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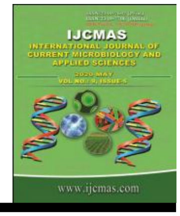

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Development of Probiotic Soymilk (Soy Water Extract) Chocolate Using Response Surface Methodology (RSM) with Incorporation of *Lactobacilli helveticus* MTCC 5463

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A B S T R A C T

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In this present investigation attempt has made to develop probiotic soymilk chocolate. The purpose of addition of soymilk was to enhance nutritional qualities of chocolate because chocolate is a confectionary item which is readily consumed by all age group of people. Both soymilk and probiotic are health promoting components. Milk chocolates were prepared by incorporating with soymilk powder and freeze-dried *Lactobacillus helveticus* MTCC 5463. The effect of various ingredients like soymilk powder, skim milk powder and cocoa powder on sensory characteristics of the prepared chocolate was studied and their levels were optimized using Response Surface Methodology (RSM) using regression equation model. Optimization of the product was done by maximising the score for sensory characteristics. The optimised probiotic soymilk chocolate constituted 14.13 (%) soy milk powder, 15 (%) skim milk powder, 15 (%) cocoa powder, 30 (%) sugar and 30 (%) cocoa butter and 0.5 (%) lecithin. After optimization freeze dried concentration of *Lactobacillus helveticus* MTCC 5463 (2% w/w) was incorporated into the probiotic soymilk chocolate and was tested for probiotic viability and organoleptic parameters. Lactobacillus counts were remained above 8.0 log cfu/g until 30 days under refrigerated condition.

Introduction

Food products which contain probiotics are in demand in by consumers (Stanton *et al.*, 2001) as these foods are claimed to have a positive effect on health (Lee and Salminen, 1995) and different type of non-fermented probiotic functional foods have been developed all over the world. In the course of their proliferation and survival in gastrointestinal tract, probiotics produce metabolites such as lactic acid and antibiotic-like substances called bacteriocins that suppress the growth of putrefactive microorganisms. Their metabolic activities also help in the pre-digestion of food components, production of vitamins, and improve the bioavailability of minerals and other nutrients (Rekha and Vijayalakshmi, 2010).

Chocolate is a luxury food product universally popular among all age groups. It is reported that in 2015/2016 about 7.3 million tons of retail chocolate confectionery were consumed worldwide, with consumption expected to reach approximately 7.7 million tons by 2018/2019 (Statista, 2018). Probiotics incorporated chocolate could offer a good alternative to several dairy products broadening the health claims of chocolate based food products. Chocolate provides an encouraging role to human nutrition through provision of antioxidants, chiefly polyphenols (Hii *et al.,* 2009). Presence of biologically active phenolic compounds in cocoa has stimulated research on its effects in oxidative stress, ageing, blood pressure regulation, reduce heart disease risk, stroke, and atherosclerosis (Buitrago-Lope, *et al.,* 2011). Chocolate is a rich source of minerals and has been identified as an ideal carrier for probiotics (Maillard and Landuyt, 2008); found to absorb more probiotics compared to yoghurt (Deanna, 2009). Chocolate being rich in cocoa butter provides protective passage for probiotics through the upper intestine to deliver them to the colon in sufficient numbers (Lahtinen *et al.,* 2007). Specifically, the rheological behaviour and sensory perception of Lactic acid bacteria (LAB) possess many beneficial effects to promote human health. In the course of their proliferation and survival in gastrointestinal tract, probiotics produce metabolites such as lactic acid and antibiotic-like substances called bacteriocins that suppress the growth of putrefactive microorganisms. Probiotic lactobacilli has many health benefits like anti-pathogenic (Kumari and Vij, 2015), anti-oxidative and digestive health promoting. Indigenous strain *L. helveticus* MTCC 5463 has been proven to possess probiotic potentials with their specific beneficial role in the gastrointestinal tract and ability to reduce cholesterol and stimulate immunity (Prajapati *et al.,* 2011. Considering the popularity of chocolates and its rich nutrient content, the main objectives of our research were to standardize and optimize the process of manufacturing probiotic chocolate; and to evaluate the finished product based on physicochemical, sensory, and microbiological parameters followed by evaluating shelf life of the standardized product.

Soymilk is best alternative in case of plant milk as its compositions resembles to cow milk and also it contains some major and minor components like protein, fat, carbohydrate, calcium, iron, sodium, carotene, vitamin-E and riboflavin (Deshpande *et al.,* 2008). The soymilk is used in various products like paneer, yogurt, cheese, tea and coffee whiteners, shrikhand, rasogolla and various indigenous milk sweets, confectionary etc. (Wang *et al.,* 2001).soymilk made them more favourable choice for food technologist to develop soy incorporated milk chocolate especially for protein disease patients. Therefore, the aim of the present study is to verify the effects of replacing the dairy milk in chocolates with different combinations of soymilk on rheological properties, as well as finding the optimum levels of these soymilk, using a central composite design.

Materials and Methods

Soymilk powder was taken purchased from bionutrients, Mandideep, Madhya Pradesh (India). Cocoa powder, Cocoa butter and sugar were procured from local market of Bhopal, Madhya Pradesh (India). Skimmed milk powder Amul All the chemicals were procured from Sigma-Aldrich (USA), Merck and Himedia (Mumbai, India). Soy lecithin was purchased from Urban Platter. The probiotic *Lactobacillus helveticus* MTCC 5463 culture was acquired from Dairy Microbiology Department, Anand Agricultural University, Anand. The lyophilized culture was propagated in sterilized reconstituted skim milk-RSM (10% T.S.) by incubation at 37°C for 8–12 h and stored at 5±2°C. Prior to use, three successive transfer of culture was administered into sterilized RSM following incubation at 37°C for 8–12 h to keep it active.

Preparation of freeze-dried probiotic culture

About 2% of activated culture of *L. helveticus* MTCC 5463 was inoculated in 100 ml of de Man Rogosa Sharpe (MRS) broth and incubated at 37°C for 12 h in order to obtain sufficient cell mass. The cells were harvested by centrifugation at 4°C, 5000 r.p.m. for 15 min, washed twice with saline water, and were stored at –20°C in case of concentrated culture.

For freeze drying, collected cells were inoculated into 10 ml sterilized skim milk containing cryoprotectant (1% glycerol), mixed thoroughly, distributed in glass vials, and frozen at –20°C for overnight. Culture was freeze-dried using a freeze-dryer (MINI LYODEL).

Optimization of probiotic soymilk chocolate

Most of the RSM applications come from area such as new/modified products, sensory quality, setting sensory standards, chemical or engineering processes, industrial research and biological investigations, which emphasizes on optimizing a process or system (Thompson, 1982). Henika (1982) described RSM as useful for analyzing experimental data from the food. Products to optimize the physical properties of food products using different levels of ingredients. The Central Composite Design (CCD) for three independent variables was performed using Design-Expert DX 8.0.7.1 version. The independent variables were soymilk powder, milk powder and cocoa powder.

The independent variables and variation levels are shown in Table1. Dependent variables were body & texture, flavor, and mouth feel of the probiotic soymilk chocolate. RSM was applied for experimental data using a commercial statistical package, Design-Expert version 8.0.7.1 for the generation of response surface plots. The second order polynomial equation of the following form was fitted to the responses. For production of chocolate the tempering temperature and stage of addition of ingredients is vital both the parameters were optimized with continuous trials.

Concentration of probiotic culture was kept 1% (conc.>10^8 cfu/gm). The protocol followed for the preparation of chocolate preparation is described in Figure.1.

Viability of probiotics

The first dilution was prepared by taking 11 g of the milk chocolate sample in 99 mL of phosphate buffer (0.1 M, pH 7.2 ± 01), containing 0.1% peptone for depolymerization of alginate capsules and release of encapsulated bacteria in solution (Sheu *et al.*, 1993), and serial dilutions were made in 9 mL normal saline. Viable lactobacilli were enumerated on MRS agar (37C for 48 h).

Analysis of developed chocolate

All the parameters such as moisture, protein, fat and ash content in soymilk chocolate probiotic chocolate were estimated by AOAC (2000) methods.

Statistical analysis

Quantitative data was expressed as mean ± SD values of 3 replicates. Data were analyzed by using completely randomized factorial design. Analysis of variance was conducted; when significant effect was detected, the means were separated by Fisher Least Square Analysis.

Optimization study data were analyzed by Completely Randomized Design as per the methods described by Steel and Torrie (1980). Storage study data were examined using Factorial CRD. The values for microbial counts were log transformed before analysis.

Results and Discussion

Body and texture of probiotic soymilk chocolate

The average body and texture score of probiotic soymilk chocolate varied from 6.8 to 8.31 (Table.2) with a significant ($p<0.0001$) soymilk powder significantly enhanced the flavour of the developed probiotic chocolate. The equation for body and texture is given below:

$$\text{Body \& texture} = +7.38 - 4.328E-004 \times A - 0.02 \times B + 0.39 \times C - 1.250E-003 \times A \times B + 0.061 \times A \times C - 0.016 \times B \times C + 0.018 \times A^2 - 8.772E-003 \times B^2 + 0.064 \times C^2$$

There was a slight effect of skim milk powder on the body and texture of probiotic soymilk chocolate (Fig.3a).

The increasing levels of soymilk powder showed a slight decrease in sensory score of body and texture of probiotic soymilk chocolate. Increasing the level of cocoa powder significantly increased sensory score for body and texture.

Flavour of probiotic soymilk chocolate

The quadratic model for flavor was found significant ($p<0.0001$) with sensory score ranging from 6.7 to 8.0: and data recorded during experimental were fitted in quadratic model as given below:

$$\text{Flavor} = +7.47+0.34\times A+0.012 \times B+0.017 \times C-0.046 \times A \times B+0.036\times A \times C-0.059\times B \times C -0.044 \times A^2 -0.012 \times B^2 -1.815E-003 \times C^2$$

Soy milk powder, skim milk powder and cocoa powder significantly enhanced the flavour of the guava milk chocolate (Fig. 3 (c) and (d) respectively).

Mouthfeel of probiotic soymilk chocolate

The average mouth feel score of guava milk chocolate varied from 6.18 to 7.6 (Table 2). The quadratic model for mouth feel was found significant ($p<0.0001$) and the data were fitted in quadratic model as mentioned below:

$$\text{Mouth feel} = +7.28-0.031 \times A+0.37 \times B+0.029 \times C+0.020 \times A \times B-1.000E-002 \times A \times C-0.052\times B \times C0.049 \times A^2 -0.12 \times B^2 - 0.031 \times C^2$$

Fig.3 (e and f) the response surface plot for mouthfeel as influenced by skim milk powder, soy milk powder and cocoa powder levels.

From Fig. 3(e) is obvious that with an increase in levels of soymilk powder, there was decrease in sensory score for mouth feel. Contrary to this, the increasing levels of skim milk powder emphatically increased the mouth feel. Fig. 3(f) shows that with increasing levels of cocoa powder, mouth feel increased considerably.

Table.1 Process variables used in the central composite design for three independent variables

Independent variables	Factors	Units	Coded variables				
			-2	-1	0	+1	+2
Soymilk	A	%	8.30	10.001	12.50	15.00	16.70
Skim milk	B	%	13.30	15.00	17.50	20.00	21.70
Cocoa powder	C	%	8.30	10.00	12.50	15.00	16.70

Table.2 Experimental design for optimization experiments for developing probiotic soymilk chocolate using response surface methodology

Run	Soy Milk Powder (%)	Cocoa Powder (%)	Skim Milk Powder (%)	Body &Texture	Odour/ Flavour	Mouth feel
1	10	20	15	7.67	7.1	7.5
2	8.2	17.5	12.5	7.4	6.7	7.1
3	12.5	17.5	12.5	7.49	7.63	7.17
4	12.5	13.29	12.5	7.32	7.39	6.18
5	15	20	15	7.82	7.65	7.41
6	12.5	17.5	12.5	7.43	7.41	7.39
7	12.5	17.5	12.5	7.4	7.39	7.45
8	12.5	13.29	12.5	7.47	7.43	7.41
9	15	15	15	7.91	7.9	6.8
10	12.5	21.7	12.5	7.38	7.49	7.6
11	12.5	17.5	8.29	6.8	7.45	7.2
12	10	20	10	7.17	7.21	7.45
13	16.70	17.5	12.5	7.45	8.0	7.1
14	15	15	10	7.1	7.63	6.58
15	12.5	17.5	16.70	8.31	7.49	7.1
16	10	15	10	7.19	7.04	6.7
17	12.5	17.5	12.5	7.27	7.52	7.1
18	15	20	10	6.97	7.69	7.4
19	12.5	17.5	12.5	7.24	7.43	7.16
20	10	15	15	7.86	7.09	6.97

Table.3 Levels of responses fixed for optimization of guava milk chocolate

Name	Goal	Lower limit	Upper limit
Soy milk Powder (%)	maximize	10	10
Skim Milk Powder (%)	is in range	15	20
Cocoa Powder (%)	is in range	10	10
Body & texture	maximize	6.8	8.31
Odour/ Flavour	maximize	6.7	8
Mouthfeel	is in range	6.18	7.6

Fig.1 Protocol for preparation of probiotic soymilk-based chocolate.

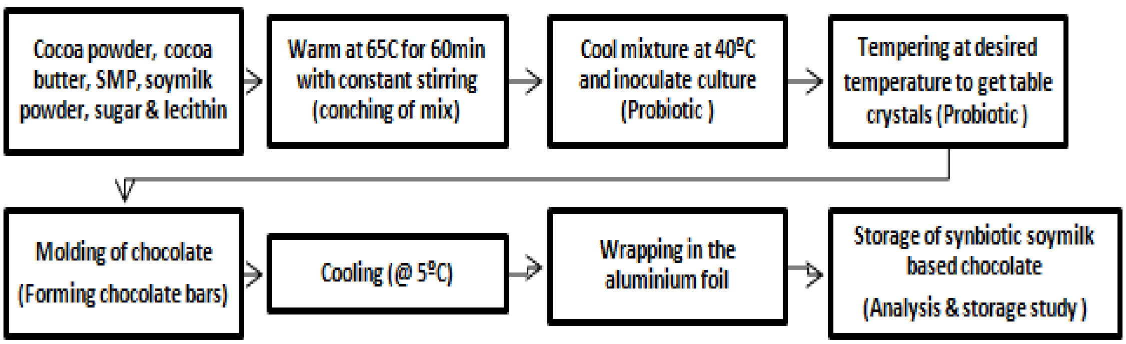


Fig.2 Survival (log cfu/g) of Lactobacillus helveticus MTCC 5463 of soymilk chocolate during storage at refrigerated (7 ± 1°c) temperature

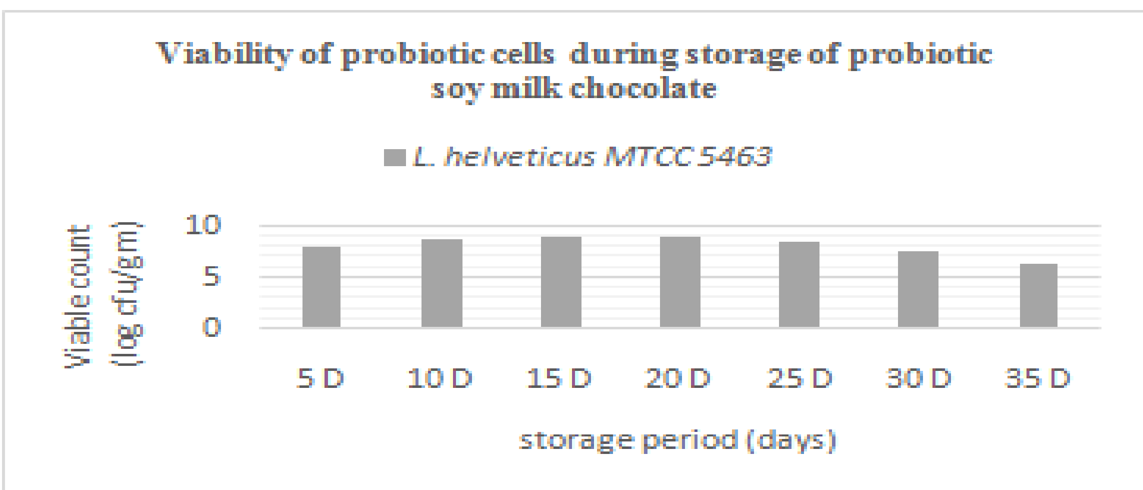
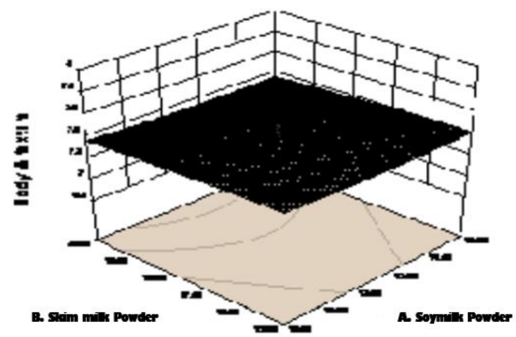
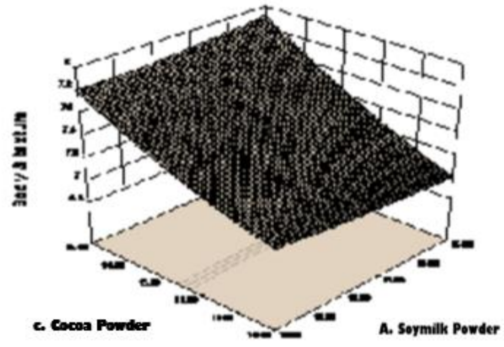


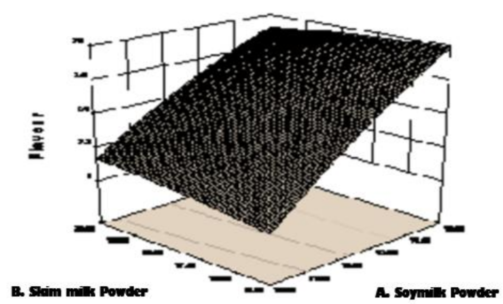
Fig.3 (a-f) Represents the Interactive between the deferent variables on body & texture, flavour and mouthfeel score of probiotic soymilk chocolate, respectively



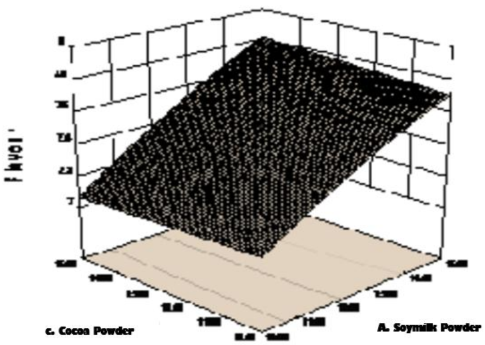
(a). Response surface of body and texture as influenced by levels of skimmilk powder and soymilk powder in probiotic chocolate



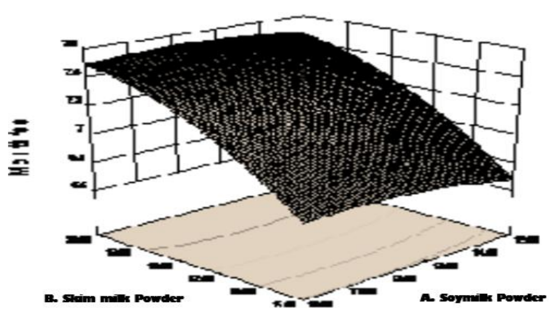
(b). Response surface of body and texture as influenced by levels of cocoa powder and soymilk powder in probiotic chocolate



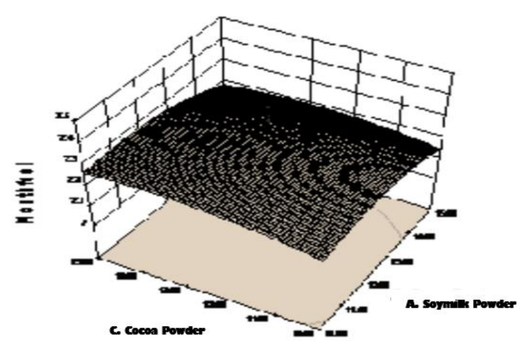
(c). Response surface of flavour as influenced by levels of skimmilk powder and soymilk powder in probiotic chocolate



(d). Response surface of flavour as influenced by levels of cocoa powder and soymilk powder in chocolate



(e). Response surface of mouthfeel as influenced by levels of milk powder and guava powder in guava milk chocolate



(f). Response surface of mouthfeel as influenced by levels of milk powder and guava powder in guava milk chocolate

Viability of probiotic *Lactobacillus helveticus* MTCC 5463

The viable counts of free as well as freeze dried *Lactobacillus helveticus* MTCC 5463 were unchanged during the storage at refrigerated conditions up to 60 days (Fig. 2) and were higher than the recommended level by International Dairy Federation guidelines, i.e., 10^7cfu/g at the end of the product shelf.

Developed product using the optimization process through response surface methodology

Proximate composition of optimized guava milk chocolate Optimized guava milk chocolate showed 02.81 % moisture, 01.87 % Protein, 30.04 % fat, 04.10 % ash and probiotic count was observed > 10^8cfu/gm in freshly prepared probiotic soymilk chocolate.

Levels of different responses were fixed as shown in the Table.3. The solution was obtained for optimized guava milk chocolate condition having value as 14.13 % guava powder, 15 % milk powder and 15 % cocoa powder and atthis level the score of graininess, body & texture, color, flavor, mouth feel & hardness were as 7.21, 7.91, 7.75, 7.77, 6.77 and 3937 gm, respectively.

In present time trend of diversification of probiotic foods is in demand towards the non-fermented and heat-treated food products; probiotic milk chocolate is a relatively innovative addition to the growing list of functional foods. RSM was successfully optimized the formulation of probiotic soy milk chocolate containing soy milk, skim milk, cocoa butter cocoa powder and probiotic. It was observed that probiotic cells were significantly survived up to 30 days of storage and after 30 days decline in cell count was observed in lactobacilli in probiotic soy milk chocolate during storage at refrigeration conditions. Therefore, soy milk powder and *Lactobacillus helveticus* MTCC 5463 was successfully incorporated to milk chocolate, though some degree of sandiness persisted. The sensory characteristics viz., flavor, body & texture and mouth feel were comparable to traditional milk chocolate.

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