DEVELOPMENT OF PROBIOTIC BEVERAGE CONTAINING WHEY PROTEIN AND BETEL LEAF EXTRACT

MINOR RESEARCH PROJECT REPORT

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Introduction:

The primary role of diet is to provide sufficient nutrients to meet metabolic requirements while giving the person a feeling of satisfaction and well-being. Recent reviews, however, support the fact that, beyond meeting nutritional needs, diet may modulate various physiological functions and may play detrimental or beneficial roles in some diseases. Today concepts in nutrition are expanding emphasizing on the use of foods to improve health and reduce the risk of diseases.

Aim of the Study:

The present study was carried out to evolve a protocol for the development of probiotic beverage powder containing whey protein (WP) and betel leaves extract (BLE).

Materials and Methodology:

Bangla variety of betel leaves were procured from a wholesale market in Mumbai. Whey protein concentrate (WPC) 70 was purchased from Mahaan Proteins Limited, New Delhi. 1 ampoule freeze dried probiotic culture (Bacillus coagulans 322: DSM-1) was procured from National Collection of Dairy Cultures, Dairy Microbiology Division, National Dairy Research Institute, Karnal- 132 001.

1. Preparation and Spray Drying of Betel leaves extract

1.1 kg of *Bangla* variety of betel leaves were treated in air convection drier at 60^{o}C. The dried leaves were ground in a mixer. 95 g of the powder was obtained which was extracted using distilled water at a ratio of 30:2.5 (ml:g) at 60^{0} C for 1 hour. The mixture was filtered and a feed solution was prepared using an encapsulating material, maltodextrin in the ratio of betel leaves extract to 5% w/v maltodextrin solution of 1:1. The final volume of the feed solution was 2400 ml. The above solution was then spray dried at the inlet drying temperature of 110.5-110.9^{0} C, outlet temperature of 60-75^{0} C and aspirator rate of 47% (LU 22 Mini Spray Drier, Labultima). Total dried betel leaves powder obtained by spray drying was found to be 32.6 g. The spray dried betel leaves extract powder (BLP) was then packed in triple laminated bags.

2. Revival and spray drying of probiotic culture

Freeze dried *Bacillus coagulans* was revived. The culture was grown using pre sterilized medium (121^{o}C, 30 mins), per 100 ml of nutrient broth. Upon cooling to 38^{o} C, the medium was inoculated with *Bacillus coagulans* and fermentation was carried out on an incubator shaker. Inoculum was built up by inoculating the culture in smaller volumes of broth thus increasing culture volume. The inoculation was done in small volumes of broth and at each step the purity of culture was tested by gram staining and by isolating on agar plate using spread plate technique. Many such cycles were repeated to build up the required cell number.

After complete sporulation, 100 ml *Bacillus coagulans* broth was centrifuged and viable count was analyzed by performing serial dilutions of the sample using sterile saline as diluents. 10 fold dilutions were performed till the sample showed an OD of 0.02 in colorimeter. The sample which showed an OD of 0.02 was used to perform 10 fold dilutions by pipetting out 0.5 ml of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} in a sterile test tube containing 4.5 ml sterile saline.

600 ml of *B. Coagulans* and saline solution (1:10, v/v) was fortified with pre sterilized calcium lactate at the ratio of 5:1 (v/g) i.e, 120 g, under aseptic conditions, on complete sporulation. According to Yadav et al (2008), calcium lactate shows 73% survival of spores of *B. Coagulans*, when it is used as an encapsulating agent [10]. The culture was spray dried at an inlet drying temperature of 110.2-110.5^{0} C, outlet temperature of 55-75^{0} C and aspirator rate of 48%. Total dried culture powder obtained by spray drying was found to be 220 g. The spray dried powder was then packed in triple laminated bags and 10 fold dilutions were performed to check for viability. The viability of spray dried probiotic powder was found to be 1265.7 x 10^{8}cfu/g.

3. Development of Spray dried powder using suitable additives

Betel leaves have a fresh pepperly taste and clove like flavor which highly dominates in the beverage. Owing to this, the strong flavor was reduced by addition of fennel seed powder (13%) and cardamom powder (3%). Natural flavoring substances are substances obtained from plant or animal raw materials, by physical, microbiological or enzymatic processes. Cardamom and fennel seeds are used for flavoring of beverages and drinks such as coffee and tea.

With an objective to boost its commercial viability and improve the acceptability of the beverage, sugar was added to 78% WPC and 5% BLP. After many trials this percentage of WPC and BLP was selected.

The resulting samples including i) PDP (Probiotic drink powder): WPC + BLP + Fennel seed powder + Cardamom powder (Control) ii) PDPS (Probiotic drink powder containing sugar): Control + Sugar (7.5 gm) were packed in triple laminated bags.

4. Microbial Analysis

4.1 Viable Count (VC) Assessment:

VC was determined to find out number of *B.coagulans* in the given sample. 10 fold dilution was performed and 0.1 ml sample was poured into sterile nutrient agar plates. Spreading was done by surface spreading. The plates were incubated at 37^{o}C for 48 hours.

4.2 Acid Tolerance Test

Culture was grown in MRS broth at 37^{0}C overnight, and subcultured in 30 ml of fresh MRS broth adjusting to different pH values (2, 2.5, 3, 4, 7) with hydrochloric acid (3.0 M). Sample was incubated overnight at 37^{0}C. Cells were serially diluted 10 fold and 0.1 ml was poured into sterile MRS agar plates. Spreading was done by surface plating. The plates were incubated at 37^{0}C for 48 hours. The survival rate was calculated as percentage of colonies grown on MRS agar compared to the initial bacterial concentration.

4.3 Bile Tolerance Test

Culture was grown in MRS broth at 37^{0}C overnight, and sub cultured in 20 ml of fresh MRS broth containing bile (sodium taurocholate) (0.1-1% w/v) in 10 flasks. Sample was incubated overnight at 37^{0}C. Cells were serially diluted 10 fold and 0.1 ml was poured into sterile MRS agar plates. Spreading was done by surface plating. The plates were incubated at 37^{0}C for 48 hours. The minimal inhibitory concentration (MIC) of bile for a strain was determined as the lowest concentration totally inhibiting the growth.

4.4 Salt Tolerance Test

Culture was grown in MRS broth at 37^{0}C overnight, and sub cultured in 20 ml of fresh MRS broth adjusting to different salt concentrations (1%, 2%, 3%, 4%, 5%, 6% and 6.5%) with sodium chloride. Sample was incubated overnight at 37^{0}C. The growth of culture was determined by the presence of turbidity.

4.5 Temperature Tolerance Test

Culture was grown in MRS broth at 37^{0}C overnight. Spreading was done by surface plating. The plates were incubated at room temperature (28-32^{0}C), refrigeration temperature (3-4^{0}C), 37^{0}C and 45^{0}C for 48 hours. The growth of culture was determined by the growth of colonies.

4.6 Antibiotic Sensitivity Test

Kirby-Bauer test was used to determine the susceptibility or resistance of the culture to various antibiotics like Ampicillin (AMP^{10}), Chloramphenicol (C^{30}), Tetracycline (TE^{30}), Norfloxacin (NX^{10}). A standardized suspension of organisms was inoculated onto Mueller-Hinton agar. Paper

disks impregnated with the above mentioned antibiotic concentrations were placed onto the agar (mcg/disc). After 48 hours of incubation, the diameters of the zones of growth were measured. The results were compared with established values to determine the organism's susceptibility or resistance to each antibiotic.

5. Sensory Evaluation

The evaluation was carried out at Research Laboratory, College of Home Science, Nirmala Niketan, Mumbai, to determine the overall acceptability of the variations of spray dried beverage at day/s 1, 8, 16 and the results were compared. The study had 15 semi trained panelists and the powder was reconstituted with 200 ml water and was served in plastic cups labeled with random digits to the panelist for sensory evaluation. The panelists were briefed about the product and the attributes to be tested before starting the evaluation. Reconstituted beverage was evaluated using a 7 point hedonic scale. Sensory scores were analyzed statistically by ANOVA to evaluate the significance at $p < 0.05$.

6. Proximate Principle Analysis

Nutritional analysis (energy, carbohydrate, protein, fat) of the spray dried probiotic powder containing whey powder and betel leaf extract powder was done at a Govt. Recognized laboratory (AnaZealAnalyticals& Research Pvt. Ltd, Navi Mumbai).

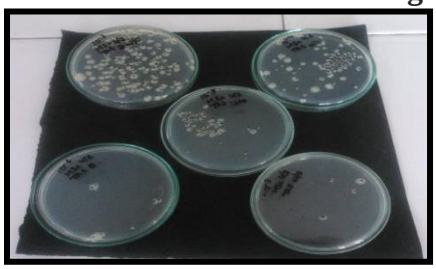
Results & Discussion:

1. Microbial Analysis

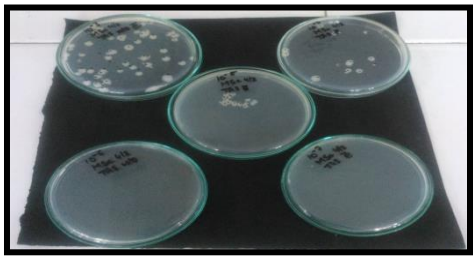
1.1 Viable Count (VC) Assessment:

In the present study, VC for the spray dried powder mixtures was estimated for a period of 6 months.

Figure 1: Estimation of Viable Count



Day 30: PDP



Day 30: PDPS

Table 1: VC of PDP and PDPS

Storage Period	PDP (x 10^8 cfu/g)	PDPS (x 10^8 cfu/g)	Storage Period	PDP (x 10^8 cfu/g)	PDPS (x 10^8 cfu/g)
Day 1	2.3	0.4	**Day 75**	0.05	0.05
Day 8	1.8	0.3	**Day 120**	0.05	0.05
Day 16	0.4	0.2	**Day 150**	0.1	0.06
Day 30	0.1	0.2	**Day 165**	0.1	0.02
Day 45	0.84	0.27	**Day 180**	0.1	0.01
Day 60	0.07	0.05			

Survival of probiotic in a spray-dried form during storage is higher at lower moisture content. The level of survival of probiotic culture remained constant during 6 months of powder storage at 4°C after spray drying.

Figure 1: VC of spray dried probiotic powder containing whey protein and betel leaves

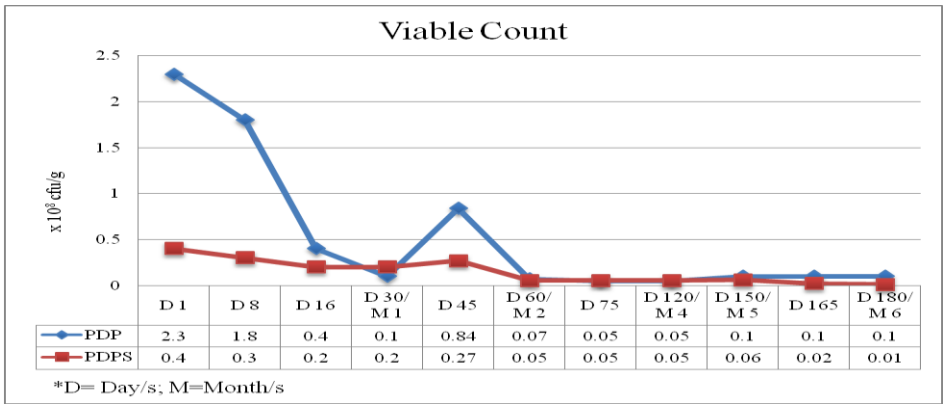


Figure 1 shows that the cell number in the probiotic drink powder containing sugar remained almost constant till 6 months. In control, there was 10 fold reduction in the number of cells. This proves that sugar stabilizes the bacterial count. So sugar containing product is stable microbiologically.

1.2 Acid Tolerance Test

The pH of gastric acid is 1.5 to 3.5 in the human stomach lumen, the acidity being maintained by the proton pump H^+/K^+ ATPase. The parietal cell releases bicarbonate into the bloodstream in the process, which causes a temporary rise of pH in the blood, known as an alkaline tide.

Table 2: Acid tolerance test of Bacillus Coagulans

pH	Survival of Bacillus coagulans ($x10^8$ cfu/ml)
2	0
2.5	0.2
3	0.00045
4	0.0022
7	14.33

As seen from table 2, *Bacillus coagulans* is sensitive to acid (low pH). The survival rate was better at pH 7.

Figure 2: Growth of *Bacillus coagulans* at pH 7

1.3 Bile Tolerance Test

The concentration of bile salts in the small intestine ranges from approximately 0.2 to 2% (wt/vol), depending upon the individual and the type and amount of food ingested.

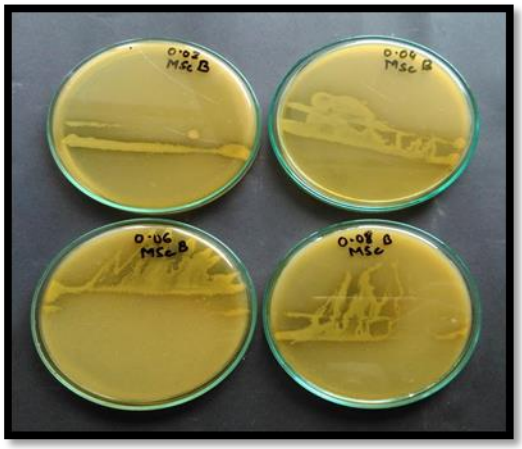
Table 3: Determination of Minimal Inhibitory Concentration (MIC) of bile

MIC Bile (%)	Results
0.1	++
0.2	++
0.3	++
0.4	++
0.5	-

MIC Bile (%)	Results
0.6	-
0.7	-
0.8	-
0.9	-
1.0	-

B. Coagulans can tolerate 0.4% of sodium taurocholate.

Figure 3: Plates showing growth of culture in 0.1-0.4% bile concentration



1.4 Salt Tolerance Test

Table 5: Salt tolerance test

Salt Concentration (%)	Presence of turbidity
0.2	++
0.4	++
0.6	++
0.8	++

Salt Concentration (%)	Presence of turbidity
1	-
1.2	-
1.3	-

Figure 4: Flasks showing presence of turbidity in 0.2, 0.4, 0.6, 0.8% salt concentration and absence of turbidity in 1, 1.2, 1.3% salt concentration

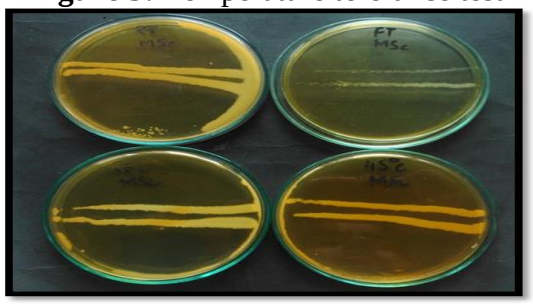
1.5 Temperature Tolerance Test

Table 4: Temperature tolerance test

Temperature (°C)	Growth of colonies
Room temperature (28-32°C)	+++
Refrigeration temperature (3-4°C)	+
37°C	++
45°C	++

The optimal temperature for growth of *Bacillus coagulans* is 30-37°C [22]. The culture was found to survive at all the temperatures however, the growth was found to be stagnated at refrigerated temperature.

Figure 5: Temperature tolerance test



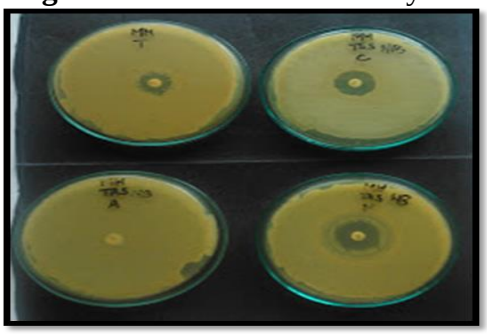
1.6 Antibiotic Sensitivity Test

Table 4: Antibiotic susceptibility profile of *Bacillus coagulans* by the disc diffusion method using zone size interpretative chart for antibiotics as per CLSI (Clinical and Laboratory Standards Institute)

Antibiotic	Inhibition diameter (mm)	Classification	Antibiotic	Inhibition diameter (mm)	Classification
Ampicillin Resistant 9mm or less): 28 Intermediate (mm): - Sensitive (mm or more): 29	9	**Resistant**	Tetracycline Resistant 9mm or less): 18 Intermediate (mm): 19-22 Sensitive (mm or more): 23	29	Sensitive
Chloramphenicol Resistant 9mm or less): 17 Intermediate (mm): 18-20 Sensitive (mm or more): 21	21	Sensitive	Norfloxacin Resistant 9mm or less): 12 Intermediate (mm): 13-16 Sensitive (mm or more): 17	25	Sensitive

Ampicillin, chloramphenicol, norfloxacin, tetracycline are common antibiotics which are used to cure intestinal infections. *Bacillus coagulans* was found to be resistant to ampicilllin however, it was sensitive to the other antibiotics.

Figure 6: Antibiotic Sensitivity test



2. Sensory Evaluation

At day 1, 8, 16 and 30, sensory evaluation scores showed that PDPS showed higher acceptability in terms of aroma, appearance, flavour, taste than control sample (Figure 3). This could be because of presence of sugar in the sample. However the results were not found to be significant. ($p > 0.05$).

Figure 7: Sensory evaluation of reconstituted PDP and PDPS

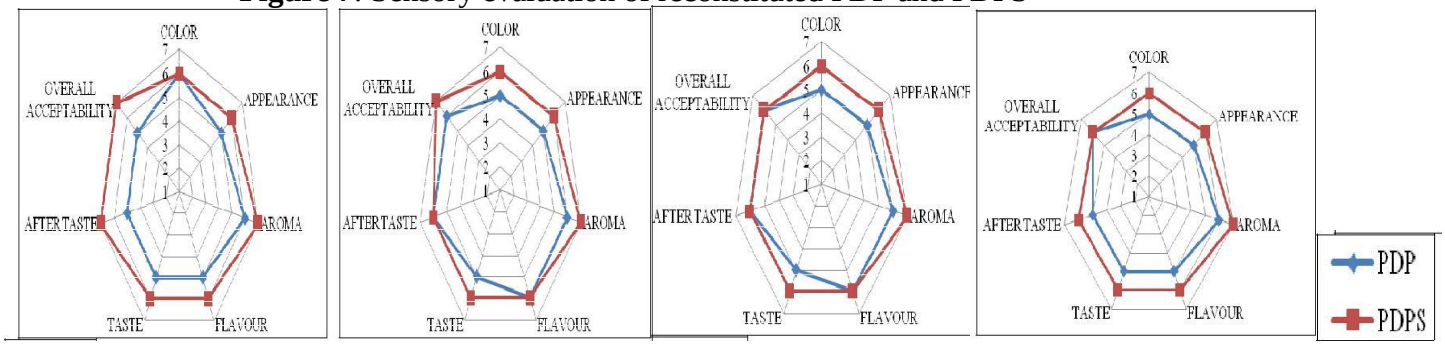


Figure A: Day 1 Figure B: Day 8 Figure C: Day 16 Figure D: Day 30

3. Nutritional Analysis:

Nutritional analysis was carried out by chemical estimation of total energy, carbohydrate, protein, fat, moisture, ash, dietary fiber content.

Table 5: Nutritional composition of PDP and PDPS

			PDP		PDPS
Sr. No.	**Parameters**	**Per 100 g**	**Per serving (15 g)**	**Per 100 g**	**Per serving (15 g)**
1.	Energy (kcal)	370	55.5	374	84.15
2.	Total Carbohydrate (g)	31.5	4.72	50.37	11.33
3.	Dietary Fiber (g)	5.71	0.85	5.6	1.26
4.	Protein (g)	53.31	7.99	38.94	8.76
5.	Total fat (g)	3.46	0.51	1.82	0.4
6.	Ash (g)	3.44	0.53	2.62	0.58
7.	Moisture (g)	7.33	1.09	5.63	1.26

It is clear from table 5 that carbohydrate and calorie content was higher in PDPS because of addition of sugar. It was observed that there was no difference found in the protein content, fat content, moisture and ash between the two samples since the concentration of WPC and BLP were constant which contributes to the macronutrients. No micronutrients (vitamins or minerals) were analyzed in the sample.

The protein content of the product developed in the study had higher protein content per serving than the commercial preparation available in the market which provides 2-6 g protein per serving. This makes it ideal to use as a high protein supplement in critical conditions. However, its micronutrient content, presence of gluten and allergens should be tested before prescribing it.

Conclusion:

Probiotic drink powder containing whey protein and betel leaves was developed with the view to impart nutritional benefits and it can be sold commercially. Based on the results obtained it can be concluded that addition of cardamom, fennel seeds enhanced the acceptability scores. PDPS had higher calories compared to PDP. The control sample showed higher viable counts as against the sample which contained sugar. However, viable count was found to be more constant in case of the sample containing sugar. The organism was not found to survive at low pH however it survived the bile concentration i.e., 0.1-0.4% which is normally found in the gastrointestinal tract. It can tolerate high temperatures, salt concentration of 0.8%. It was found to be sensitive to antibiotics except for ampicillin. The study showed that the probiotic organism survived the spray drying procedure hence it can be added to make a healthy powder containing biological benefits. Probiotic products found in the Indian market contains 6.5-7 billion beneficial bacteria (*Lactobacillus casei* strain Shirota). PDPS had higher calories compared to PDP. Further studies

are required to estimate the micronutrients and variety of betel leaves can be tested to develop the product.

References:

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