C: Incubation Time (hours)
1	2.50	3.50	60.00
2	4.00	3.50	60.00
3	2.50	5.00	60.00
4	2.50	3.50	60.00
5	2.50	2.00	60.00
6	2.50	3.50	60.00
7	1.00	5.00	96.00
8	1.00	2.00	24.00
9	2.50	3.50	96.00
10	4.00	2.00	24.00
11	4.00	2.00	96.00
12	1.00	5.00	24.00
13	4.00	5.00	24.00
14	2.50	3.50	24.00
15	2.50	3.50	60.00
16	2.50	3.50	60.00
17	1.00	2.00	96.00
18	2.50	3.50	60.00
19	1.00	3.50	60.00
20	4.00	5.00	96.00

2.5 Analysis

Physicochemical and microbiological changes in fermented samples such as pH, titratable acidity, viable cell count and proximate composition were analyzed. The tests were conducted in triplicate to guarantee the reproducibility and compared to the RSM-optimized results (Nguyen et al., 2019).

2.5.1 pH of Probiotic

The pH was measured with a calibrated digital pH meter (Mishra & Saumya, 2021).

2.5.2 Acidity of Probiotic

Percentage lactic acid by titration of 10 mL of the sample with 0.1 N NaOH using phenolphthalein (Kumar-M et al., 2020).

$$\text{Titratable Acidity (percent)} = \frac{\text{mL of NaOH used x Normality of NaOH x 0.09}}{\text{mL sample}} \text{ x } 100$$

The total acidity was expressed as a percentage of lactic acid equivalent (Boukid & Castellari, 2022).

2.5.3 Cell Count in Fermented Rice Milk

Viable yeast was counted with the use of spread plate technique on Yeast Peptone Dextrose (YPD) agar after 24- 48 hours and colonies were counted (CFU/mL) (Fitriani, Zubaidah, Susilo, & Al Muhdhar, 2020).

2.5.4 Proximate Analysis

Optimized sample was evaluated according to moisture (oven-drying), protein (Kjeldahl), fat (Soxhlet), ash (incineration), and fiber (standard digestion) (Okur et al., 2025).

2.5.5 HPLC Analysis

Bioactive metabolites were examined (organic acids, sugars, phenolics, and amino acids) (Anupma & Tamang, 2020).

2.5.6 FTIR Analysis

The 4000-400 cm^{-1} range was analyzed to study the changes of functional groups such as hydroxyl (–OH), aliphatic (–CH), carbonyl (C=O), and amide I (Sharma & Rana, 2024).

Results and Discussion

3.1 Optimization of probiotic by using Response Surface Methodology (RSM)

Central Composite Design CCD in Design-Expert 8 was used where the independent variables were date pulp concentration, inoculum size and incubation time with responses being pH, titratable acidity and viable cell count. The best conditions were reached at a compromise of maximizing the growth of microorganisms and acid production, with the low substrate, large inoculum and extended incubation (Anbalagan, Subramanian, Suresh, & Sivaperumal, 2024).

Table 3.1: ***Experimental Design Matrix and Observed Responses***

Run	Factor 1 A: Date pulp (g)	Factor 2 B: Inoculum Size (mL)	Factor 3 C: Incubation Time (Hours)	Response 1 pH	Response 2 Acidity	Response 3 Cell Count
1	2.50	3.50	60.00	5.80	0.22	7.93
2	4.00	3.50	60.00	5.30	0.31	8.05
3	2.50	5.00	60.00	5.58	0.26	8.04
4	2.50	3.50	60.00	5.80	0.22	7.94
5	2.50	2.00	60.00	5.02	0.36	8.06
6	2.50	3.50	60.00	4.19	0.51	7.96
7	1.00	5.00	96.00	4.52	0.45	7.98
8	1.00	2.00	24.00	5.30	0.31	8.04
9	2.50	3.50	96.00	4.63	0.45	8.02
10	4.00	2.00	24.00	4.63	0.43	8.02
11	4.00	2.00	96.00	5.52	0.27	8.05
12	1.00	5.00	24.00	5.52	0.27	8.05
13	4.00	5.00	24.00	5.52	0.27	8.05
14	2.50	3.50	24.00	4.80	0.40	8.03
15	2.50	3.50	60.00	5.30	0.31	8.04
16	2.50	3.50	60.00	4.30	0.49	7.97
17	1.00	2.00	96.00	3.80	0.58	7.81
18	2.50	3.50	60.00	4.02	0.54	7.91
19	1.00	3.50	60.00	5.02	0.36	8.06
20	4.00	5.00	96.00	5.52	0.27	8.05

3.1.1 Optimization factors on the pH of probiotic rice milk

The decrease of pH was caused by fermentation which breaks down carbohydrates to organic acids. Increased inoculum and increased incubation enhanced acidification, whereas increased substrate reduced acidification, making pH a good measure of microbial growth (Rafeeq & Zia, 2026; Shabbir et al., 2023).

3.1.1.1 ANOVA Table

The model was significant (F = 3.18, p = 0.0431) and lack of fit is not significant (F = 4.13, p = 0.0727). The strong response of AB and AC indicates that the decline in pH is largely influenced by the initial factor of date pulp, the inoculum size, and the incubation period as opposed to the single factors (Jena et al., 2022) (Rafeeq, Zia, Shahid, & Khan, 2025c).

Table 3.2: ***Analysis of Variance (ANOVA) pH -Response Surface 2FI Model***

Source	Sum of Squares	df	Mean Square	F-value	p-value (Prob > F)	Remark
Model	5.23	9	0.58	3.18	0.0431	Significant
A – Date Pulp	0.16	1	0.16	0.86	0.3761	
B – Inoculum Size	0.57	1	0.57	3.10	0.1089	
C – Incubation Time	0.35	1	0.35	1.90	0.1979	
AB	1.21	1	1.21	6.61	0.0279	
AC	1.04	1	1.04	5.71	0.0380	
BC	0.099	1	0.099	0.54	0.4788	
A^2	0.56	1	0.56	3.09	0.1095	
B^2	0.10	1	0.10	0.56	0.4726	
C^2	0.78	1	0.78	4.25	0.0662	
Residual	1.83	10	0.18			
Lack of Fit	1.47	5	0.29	4.13	0.0727	Not significant
Pure Error	0.36	5	0.071			
Cor Total	7.06	19				

The F-value (3.18, p = 0.0431) indicates that there is significance and the important terms are AB and AC. The F-value (4.13, p = 0.0727) is insignificant but the value is indicating a slightly poor fit and it could have been improved by making some adjustments to the model.

Table 3.3 ***Model Summary Statistics for pH Response***

Statistic	Value	Statistic	Value
Std. Dev.	0.43	R-Squared	0.7408
Mean	5.00	Adj R-Squared	0.5076
C.V. %	8.55	Pred R-Squared	0.1775

PRESS	5.81	Adeq Precision	6.949

Model summary demonstrates that model has a good fit (R^2 = 0.7408, Adj R^2 =0.5076) yet poor predictive power (Pred R^2 = 0.1775). The adequate precision (6.949 > 4) is the indication of the fact that there is enough signal but it is possible that the model will need to be improved by reducing, transforming, or managing outliers (Rafeeq, Zia, Shahid, & Khan, 2025a) .

Table 3.4 Regression *Coefficients and Multicollinearity Statistics for pH Response*

Factor	Coefficient Estimate	df	Standard Error	95% CI (Low)	95% CI (High)	VIF
Intercept	5.19	1	0.13	4.90	5.48	
A Date Pulp	0.14	1	0.15	-0.19	0.46	1.00
B Inoculum Size	-0.26	1	0.15	-0.59	0.069	1.00
C Incubation Time	0.20	1	0.15	-0.12	0.53	1.00
AB	0.39	1	0.15	0.052	0.73	1.00
AC	0.36	1	0.15	0.024	0.70	1.00
BC	-0.11	1	0.15	-0.45	0.23	1.00
A^2	-1.74	1	0.99	-3.95	0.47	24.18
B^2	-0.74	1	0.99	-2.95	1.47	24.18
C^2	2.04	1	0.99	-0.17	4.25	24.18

It was found through regression analysis that date pulp (A), incubation time (C), and inoculum size (B) have a significant, albeit minor, effect on pH. The positive interactions (AB, AC) are very strong and the negative quadratic effects of A^2 and B^2 are curvilinear trends. The model is a moderate predictor of variation (R^2 = 0.409) and has a good fit (lack-of-fit p =0.9924) with a sufficient precision (5.054), but its predictive power is low (Pred R^2 = -0.121) (Rafeeq, Zia, Shahid, & Khan, 2025b).

3.1.1.2 Interaction between Variables

In the 3D response surface plot (Figure 3.1), low concentration of inoculum led to slower rate of pH decrease whereas longer fermentation led to lower pH rate caused by higher production of acid by microbes. The effect however stabilized after 60-72 hours.

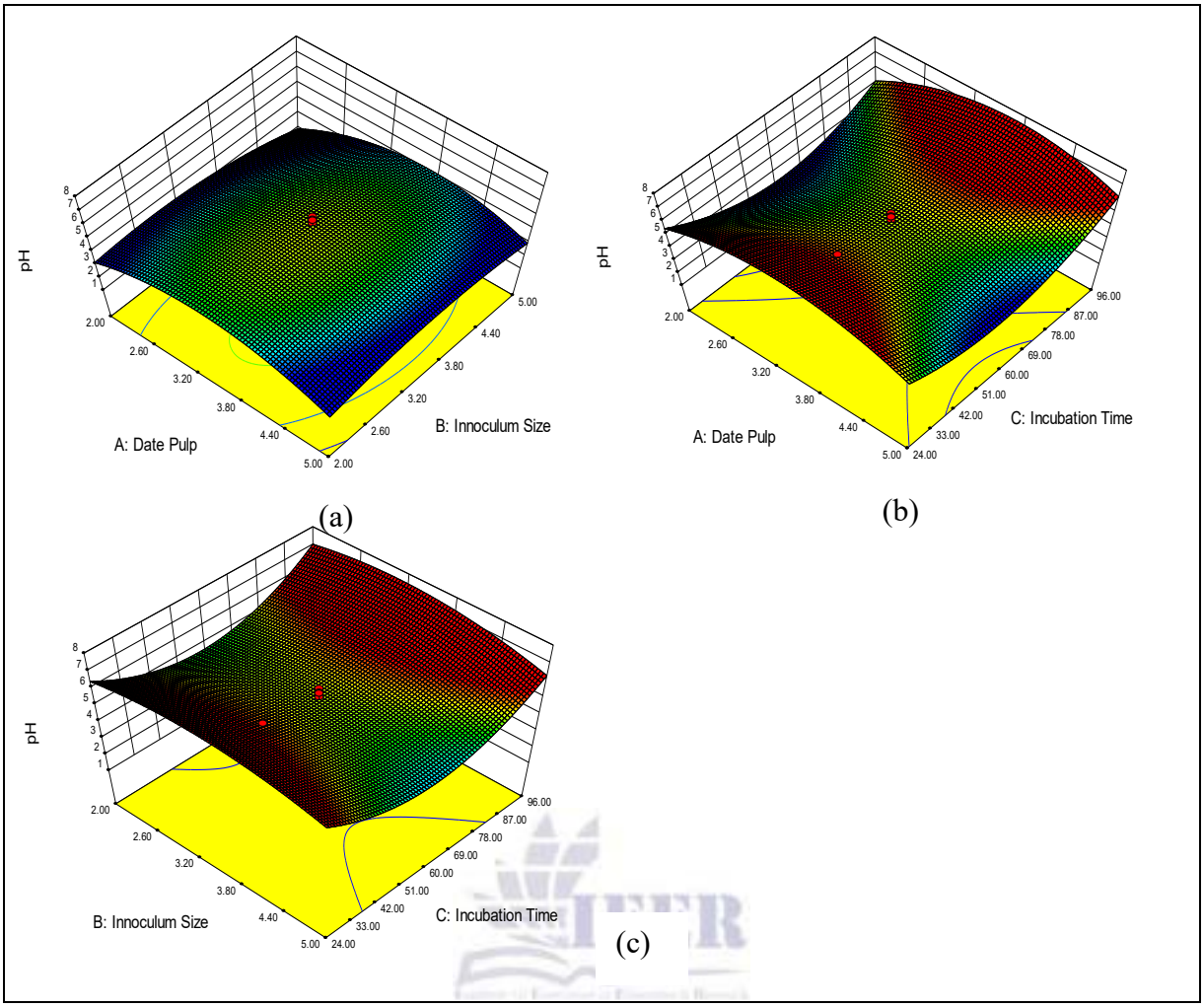

Figure 3.1: *The correlation of variables of fermentation on pH of probiotic, Date pulp vs Ioculum Size (a), Date Pulp vs Icubation Time (b) and Inoculum Size vs Incubation Time (c)*

ANOVA revealed that interactions between AB and AC had a significant influence on pH implying that the acidity is dependent on the combined effect of date pulp, inoculum size, and incubation time as observed in other studies on probiotic fermentation.

3.1.1.3 Relation of the predicted value and actual value

Predictions of RSM were quite close to experimental pHs, which prove that the quadratic regression model is an effective model to describe the fermentation process. To optimize fermentation conditions, RSM-CCD was justified by R^2 = 0.7408 and sufficient precision = 6.949.

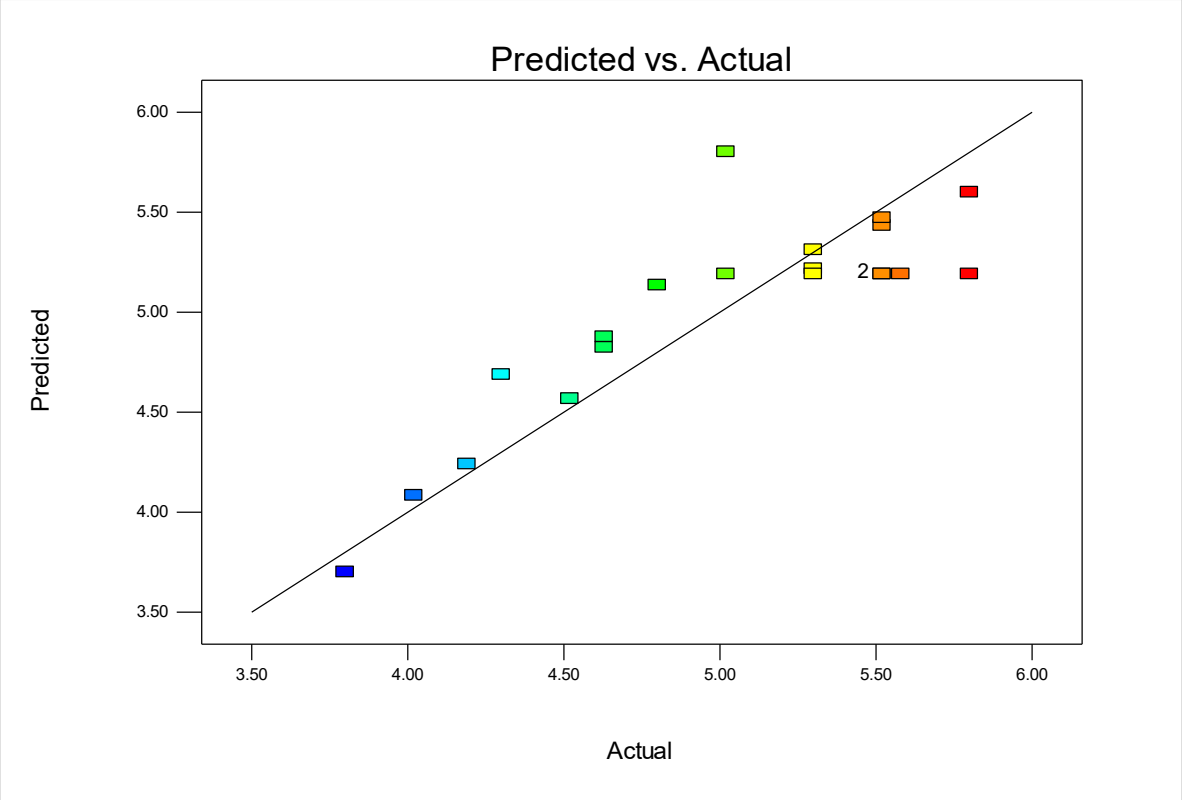

Figure 3.2: Pricicted vs actual values graph

3.1.1.4 Regression Equation

pH = 5.30 + 0.12A + 0.13B — 0.08C + 0.001AB + 0.36AC + 0.05BC

Where: A = Date Pulp (g)
B = Inoculum Size (mL)
C = Incubation Time (Hours)

3.1.2 Effect of Probiotic Fermentation on Acidity

Titratable acidity was found to increase during fermentation because of the formation of organic acids, and the presence levels of the date pulp were higher, indicating that date pulp is a viable fermentable material (Kamal et al., 2023).

3.1.2.1 ANOVA Table

A good model fit as it provides a significant model (F = 3.13, p = 0.0450), and insignificant lack of fit (p = 0.0681). The presence of significant interactions (AB and AC) suggests that the effect of acidity is primarily determined by the combined effect of date pulp, inoculum size, and incubation time and not necessarily by the factors separately.

Table 3.5: ***Analysis of Variance (ANOVA) for Acidity Response***

Source	Sum of Squares	df	Mean Square	F-value	p-value (Prob > F)	Remark
Model	0.17	9	0.019	3.13	0.0450	Significant
A – Date Pulp	0.004706	1	0.004706	0.78	0.3992	
B – Inoculum Size	0.018	1	0.018	3.02	0.1127	
C – Incubation Time	0.011	1	0.011	1.86	0.2022	
AB	0.039	1	0.039	6.46	0.0293	

Source	Sum of Squares	df	Mean Square	F-value	p-value (Prob > F)	Remark
AC	0.034	1	0.034	5.57	0.0400	
BC	0.0032	1	0.0032	0.53	0.4844	
A^2	0.021	1	0.021	3.53	0.0895	
B^2	0.002645	1	0.002645	0.44	0.5241	
C^2	0.027	1	0.027	4.46	0.0608	
Residual	0.061	10	0.00607			
Lack of Fit	0.049	5	0.009843	4.29	0.0681	Not significant
Pure Error	0.011	5	0.002297			
Cor Total	0.23	19				

The model F-value (3.13) was significant (p = 0.0450) and there was only 4.50% probability of it occurring by chance because of noise or otherwise. There were important terms such as AB and AC (p < 0.05). The F-value (4.29) was not significant (p=0.0681) showing that the fit to the model was acceptable.

Table 3.6: ***Model Summary Statistics for Titratable Acidity Response***

Statistic	Value	Statistic	Value
Std. Dev.	0.078	R-Squared	0.7380
Mean	0.36	Adj R-Squared	0.5022
C.V. %	21.40	Pred R-Squared	0.1807
PRESS	0.19	Adeq Precision	6.883

Pred R^2 (0.1807) was smaller than the Adj R^2 (0.5022), which showed that it had a low predictive power and there may be a problem with the model. Nevertheless, Adeq Precision (6.883 > 4) indicated that there was enough signal, which indicated that the model would be adequate in navigating the design space.

Table 3.7: ***Regression Coefficients and Multicollinearity Results of Titratable Acidity Response***

Factor	Coefficient Estimate	df	Standard Error	95% CI (Low)	95% CI (High)	VIF
Intercept	0.33	1	0.023	0.28	0.38	
A Date Pulp	-0.024	1	0.027	-0.083	0.036	1.00
B Inoculum Size	0.046	1	0.027	-0.013	0.11	1.00
C Incubation Time	-0.036	1	0.027	-0.096	0.023	1.00
AB	-0.070	1	0.028	-0.13	-0.008626	1.00
AC	-0.065	1	0.028	-0.13	-0.003626	1.00
BC	0.020	1	0.028	-0.041	0.081	1.00

Factor	Coefficient Estimate	df	Standard Error	95% CI (Low)	95% CI (High)	VIF
A^2	0.34	1	0.18	-0.063	0.74	24.18
B^2	0.12	1	0.18	-0.28	0.52	24.18
C^2	-0.38	1	0.18	-0.78	0.021	24.18

3.1.2.2 Association between Variables

The 3D plots revealed that the more the concentration of inoculum was used and length of incubation the more acid was produced, and this was affected by substrate concentration. Significant interactions (AB and AC) show that acidity is dependent on joint effects of variables, which is consistent with probiotic fermentation research.

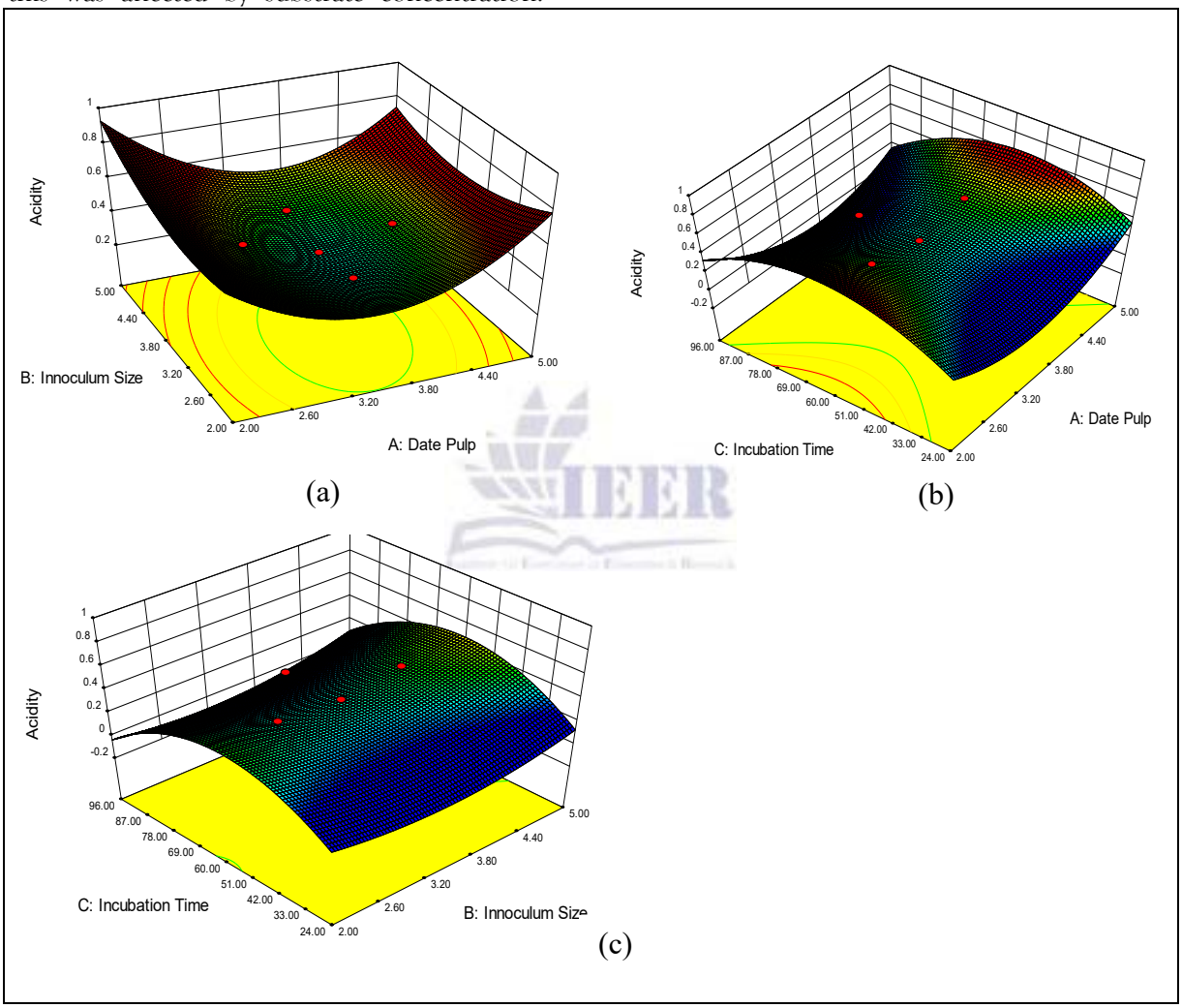

Figure 3.3: *The correlation of variables of fermentation on acidity of probiotic, Date pulp vs Ioculum Size (a), Date Pulp vs Icubation Time (b) and Inoculum Size vs Incubation Time (c).*

Increased inoculum and incubation augmented acidity, which was regulated by date pulp concentration.

3.1.2.3 Predicted vs Actual Values

There were no significant differences between predicted and actual values (R^2 = 0.7380; sufficient precision = 6.883), which substantiates that the RSM-CCD model is applicable in optimization of fermentation.

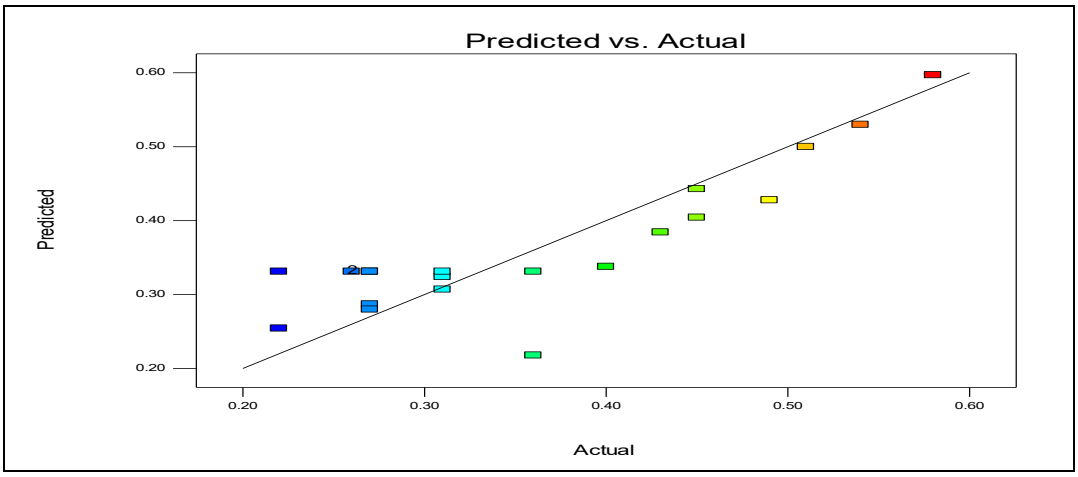

Figure 3.4: ***Predicted versus Actual values titratable acidity***

3.1.2.4 Final Regression Equation, as a function of the Actual Factors

Regression equation in terms of actual factors:

$$Y=0.33-0.0092A+0.012B-0.0085C-0.028(AB)-0.026(AC)+0.008(BC)+0.14A2+0.05B2-0.15C2$$

Regression equation enables a direct prediction of titratable acidity with variables of fermentation incorporating both linear, interaction, and quadratic effects to demonstrate how each variable and combination of variables affect acid production.

3.1.3 Analysis of Variable Count Cell Count

Cell count analysis revealed that the best conditions that yielded the best viable probiotics (log CFU/mL) were the optimized conditions. The growth was a characteristic microbial curve: 24 h had a moderate growth, 36-72 h exhibited exponential growth and 84-90 h kept growth constant which proves that RSM was effective in maximizing cell viability.

3.1.3.1 ANOVA Table

ANOVA revealed the model to be significant ($F = 4.13$, $p = 0.0153$) and that the interactions between the probiotics A and C play a significant role ($p < 0.05$) in influencing probiotic growth. The lack of fit was negligible ($F = 0.85$), and high precision ratio (8.782) was used to verify that the model could be used to optimize cell growth.

Table 3.8: ***ANOVA Cell Count Response Surface 2FI Model***

Source	Sum of Squares	df	Mean Square	F Value	Prob > F	
Model	0.054	6	8.923E-003	4.13	0.0153	significant
A-Date Pulp	2.826E-003	1	2.826E-003	1.31	0.2733	
B-Innoculum Size	7.650E-003	1	7.650E-003	3.54	0.0824	
C-Incubation Time	1.424E-003	1	1.424E-003	0.66	0.4315	
AB	0.019	1	0.019	8.80	0.0109	
AC	0.017	1	0.017	7.92	0.0146	
BC	5.512E-003	1	5.512E-003	2.55	0.1342	
Residual	0.028	13	2.160E-003			
Lack of Fit	0.016	8	2.025E-003	0.85	0.6008	not significant
Pure Error	0.012	5	2.377E-003			
Cor Total	0.082	19				

The high AB and AC interactions demonstrate that the effect of date pulp combined with inoculum size and incubation time has a strong influence on the probiotic growth. The model is important (F = 4.13, p = 0.0153) that lack of fit is insignificant (F = 0.85, p = 0.6008).

Table 3.9: ***Model Summary Statistics Cell Count Response Surface 2FI Model***

Statistic	Value
Standard Deviation	0.046
Mean	8.00
Coefficient of Variation (C.V. %)	0.58
R-Squared	0.6559
Adjusted R-Squared	0.4971
Predicted R-Squared	-0.5152
PRESS	0.12
Adequate Precision	8.782

The RSM model of probiotic cell count demonstrated a high level of reliability with low standard deviation (0.046), low C.V (0.58%), and R^2 = 0.6559 (Adj R^2 = 0.4971) denoting that it was a good fit. Despite negative Pred R^2 (-0.5152), there is strong interaction between the terms and the adequate precision (8.782 > 4) of the model is high, which indicates its appropriateness in maximizing fermentation conditions.

Table 3.10: ***Coefficient Estimates Response Surface 2FI Cell Count***

Factor	Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	8.00	1	0.010	7.98	8.03	1.00
A – Date Pulp	0.018	1	0.016	-0.016	0.053	1.00
B – Innoculum Size	-0.030	1	0.016	-0.064	0.00444	1.00
C – Incubation Time	0.013	1	0.016	-0.021	0.047	1.00
AB	0.049	1	0.016	0.013	0.084	1.00
AC	0.046	1	0.016	0.011	0.082	1.00
BC	-0.026	1	0.016	-0.062	0.00925	1.00

Final Equation as a Function of Coded Factors:

$$\text{Cell Count} = 8.00 + 0.018A - 0.030B + 0.013C + 0.049AB + 0.046AC - 0.026BC$$

Where:
A=Date Pulp
B=Inoculum Size
C= Incubation Time

Final Equation as a Function of Real Factors:

$$\textit{Cell Count} = 8.35208 - 0.11507A - 0.066667B - 0.0009368C + 0.021667(AB) + 0.00085648(AC) - 0.00048611(BC)$$

Where:
A= Date Pulp concentration
B= Inoculum Size
C= Incubation Time

3.1.3.2 Interaction Among Variables

The cell counts of the predicted and experimental cells were closely matched with the majority of the points showing along the 45 degree diagonal indicating that the RSM model is good at estimating viable cells despite small biological or experimental factors.

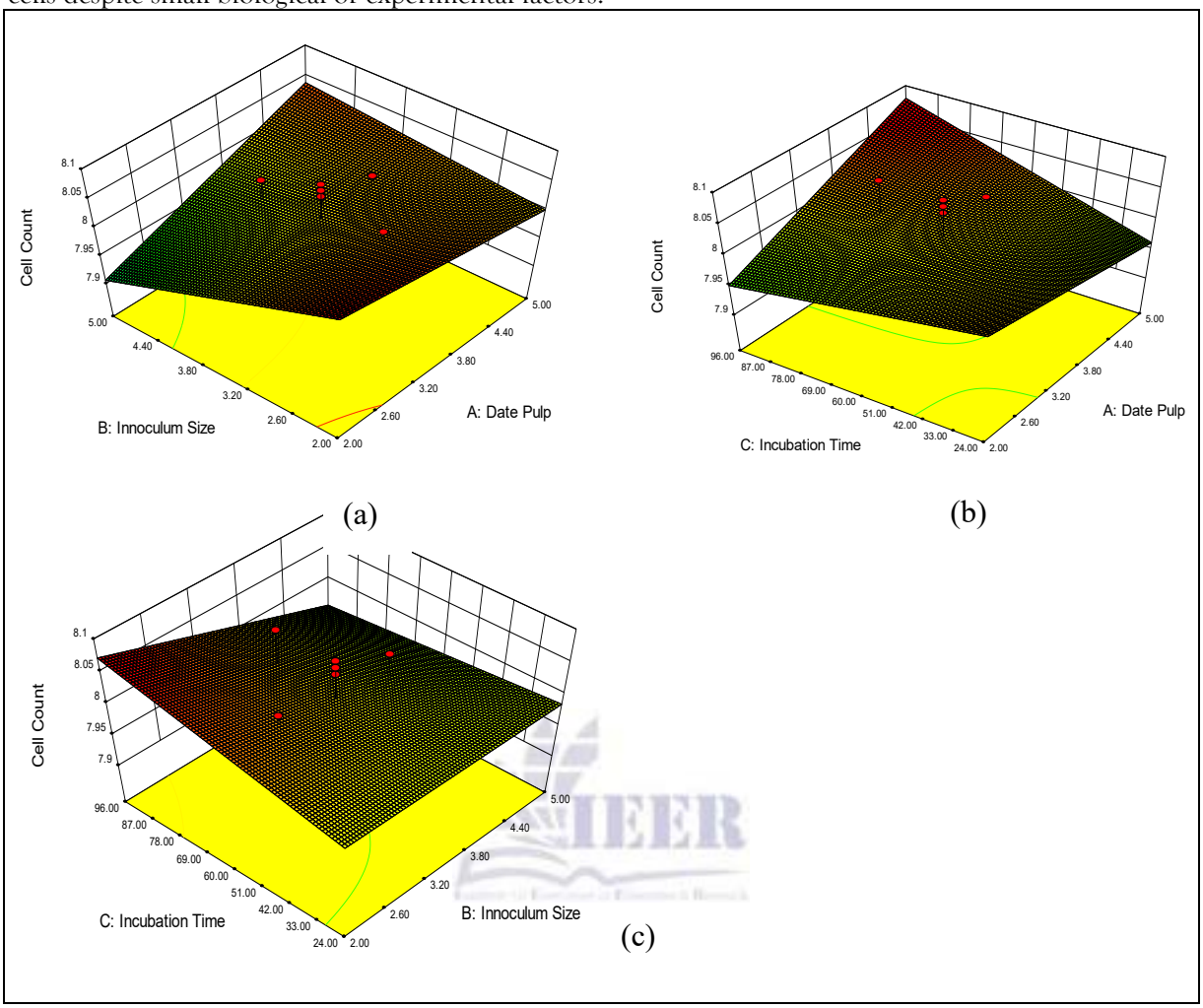

Figure 3.5: *Correlation of fermentation variables with probiotic cell count: (a) Date pulp concentration versus inoculum size, (b) Date pulp concentration versus incubation time, and (c) Inoculum size versus incubation time*

3.1.3.3 Predicted vs Actual Values Correlation

There is high positive relationship between predicted and actual cell counts, which proves that the model can be used to optimize fermentation with small deviations likely to occur owing to biological variability.

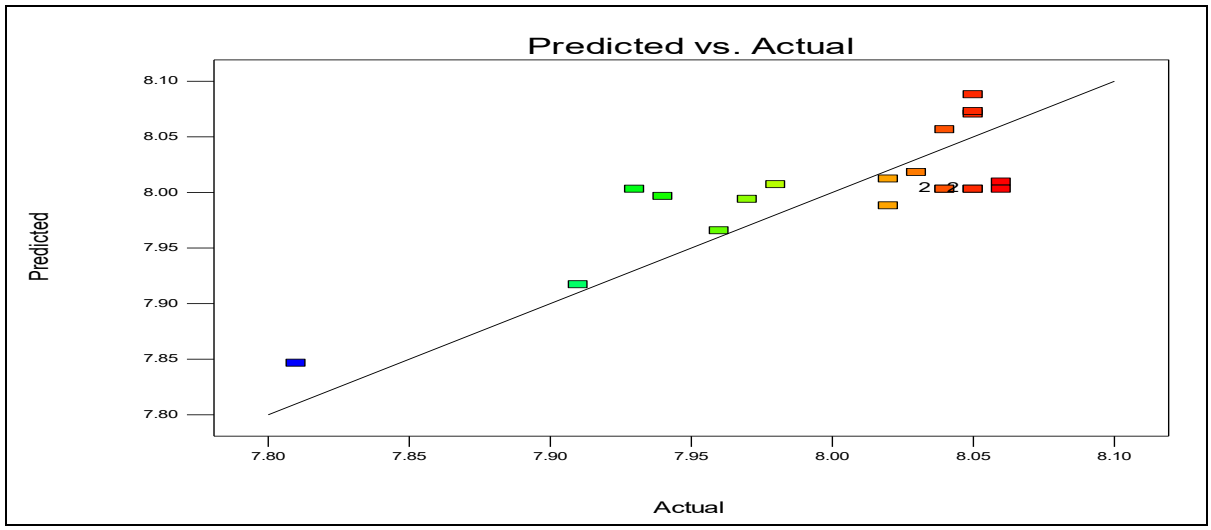

Figure 3.6: ***Predicted vs Actual Values graph of cell count***

3.1.3.4 Regression Equation

The regression equation involved in the relation between the date pulp, inoculum size and incubation time with the cell count demonstrates that the three factors considered individually and also as a combination have effects on the growth of the probiotics.

Cell Count=8.00+0.018·A-0.030·B+0.013·C+0.049·AB+0.046·AC-0.026·BC

End Equation as a **Function of Coded Factors:**

Cell Count=8.35208-0.11507*A*-0.066667*B*-0.000936819C
+0.021667(A·B)+0.000856481(A·C)-0.000486111(B·C)

The cell growth was improved through positive interaction (AB, AC) and deterred through negative interaction (BC), thus making the model useful in predicting the cell counts and optimal conditions (fermentation) to attain these results.

3.2 Evaluation of Probiotic by using HPLC and FTIR

The FTIR spectrum had a broad peak at 3281 cm -1 equivalent to OH and NH groups and a peak at 1636 cm -1 equivalent to carbonyl (C=O) groups. Peaks of 2927 and 2856 cm -1 displayed the aliphatic constituents and a small peak of 2154 cm -1 reflected the little functional groups that proved the existence of proteins, organic acids, and fermentation metabolites (Panesar, Anal, & Kaur, 2022).

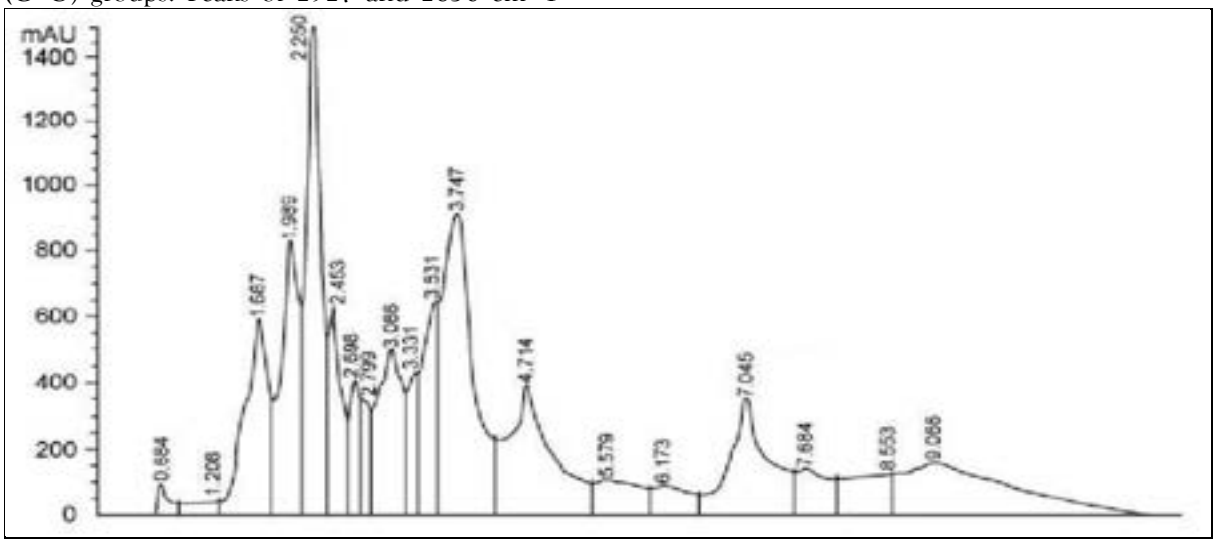

Figure 3.7: ***HPLC graphic results***

The FTIR spectrum had a broad peak at 3281 cm -1 equivalent to OH and NH groups and a peak at 1636 cm -1 equivalent to carbonyl (C=O) groups. Peaks of 2927 and 2856 cm -1 displayed the aliphatic constituents and a small peak of 2154 cm -1 reflected the little functional groups that proved the existence of proteins, organic acids, and fermentation metabolites (Krishnan, Krishnan, & Kumar, 2024).

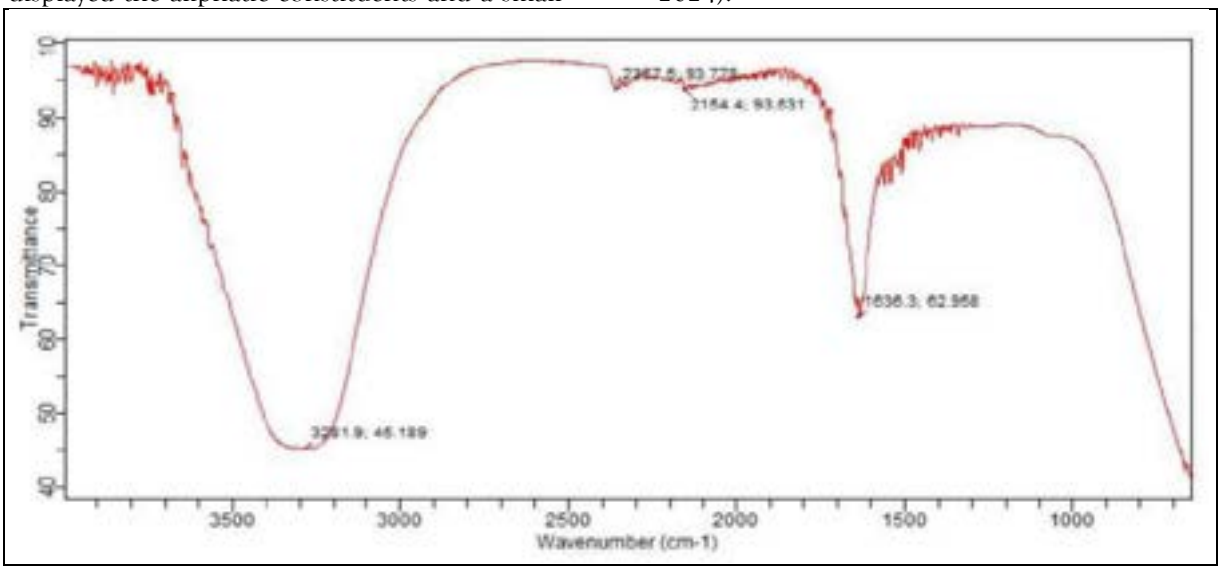

Figure 3.8: ***Graphic findings of FTIR spectroscopy***

3.3 The proximal analysis of Probiotic

The fermentation with Saccharomyces boulardii enhanced moisture (70.5%) and crude protein (8.5 → 12.8%), marginally lowered crude fat (3.2→ 2.8%), and carbohydrates (84.5 → 80.2%), and marginally augmented ash (3.8 → 4.2%). The gross energy decreased slightly (385 378 kcal/100 g DM). These shifts suggest protein enrichment through the microbial biomass and the usage of sugars and lipids to promote yeast growth and development in accordance with previous researchers on yeast-fermented plant material (Jiang et al., 2023).

Table 3.11: ***Proximate Analysis report***

Component	AOAC Method (2019)	Unfermented Mixture (Control)	Fermented Mixture (with *S. boulardii*)	Net Change & Biological Explanation
Moisture (as-is basis)	934.01	65.0 ± 2.5	70.5 ± 2.0	Increased due to water production (metabolic water) during respiration.

Dry Matter	(By difference)	35.0 ± 2.5	29.5 ± 2.0	Corresponding decrease due to higher final moisture.
Composition of DRY MATTER				
Crude Protein	990.03 / 2001.11	8.5 ± 0.4	12.8 ± 0.6	↑ +4.3%. Significant increase due to synthesis of microbial single-cell protein (yeast biomass).
Crude Fat (Ether Extract)	920.39	3.2 ± 0.3	2.8 ± 0.2	↓ Slightly reduced as lipids are utilized for membrane synthesis and energy.
Crude Ash (Total Minerals)	942.05	3.8 ± 0.2	4.2 ± 0.2	↑ Slight increase; yeast cells concentrate minerals and the ash includes microbial minerals.
Crude Fiber	978.10	6.0 ± 0.5	5.5 ± 0.5	↓ Minor reduction; hemicellulose in rice bran and date pulp may be partially solubilized.

Nitrogen-Free Extract (NFE)[1]	(By difference)	78.5 ± 0.8	74.7 ± 0.9	↓ -3.8%. Substantial reduction. This digestible carbohydrate fraction (sugars, starch) is the primary carbon & energy source for S. *boulardii*.
Total Carbohydrates[2]	(By difference)	84.5	80.2	↓ Sum of Crude Fiber and NFE. Overall reduction confirms substrate consumption.
Calculated Gross Energy[3]	-	385 ± 8 kcal/100g DM	378 ± 8 kcal/100g DM	

4. Conclusion

A rice milk-based functional, non-dairy probiotic drink was made by fermentation of date pulp with Saccharomyces boulardii, a high gut-colonizing, high acid-tolerant yeast. Date pulp furnished natural sugars, minerals and bioactive compounds which boosted the growth of probiotics. The optimal fermentation conditions were determined using RSM-CCD on date pulp concentration, inoculum size, and incubation time and responses were pH, titratable acidity, and viable cell count. The best concentration was 1 g date pulp, 5 mL inoculum and 96 hours which produced pH 4.52, 0.45 acidity and 7.98 log CFU/mL indicating stable probiotic activity. There was a nutritional improvement indicated by proximate analysis: protein levels rose to 12.8 0, ash levels reduced to 4.2 0, carbohydrates, nitrogen-free extract, fat, and fiber were all lower because of microbial metabolism. The HPLC has verified the generation of bioactive metabolites, such as organic acids and phenols, which are advantageous to the gut health and immunity. The structural changes of macronutrients were observed by analysis of FTIR which were evidence of the enzyme activity and the availability of nutrients. In general, the beverage had better functional and nutritional values.

Acknowledgement

The authors would like to express their sincere gratitude to the Department of Biochemistry, Riphah International University, Faisalabad, for providing the essential resources, laboratory facilities, and technical support that made this research possible.

Conflict of Interest

All authors have no Conflict of interest

Funding Source

There is no funding sources

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