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MICROBIOLOGICAL EVALUATION OF SOY FORTIFIED *SHRIKHAND* BY USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT : Soy fortified shrikhand samples were prepared with various levels of soymilk, cane sugar and yoghurt culture (*Lactobacillus* delbrueckii subsp. *bulgaricus* and *Streptococcus thermophilus* in the ratio of 1:1) examined for selective enumeration of health beneficial microorganisms. The present investigation was carried out to optimize the process for manufacturing of soy fortified shrikhand by its response with employing the 3-factor Central Composite Rotatable Design (CCRD). The formulated soy fortified shrikhand with 45.0% soymilk, 30.0% cane sugar and 3.0% yoghurt culture was found highly suitable ($P<0.05$) for obtaining the maximum viable count of *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus* whereas the desirability index was 0.985.

Key Words: Soymilk, Shrikhand, Yoghurt culture, Central Composite Rotatable Design.

INTRODUCTION

Soybean, (Glycine max L.) is economically the most important oilseeds in the world and can be used in solving protein deficiency problems (Ikya *et al.,* 2013). Soymilk is intended for population who cannot digest milk for reasons like Lactose intolerance, allergy to milk proteins, or vegetarian way of diet (Božanić *et al.,* 2011). Lactic acid fermentation may be used as a means to reduce beany flavours (Wang *et al.,* 2006) and antinutritional factors such as phytic acid in soybean products (Donkor *et al.,* 2007). In fermented soymilk products soy yoghurt is gaining more popularity in the developing countries (Ghorbani *et al.,* 2012). The yoghurt or curd (*dahi*) is a basic material for the preparation of shrikhand (Nigam *et al*., 2009). *Shrikhand* is an indigenous semi-soft, sweetish-sour, whole milk delicious and healthful dessert, particularly in western part of India and prepared from lactic acid fermentation. It is made with chakka (strained yoghurt/curd) which is finely mixed with sugar and flavoring agents. It is popular because of its characteristics flavour, taste, palatable nature and possible therapeutic value (Nigam *et al*., 2009). Patel and Chakraborty (1985) suggested that yoghurt is preferred for shrikhand making than curd (*dahi*) because curd takes longer time to form coagulum than yoghurt. To reduce the cost and enhance nutritive value of yoghurt some vegetable product has also been incorporated in the milk (Patel *et al*., 2009, 2010). The objective of the present research was to investigate the effect of soy fortification on the viable count of *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus* in *shrikhand*.

MATERIALS AND METHODS

The standard yoghurt culture (*Lactobacillus delbrueckii ssp. bulgaricus* NCDC 009 and *Streptococcus thermophilus* NCDC 074) were obtained separately from National Collection of Dairy Culture (NCDC), Dairy Microbiology Division at NDRI Karnal, Haryna, India. The rest of the materials were procured from the Department of A.H. & Dairying, BHU, Varanasi, India.

Preparation of soymilk

Soybean was soaked into water (1: 3) with 0.3% Sodium Bi Carbonate ($NaHCO_3$) for 6 to 8 hrs and dehulled. Dehulled soybean was further used to prepare soymilk by using soy cow machine (SSP Pvt Ltd).

Manufacturing of soy fortified *shrikhand*
A detailed flowchart with mass balance and process details are given in Figure 1.

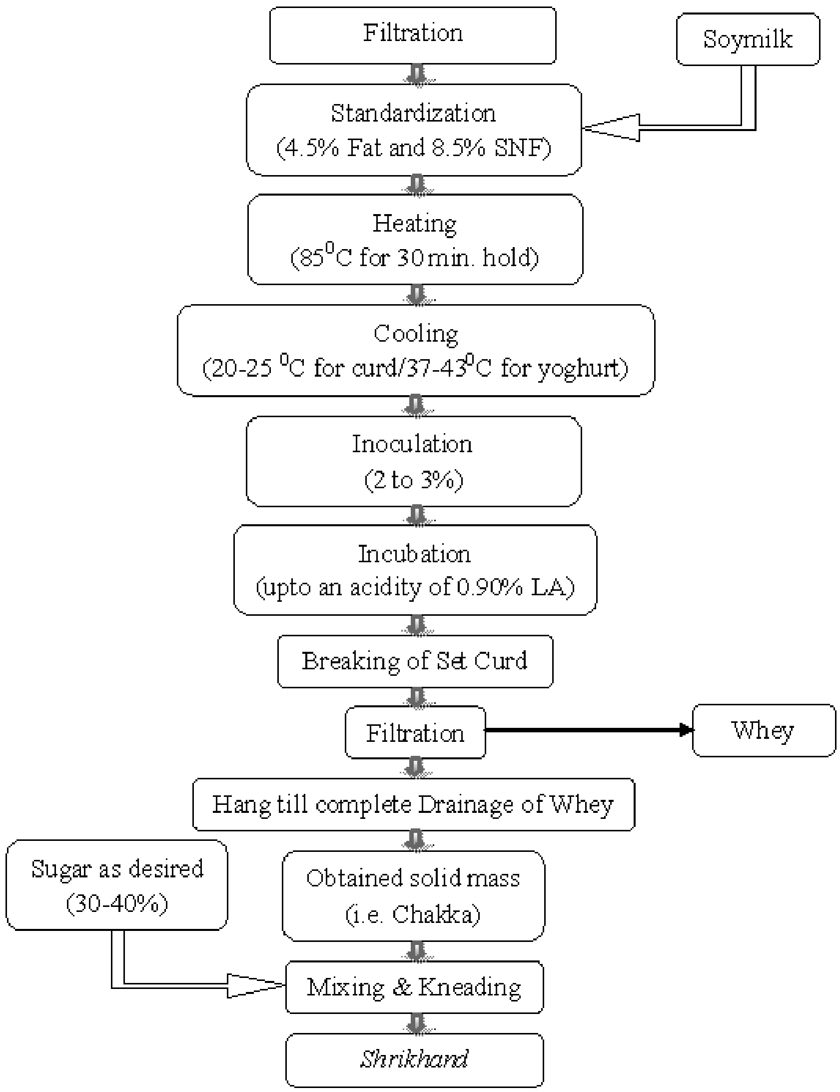


Figure 1: Flow-diagram for the manufacture of soy fortified *shrikhand*

Analysis of microflora of soy fortified shrikhand:

Selective enumerations of *Lactobacillus delbrueckii ssp. bulgaricus NCDC 009* and *Streptococcus thermophilus NCDC 074* were done according to Shah (2000).

Design of experiments

Response surface methodology which involves design of experiments, selection of levels of variables in experimental runs, fitting mathematical models and finally selecting variables' levels by optimizing the response (Khuri and Cornell, 1996) was employed in the study. A Central Composite Rotatable Design (CCRD) was used to design the experiments comprising of three independent processing parameters. The total number of experiments with three factors was twenty. Fourteen experiments were augmented with six replications at the centre points to evaluate the pure error. The Table 1 show run number and experimental conditions of the runs arranged by CCRD. Product formulation was optimized by analyzing the response.

Analysis of data

The data obtained during the present investigation were suitably analysed and optimized by employing response surface methodology (RSM) to the various parameters viz., canesugar, soymilk and yoghurt culture. ANOVA was performed to validate the RSM optimization. The experimental data obtained from the design were analysed by the response surface regression procedure using the following second order polynomial equation:

$Y_i = \beta_o + \sum\beta_i X_i + \sum\beta_{ii} X_i^2 + \sum\beta_{ij}X_iX_j$ .... (1)

Where, Y_i was the predicted response, β_o was a constant, β_i was the i^{th} linear coefficient, β_{ii} was the i^{th} quadratic coefficient and β_{ij} was ij^{th} interaction coefficient and X_i X_j were independent variables. Numerical optimization technique of the Design-Expert software (8.0.7.1) was used for optimization of response. The desired goals for each factor and responses were chosen (Table 2). Responses obtained after each trials were analyzed to visualize the interactive effect of various parameters on microbial properties of soy fortified shrikhand.

RESULTS AND DISCUSSION

Selective enumeration of *Lactobacillus delbrueckii ssp. bulgaricus*

The average viable count of Lactobacillus delbrueckii ssp. bulgaricus (10^8 cfu/g) varied from 5.9 to 8.9 (Table 1). The quadratic equation obtained by the Response Surface Application (RSA) of the data showing the effect of cane sugar, soymilk and yoghurt culture could be presented as follows:

Lactobacillus delbrueckii ssp. bulgaricus = $+7.68-0.23*A+0.79*B+0.37*C-0.12*A*B +0.019*A*C-0.056*B*C-0.081*A^2-0.098* B^2- 0.063*C^2$

Here A, B and C are coded terms for the three variables viz., cane sugar, soymilk and yoghurt culture respectively. ANOVA F-value was determined to examine the goodness of fit for the developed model (Table 5).The F-value for Overrun was significant ($P<0.0001$). The coefficient of determination (R^2) of Lactobacillus delbrueckii ssp. bulgaricus (10^8 cfu/g) showed that quadratic model terms (A, B and C) were significant ($P<0.05$) (Table 3), however, quadratic interactive model term (AB, AC and BC) and quadratic model term (A^2, B^2 and C^2) were found to be not significant ($P>0.0001$) (Table 5). The coefficient of determination was found to be 0.9599, indicating that 95.99% of the variability in the response could be explained by the model. The model F-value of 26.65 implies the model is significant. The 'Pred R-squared' of 0.79635 was in reasonable agreement with the 'Adj R- squared' of 0.9239. "Adeq Precision" was 18.074 which was greater than 4, indicating an adequate signal. Hence, the model could be used to navigate the design space.

Table 1: Experimental runs and actual values of factors used in central composite rotatable design

Run	A:Cane Sugar %	B:Soymilk %	C:Yoghurt Culture cfu/gm	*Lactobacillus bulgaricus* cfu/gm	*Streptococcus thermophilus* cfu/gm
1	30	15	3	7.1	6.4
2	35	30	2.5	7.5	6.7
3	35	30	2.5	7.9	6.8
4	30	45	3	8.9	8.5
5	35	30	2.5	7.5	6.8
6	30	15	2	6.5	6.1
7	35	4.77	2.5	5.9	4.9
8	40	45	3	8.2	7.9
9	35	30	2.5	7.8	6.8
10	43.41	30	2.5	6.8	6.5
11	35	30	2.5	7.9	6.6
12	35	30	3.34	8.1	7.6
13	35	55.23	2.5	8.7	8.25
14	26.59	30	2.5	7.9	7.2
15	40	15	2	6.2	5.1
16	30	45	2	8.2	7.8
17	35	30	1.66	6.7	6.2
18	35	30	2.5	7.5	6.5
19	40	15	3	7.2	6.6
20	40	45	2	7.75	6.7

Table 2: Constraints selected in the range for optimization of soy fortified *shrikhand*

Constraints	Goal	Lower Limit	Upper Limit
A:Cane Sugar	is in range	30	40
B: Soymilk	is in range	15	45
C:Yoghurt Culture	is in range	2	3
Lactobacillus delbrueckii subsp. bulgaricus (L.B.)	maximize	5.9	8.9
Streptococcus thermophilus (S.T.)	maximize	4.9	8.5

Lower weight = 01, Upper weight = 01, Importance = 03.

Table 3: Coefficient of estimates of coded factors for different levels of ingredients in soy fortified shrikhand

Factor	*Lactobacillus delbrueckii subsp. bulgaricus*	*Streptococcus thermophilus*
Intercept	7.677635	6.7
A:Cane Sugar	-0.23431	-0.27
B: Soymilk	0.787811	0.9
C:Yoghurt Culture	0.373769	0.44
AB	-0.11875	-0.11
AC	0.01875	0.21
BC	-0.05625	0.013
A^2	-0.08061	0.076
B^2	-0.09829	-0.021
C^2	-0.06293	0.093

Table 4: Optimized solutions of soy fortified shrikhand with desirability by Design of Expert 8.0.7.1

Number	Cane Sugar	Soymilk	Yoghurt Culture	L.B.	S.T.	Desirability
1	30	45	3	8.87544	8.37263	0.9854
2	30.11	45	3	8.87154	8.36551	0.9843
3	30	45	2.99	8.87302	8.36665	0.9822
4	30	44.69	3	8.86172	8.35196	0.9819
5	30.47	45	3	8.85849	8.3432	0.9807
6	30	45	2.99	8.8705	8.36057	0.9790
7	30.76	45	3	8.84714	8.32542	0.9778
8	30	45	2.98	8.8688	8.35647	0.9769
9	30	44.51	2.99	8.85156	8.33446	0.9768
10	30	44.2	3	8.84001	8.31942	0.9762

The response surface plot shown as Fig.2 (a) shows that with the increase in the level of soymilk, there was a considerable increase in the viable count of *Lactobacillus delbrueckii ssp. bulgaricus*. However, the viable count of *Lactobacillus delbrueckii ssp. bulgaricus* tended to decrease with increasing levels of sugar in the formulation. A significant decrease in viable count of *Lactobacillus delbrueckii ssp. bulgaricus* was observed with an increase in the levels of cane sugar in the formulation (Fig. 2b). Higher levels of soymilk exerted more positive influence on the viable count of *Lactobacillus delbrueckii ssp. bulgaricus* than the levels of yoghurt cultures (Fig. 2c). From figure 2 (a, b and c) clearly depicts that increased in levels of soymilk and yoghurt culture the viability of *Lactobacillus delbrueckii ssp. bulgaricus* was increased with irrespective the cane sugar levels.

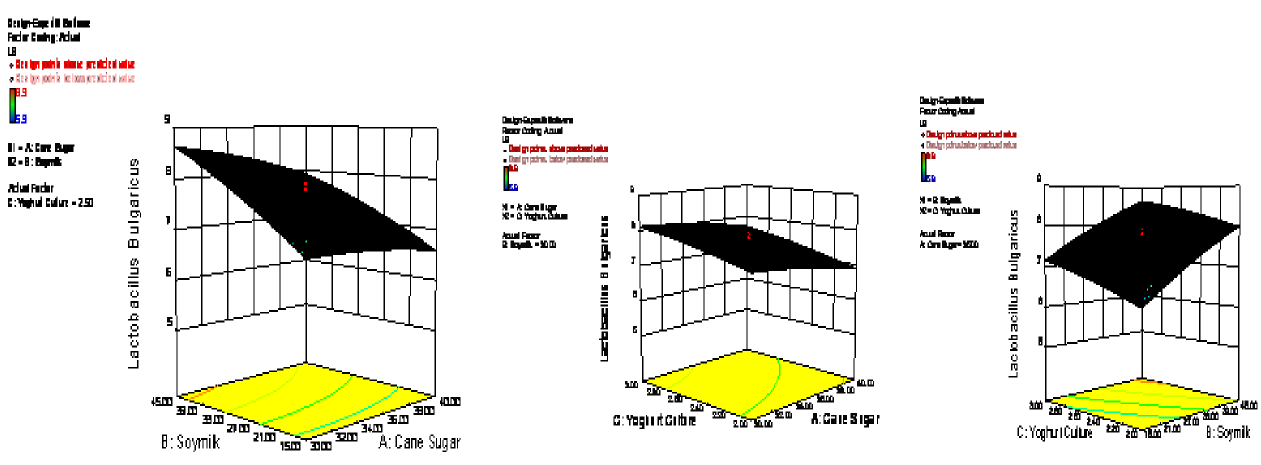
Selective enumeration of *Streptococcus thermophilus*

The average viable count of *Streptococcus thermophilus* (10^8 cfu/g) varied from 4.9 to 8.5 (Table 1), from figure 2 (a, b and c) clearly depicts that increased in levels of soymilk and yoghurt culture the viability of *Streptococcus thermophilus* was increased whereas the cane sugar had irrespective effect. The quadratic equation obtained by the Response Surface Application (RSA) of the data showing the effect of canesugar, soymilk and yoghurt culture could be presented as follows:

Streptococcus thermophilus = $+6.70-0.27*A+0.90*B+0.44*C-0.11*A*B+0.21*A*C+0.013*B*C+0.076*A^2-0.021*B^2+0.093*C^2$

Table 5: ANOVA results showing the variables as a linear, quadratic and interaction terms on each response variable and coefficients for the prediction models

Source	df	*Lactobacillus delbrueckii subsp. bulgaricus*		*Streptococcus thermophilus*	
		F-value	p-value Prob>F	F-value	p-value Prob>F
Model	9	26.65	< 0.0001	56.44	< 0.0001
A-Cane Sugar	1	15.61	0.0027	32.48	0.0002
B-Soymilk	1	176.49	< 0.0001	365.37	< 0.0001
C-Yoghurt Culture	1	39.73	< 0.0001	88.04	< 0.0001
AB	1	2.35	0.1564	3.32	0.0984
AC	1	0.059	0.8137	11.85	0.0063
BC	1	0.53	0.4845	0.04	0.8436
A^2	1	1.95	0.1928	2.72	0.1303
B^2	1	2.90	0.1195	0.22	0.6515
C^2	1	1.19	0.3012	4.13	0.0695
Residual	10				
Lack of Fit	5	1.31	0.3886	2.81	0.1406
Pure Error	5				
Cor Total	19				
R-Squared		0.9599		0.98069	
Adj R-Squared		0.9239		0.96332	
Pred R-Squared		0.79635		0.8786	
Adeq Precision		18.074		26.368	
C.V. %		2.9171		2.57	
PRESS		2.4437		1.91798	



a b c

Figure 2: Response surface for the effect of (a) Soymilk and Cane sugar (b) Yoghurt culture and Cane sugar (c) Yoghurt culture and Soymilk on selective enumeration of *Lactobacillus delbrueckii subsp. bulgaricus* of soy fortified *shrikhand.*

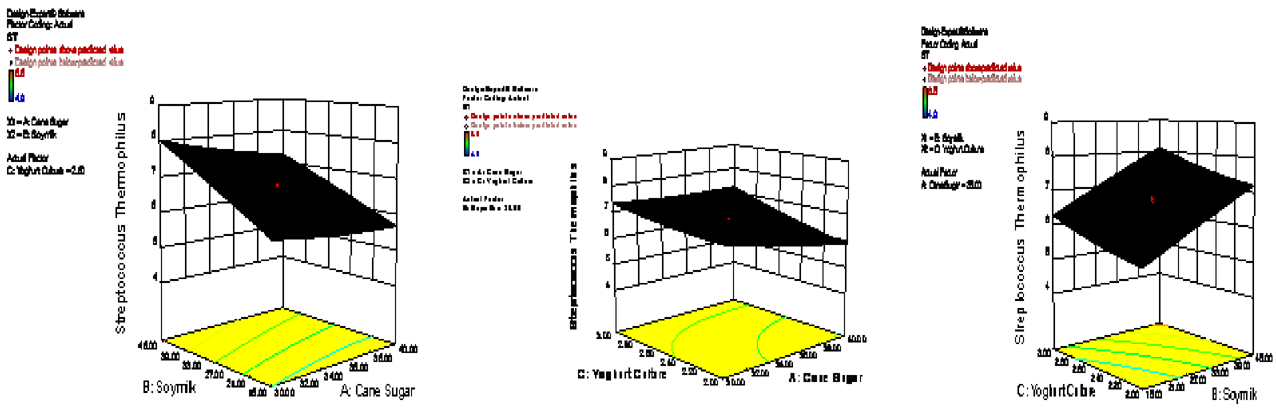


Figure 3: Response surface for the effect of (a) Soymilk and Cane sugar (b) Yoghurt culture and Cane sugar (c) Yoghurt culture and Soymilk on selective enumeration of *Streptococcus thermophilus* of soy fortified *shrikhand.*

Here A, B and C are coded terms for the three variables, i.e cane sugar, soymilk and yoghurt culture respectively. ANOVA F-value was determined to examine the goodness of fit for the developed model (Table 5).The F-value for Overrun was significant ($P<0.0001$). The coefficient of determination (R^2) of Streptococcus Thermophilus showed that quadratic model terms (A, B and C) were significant ($P<0.05$) (Table 3). However, quadratic interactive model term (AB AC, BC) and quadratic model term (A^2, B^2, C^2) were found to be not significant ($P>0.0001$) (Table 5). The coefficient of determination was found to be 0.9807, indicating that 98.07% of the variability in the response could be explained by the model. The model F-value of 56.44 implies the model is significant. The 'Pred R-squared' of 0.8786 is in reasonable agreement with the 'Adj R- squared' of 0.96332. "Adeq Precision" was 26.368 which was greater than 4, indicating an adequate signal. Hence, the model could be used to navigate the design space. The response surface plot shown as Fig.3 (a) shows that with the increase in the level of soymilk, there was a considerable increase in the viable count of Streptococcus thermophilus. However, the viable count of Streptococcus thermophilus tended to decrease with increasing levels of sugar in the formulation. The decrease in viable count of Streptococcus thermophilus was found not significant when the levels of cane sugar increases with yoghurt culture in the formulation (Fig. 3b). Higher levels of soymilk exerted more positive influence on the viable count of Streptococcus thermophilus than the levels of yoghurt cultures (Fig. 3c). From figure 3 (a, b and c) clearly depicts that increased in levels of soymilk and yoghurt culture the viability of Streptococcus thermophilus was increased with irrespective the cane sugar levels. The highest desirability index was 0.9854 compared to all other formulations (Table 4). The results (Fig. 2 and 3) clearly depicts that the viable count (cfu/g) of both Lactobacillus delbrueckii ssp. bulgaricus and Streptococcus thermophilus was increasing with increasing the levels of soymilk and yoghurt culture irrespective with the cane sugar levels. However, the viable count of Lactobacillus delbrueckii ssp. bulgaricus was higher than Streptococcus thermophilus which may be due to the high acid producing properties of culture. De (1989) also reported the same. We have found that soymilk increases the growth of yoghurt culture which was presence of soy-oligosacchride, a prebiotic, in soymilk. Similar findings reported in Petra et al. (2013) and Cuenca et al. (2005). The results also clearly depicts that as the cane sugar levels increases the product becomes more concentrated with sugar and the viable count of both culture was decreases. Similar findings showing influence of cane sugar on the bacterial count of *Misti Dahi* (Sweet Yoghurt) has been reported by Akter et al. (2010).

CONCLUSION

The optimized levels of soymilk, cane sugar and yoghurt cutlure for the manufacture of the soy fortified *shrikhand* were predicted based on viable count of Lactobacillus delbrueckii ssp. bulgaricus and Streptococcus thermophilus using RSM. Out of 10 suggested formulations, formulation no. 1 had maximum viable count and highest desirability index (0.9854) compared to all other formulations. Hence, the formulation with soymilk (45.0%), cane sugar (30.0%) and yoghurt culture (3.0%) was considered most suitable formulation for manufacturing soy fortified *shrikhand*, which could be suggested as a suitable combination. The developed such value-added products with different levels of soymilk, cane sugar and yoghurt culture are highly palatable and provide nutritional benefits to consumers.

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