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Research Article

ISOLATION AND FREEZE DRYING OF CERTAIN PROBIOTIC STRAINS

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ABSTRACT

The aim of the present study was to evaluate few probiotics strains available commercially in Lahore city and isolation and identification of strains from these commercial probiotics. The identified and isolated probiotic strains were freeze dried. These strains included *Lactobacillus acidophilus, Bifidumbacterium bifidus* and *Saccharomyces boulradii.* Both the commercial and laboratory freeze dried probiotic micro-organisms were subjected to microbial studies. The isolation of the microbes was done by using different types of growth media and identification of microbes was based on fermentation of different carbohydrates, gas production from glucose, growth at various temperature and tolerance to sodium chloride, colony characteristics and staining. The actual microbial count in laboratory of commercial probiotics indicated that there was a discrepancy in the viable count stated on the label of *Lactobacillus acidophilus, Bifidumbacterium bifidus* i.e., the actual viable count is less than the stated viable count on the label. This laboratory evidence suggest that bacteria cannot survive longer time on shelves which is probably the main reason that probiotics inspite of their *in vivo* effectiveness have not found a good way in physician prescription pattern. However, such discrepancy was not observed in case of yeast. The microbiological studies suggested that it is feasible to isolate, identify and freeze dry the microbes in the laboratory and pilot plant scale. The microbial count after freeze drying of laboratory prepared probiotics had shown that anaerobic bacteria (*Bifidumbacterium bifidus)* survived more during the freeze drying as compared to *Lactobacillus acidophilus* and yeast survive more than the both bacterial strains.

Keywords: Probiotics, fermentation and freeze drying.

INTRODUCTION

As defined by the Food and Agriculture Organization of the United Nations and the World Health Organization in October, 2001, probiotics "are live microorganisms which when administered in adequate amounts confer a health benefit on the host". The word Probiotic was coined in the 1960s to name substances produced by microorganisms which promoted the growth of other microorganisms[1]. The probiotic bacteria used in commercial products today are mainly members of the genera *Lactobacillus, Bifidobacterium* and Yeast but other microbes are also used. *Lactobacillus* species from which probiotic strains have been isolated include are *L. acidophilus* BG2FO4, INT-9, NCFB 1748 NCFM and DDS-1, *L. plantarum* ST31, *L. johnsonii* LA1, *L. casei Shirota, L. fermentum* and *L. rhamnosus* strain such as GG 60 .Specific strains of bifidobacteria used as probiotics include *B. breve* strain Yakult and RO7O, *B. lactis* Bb12, *B. longum* RO23*, B. bifidum* RO71, *B. infantis* RO33, *B. longum* BB536 and SBT-292811; *Streptococcus thermophiles* a probiotic micro-organism. *Streptococcus salivarus* subspecies thermophilus type 1131 is another strain used in commercial probiotics[2]. The principal probiotic yeast is *Saccharomyces boulardii*[3]. Probiotics exhibit antimicrobial, immunomodulatory, anti carcinogenic, anti diarrheal and antioxidant activities. The antimicrobial activity of probiotics is thought to be accounted for, in large part, by their ability to colonize the colon and reinforce the barrier function of the intestinal mucosa. In addition, some probiotics have been found to secrete antimicrobial substances. These substances are known as bacteriocins. Such bacteriocin has been isolated from *Lactobacillus plantarum* ST31, a probiotic derived from sour dough[4]. *Saccharomyces boulardii* has been found to secrete a protease which digests two protein exotoxins, toxin A and B, which appear to mediate diarrhea and colitis caused by *Clostridium difficile*. The proposed uses of probiotics are, in the treatment of various digestive illnesses such as ulcer due to *Helicobacter pylori*[5], clearing up constipation[6], reducing episodes of chronic diarrhea[7], antibiotic associated diarrhea, alleviation of symptoms of lactose malabsorption[8], acute diarrhea[9], candiasis[10], antibiotic effects such as antibacterial effects against *E. coli*[11], Crohn's disease[12], liver injury[13], food poisoning[14], ulcerative colitis[15], act as anti hypertensive[16], reduce the risk of heart attack[17] and reduce cholesterol level in the blood[18], can prevent urinary tract and vaginal infections, can be used against mucosal infections[19] and antiviral effects such as gastroenteritis in children[20]. Today there are more than 70 lactic acid bacteria containing products worldwide. More than 53 different types of probiotic milk products are marketed in Japan alone, including frozen desserts like frozen yogurt[21], frozen dough[22], sour cream and ice cream[23]. Probiotics have been incorporated into drinks e.g., kefir like beverages[24] and whey beverages[25] and effervescent preparations[26]. Those are also being marketed as tablets such as enteric soluble royal jelly tablets[27], enteric coated tablets[28] and chewable tablets[29], as capsules such as gel encapsulated probiotics[30,31] and as microcapsules[32]. These have been marketed as mineral water composition[33]. Probiotics are also marketed in combination with antibiotics[34], vitamins and prebiotics[35]. The Fermented Milks and Lactic Acid Beverages Association in Japan have introduced a standard of a minimum of 107 CFU/ml viable probiotic cells for fresh dairy products (minimum concentration of probiotic required for beneficial effect). The scientist's have[36] suggested a minimum viable number of 106 CFU/ml or gram but recommends 108 CFU/g to compensate for reduction through passage through the gut. A typical daily dose should supply about 3 to 5 billion live microorganisms[37]. The typical dose of *Saccharomyces boulardii* yeast is 250 mg twice daily[38].

Aims and objective

The aim of the present study was to evaluate some of the probiotics for their qualitative and quantitative counts available commercially in Lahore city, identification, isolation and freeze drying of the isolated strains in pure cultures.

MATERIALS AND METHODS

In the Lahore city the probiotics were available only as capsules and powder for oral administration. Probiotics of three different brands were purchased and isolated in pure cultures, identified and freeze dried. These probiotics were designated as "commercial probiotics". Details related to brand, type of strain, dosage form, packaging etc., can be seen in Table 3.

Isolation of Strains

Viable count for bacteria and yeast

Pour plate method was used for the quantitative determination of bacteria and yeast[39]. The seal of the packing was broken near the burner and a capsule was transferred with help of a sterilized forceps to a test tube containing 9 ml sterile water (water blank) to make 10^{-1}. It was labeled as dilution -I along with the brand name of the probiotic and from this further six serial dilutions were prepared for bacteria. 1 ml of the last three dilutions was poured aseptically in sterilized petri dishes separately and then added molten nutrient agar medium (N.A) maintained at 40^0C and mixed. It was allowed to solidify and then incubated at 37^0C under anaerobic conditions for 24 hours. For yeast an amount of 1 g (4 sachet, each contain 250 mg) was used and dilutions were prepared in the similar manner as for bacteria and last three dilutions were poured in sterilized petri dishes separately and then added molten Sabouraud's Dextrose Agar (SDA) medium, maintained at 40^0C and mixed. It was allowed to solidify and then incubated at 22^0C for 48 hours. For the separation of the microbe's dilution -I of the three probiotics was streaked on the surface of three NA agar plates /SDA agar plates. Also NA agar slants/SDA agar slants were streaked from the dilution-I of the three probiotics. The plates and slants for bacteria and yeast were incubated as described earlier. The petri dishes and the slants for bacteria were placed in the desiccators, a enlighten candle was inserted to convert the oxygen to CO_2[40].

Identification of bacteria

The isolated 24 hours old pure cultures of bacteria were stained and examined for their morphological characteristics. The original method of Gram[41] modified by Conn[42] was used to stain the bacteria. Bacterial cultures were also stained for presence of spores. The identity of the cultures was based on the characteristics of the bacteria as described in Bergey's Manual of Determinative Bacteriology[43], fermentation of different carbohydrates, gas production from glucose, growth at various temperature and tolerance to sodium chloride.

Identification of yeast

The colonies of the yeast on SDA medium were examined after incubation and stained with Lactophenol blue and Safranin for one minute separately[44]. The slides were examined under the microscope to study the morphological characteristics of the yeast.

Preparation of Probiotic

The isolated strains of probiotics (*Lactobacillus acidophilus, Bifidumbacterium bifidus* and *Saccharomyces boulradii*) were freeze dried at Veterinary Research institute, Lahore. In the present experiment a pilot freeze dryer (Edwards Fast 3400) was used. The details of the preparation were as follows:

Cultivation process

The three types of isolated microorganisms had been grown in Roux culture bottles. De MAN Rogosa Sharpe agar (MRS) culture medium[45] was used for cultivation of bacteria and Sabourad's Dextrose agar for cultivation of yeast. The ingredients/ formula of the media is described in appendix A. L-cysteine was used the as growth factor for the cultivation of *B. bifidum*. The optimized cultivation conditions for *B. bifidum* were temperature 40^0C[46], yeast extract concentration 35 g/liter and glucose concentration 20 g/liter and 0.1 ml of 5 % L-cysteine / 100 ml for 24 hours under anaerobic conditions[47]. The growth parameters for *L. acidophilus* included temperature 42^0C[48]. The growth conditions for *S. bouraldii* temperature were 28^0C[49] for 48 hours under aerobic conditions.

Preparation of inoculum

For the inoculation of medium an amount of 50 ml of sterile respective broth for each type of microorganism was inoculated with a loop full (diameter 3 mm) of 24 hours old culture of bacteria and incubated according to the conditions as described earlier. For yeast inoculation 48 hours old culture was used.

Preparation of suspension

After the incubation, 20 ml of sterile water was added in each of the Roux culture bottle to suspend the growth. The time taken to suspend the growth was 30 minutes approximately. The suspension was transferred aseptically in a sterile 500 ml conical flask. To determine the dry cell weight an amount of 10 ml and 50 ml of the suspension was with drawn aseptically in separate centrifuge tubes of different sizes (10 and 50 ml). The procedure used to determine dry cell weight is described below. In order to determine the viable count before freeze drying 1 ml was withdrawn aseptically from each suspension of either bacteria or yeast. To determine the density of the microbial suspension an amount of 30 ml (10 ml separately for three test tubes) of the suspension was withdrawn aseptically. The density was determined by opacity tube method. The procedure is described below.

Centrifugation

The remaining 89 ml of the suspension was centrifuged in 50 ml centrifugation tube at 10,000 rpm for 30 minutes and washed three times with sterile water (the supernatant was discarded and the packed cells were resuspended in sterile water) and centrifuged. The packed cells in the centrifuge tube were transferred aseptically in a sterile flask (with a capacity of 250 ml) and then filled in the ampoules for freeze drying.

Filling of the ampoules

An amount 27 ml of 30 % skimmed milk[50] was added to the collected mass of cells aseptically as a cryopreservative[51]. The cryopreservative was added before freeze drying to protect the microbial cell from tearing by the crystals formed during freezing stage of freeze drying. The 30 % skimmed

milk was prepared by dissolving 30 g of dehydrated skimmed milk in 100 ml distilled water and then sterilized at 110^0C for 20 minutes at 10 Ib pressure/ sq. in. A total amount of 116 ml (89 ml suspension + 27 ml skim milk, 30 %) was poured aseptically with the help of sterilized pipette in 5 ml sterile glass ampoules, 58 ampoules (2 ml in each ampoule). A separate pipette was used for each type of microbe.

Freeze Drying

The ampoules were freeze dried in the pilot freeze dryer (Edwards Fast 3400) Conditions of freeze drying are given in the Table 1.

Determination of dry weight

As the dose of probiotic bacteria is in billions so it was necessary to determine the dry cell weight to find out the quantity of dry cell mass of bacteria. A sterile centrifuge tube was taken and weighted (W1). An amount of 10 ml and 50 ml sample of the suspension was withdrawn aseptically and transferred to the weighted centrifuged tube. It was then labeled with name of respective microorganism and then centrifuged at the required speed for each micro-organism. The settled mass of microorganisms was washed with sterile water and centrifuged again. This procedure was performed twice. The supernatant was discarded and the collected mass was dried at room temperature (25^0C), after drying the tube was weighted (W2). Then the difference of W1 and W2 were taken to determine the dry cell weight (W3).

Measurement of the Density of the Suspension
Opacity Tube Method

Opacity tube method was use to determine cell density of suspension before the freeze drying 52.

Procedure

- A sample of each of the suspension was drawn out in 10 ml amount aseptically and centrifuged for 30 minutes. The supernatant was discarded. Packed cells collected from the bottom and re suspended in an equal amount of normal saline solution and was again centrifuged to wash the cells. The supernatant discarded and packed cells at the bottom were re suspended in 10 ml normal saline and transferred to labeled test tubes.
- Three of the sealed test tubes were compared with McFarland's Nephalometer opacity tubes to check the density and approximate count of microbes per ml.
- The ten opacity tubes were arranged in a rack and labeled from 1 to 10. Each tube had a different density.
- Before comparison each opacity and the test tubes were thoroughly shaken. The standard approximate density in each McFarland's Nephalometer opacity tube can be seen in Table 2.

Determination of Viable Count before Freeze Drying

Pour plate method was used to determine the viable count before freeze drying. An amount I ml of sample drawn aseptically from the prepared suspension and transferred to a test tube containing 9 ml sterile water to make 10^{-1} and from this eight serial dilutions were prepared. The last three dilutions were poured in 1 ml quantity in sterilized petri dishes separately and mixed with the molten medium required for each type of microbe. The petri dishes were allowed to solidify and then incubated. Results can be seen in Table 7.

Determination of Viable Count after Freeze Drying

After freeze drying the viable count was determined again by the pour plate method. A quantity of one g of freeze dried powder of each microorganism was added in 9 ml sterile water to make 10^{-1} and from this 8 serial dilutions were prepared. The last three dilutions were poured aseptically in 1 ml quantity in sterilized Petri dishes containing molten medium and mixed. It was allowed to solidify and then incubated. Results can be seen in Table-6.

Table 1: Conditions of the Freeze Drying

	Temperature	Time in hour	Pressure	Lyophilization time
Freezing	-30 to -35 ^{O}C	6		
Condensing	-45 to – 50 ^{O}C	1		
Vacuum		1	200 m torrs.	
Heating	0 to 35^{o}C (inner guage)	7-9		
Total time		15		15 to 17 hours.

*The total time taken for freeze drying varies but usually it is 15 to 17 hours.

Table 2: Approximate Density in Each Mcfarlad's Nephalometer Opacity Tube

No of tubes.	No. of approximate cells in each opacity tube
1	300,000,000
2	600,000,000
3	900,000,000
4	1,200,000,000
5	1,500,000,000
6	1,800,000,000
7	2,100,000,000
8	2,400,000,000
9	2,700,000,000
10	3,000,000,000

Table 3: Observations of Physical Characteristics and Viable Count of the Commercial Probiotics

Brand name	Probiotic Strain	Manufacturer	Quantity per sachet or capsule	Quantity Per container.	Actual viable count in the laboratory
Enflor	*Saccharomyces boulradii*	Hilton Pharma, Karachi Pakistan	250 mg / Sachet	10 sachet	2.1×10^3 / Sachet
Probiotic DDS-1	*Lactobacillus acidophilus*	Walnut Creck, California	1.5 billion / capsule	30 capsules	1.3×10^3 / capsule
Acidophilus and *bifidum.*	*Lactobacillus acidophilus, Bifidobacterium bifidus L. rhamnosus.*	Natural factor, Canada.	0.5 billion 0.5 billion 4.0 billion (5 billion/ capsule)	90 capsules	2×10^3 / capsule

Table 4: Characteristics of Bacteria Isolated From Commercial Probiotics

No. of tests	Characteristics	*L. acidophilus*	*B. bifidus*
1.	Gram's Reaction[58]	+	+
2.	Endospore[59]	-	-
3.	Motility	-	-
4.	Urease[60]	-	-
5.	Simmon's Citrate	+	+
6.	Gelatin Liquefaction	-	-
7.	Potassium Cyanide	0	0
8.	Methyl Red	A	A
9.	Voges – Proskauer	+	+
10.	Indole[61]	-	-
11.	Decarboxylase (Lysine)[62]	-	-
12.	Decarboxylase (Arginine)[62]	-	-
13.	Sodium Chloride 6.5 %, 7.5 %, 15 %	0	0
14.	Huge and Leifson Test[63]	O/F	O/F
15.	Triple Sugar Iron Agar	+	+
16.	Litmus Milk Test	C	C

O/F = Oxidative Fermentation, O = Oxidation, F = Fermentive, V = Variable Result, C= Coagulated, A = Acid, 0 = no growth

Table 5: Observations for Determination of Dry Cell Weight

Type of the Microorganism	Weight of centrifuged tube W1		Weight of centrifuged tube + microbial mass W2		Dry weight W3	
	For 10 ml	For 50 ml	For 10 ml	For 50 ml	For 10 ml	For 50 ml
Lactobacillus acidophilus	7.246 g	13.944g	7.280 g	69.72 g	0.04 g	55.776 g
Bifidumbacterim bifidus	7.220 g	14.077 g	7.239 g	70.385 g	0.019 g	56.308 g
Saccharomyces boulardii	7.192 g	15.689 g	7.197 g	78.445 g	0.05 g	62.756 g

Table 6: Average Viable Count before and after Freeze Drying

Type of microorganism	Average viable count before freeze drying (cfu/ml)	Average viable count after freeze drying (cfu/g)
Lactobacillus acidophilus	2.6×10^8	2.1×10^8
Bifidumbacterium bifidus	2.8×10^8	2.6×10^8
Saccharomyces boulradii	2.5×10^8	2.3×10^8

RESULTS AND DISCUSSION

Isolation and Identification of Microbes

Viable counting for bacteria and yeast

Table 3 can be seen for the actual viable count in the laboratory from commercial probiotics. The microbiological evaluation declared that there was a discrepancy between the quantitative viable count CFU/ capsule and viable count stated on the label of the commercial probiotics, Probiotic DDS-1 and *acidophilus* and *bifidus* i.e. the viable count was less than the count stated on the label of the products. This is indicating that probiotics containing bacteria are not stable on shelf for a long time.

Characteristics of Micro-Organisms Identified

Lactobacillus acidophilus

When dilutions of commercial probiotics were inoculated on nutrient agar medium, a bacterium was isolated after an incubation period of 24 hours. Its colonies were flat and pale off-white in colour[53]. The colonies become rough after 48 hours of incubation and twisted filaments were seen. When the cultures were incubated for 72 hours a deep felt mass in the centre of the colony could be seen. No pigment formation was observed[53]. The bacterium was gram +ve, rods had round ends occur singly, in pairs and very short chains. It had a mean diameter of 1.05 um. The bacterium was non motile[54]. Growth occurred both in aerobic and anaerobic conditions but profuse growth was seen under anaerobic condition. Growth did not occur at 15^0C and 25^0C. But growth was observed at 35^0C. The bacterium did not show growth above 45^0C. The bacterium did not form spores[54]. The biochemical studies (Table 4) of the bacterium showed no liquefaction of gelatin, citrate was utilized as a source of carbon, while urea was un-affected[55]. Methyl red test was positive[56]. Voges-Proskauer test was positive. The bacterium fermented all the carbohydrates except rhamnose, ribose and starch without the production of gas. Milk was coagulated. Did not show growth in sodium chloride broth at various concentrations (6.5, 7.5 and 12.5 %)[57]. In Triple Sugar Irion Agar medium

both slant and butt were acidic showing the fermentation of both glucose and lactose. On the above basis the bacterium was identified as *Lactobacillus acidophilus*. The identified strain of *L. acidophilus* was lyophilized at Veterinary Research Institute Lahore, Pakistan.

Bifidumbacterium bifidus

When the dilutions of commercial probiotics were inoculated on nutrient agar medium, anaerobically, a bacterium was isolated after 24 hours of incubation. Its colonies on nutrient agar medium were circular, smooth and whitish. Morphologically they were gram +ve, non-motile rods. The rods were bifurcated (Y and V form), branches were also seen. The bacterium did not form spores. The bacterium did not grow at 15^0C and showed limited growth at 25^0C. A good growth rate was observed at 37^0C. The bacterium could grow only under anaerobic conditions. No growth was observed under aerobic conditions. Biochemical studies (Table 4) showed that the bacterium did not liquefy gelatin and used citrate as carbon source, but urea was not utilized. Methyl red test was positive. The Voges – Proskauer test was positive. The bacterium fermented all the carbohydrates except rhamnose, ribose and starch without the production of gas. Milk was coagulated. Arginine test was negative. Did not show growth in sodium chloride broth at various concentrations (6.5, 7.5 and 12.5 %). Triple sugar Iron Agar medium both slant and butt were acidic showing the fermentation of both glucose and lactose. On the above mentioned basis the bacterium was identified as *Bifidumbacterium bifidus*. The identified strain of *B bifidus* was lyophilized at Veterinary Research Institute Lahore, Pakistan.

Saccharomyces boulradii

When the dilutions of the Enflor Sachet (Commercial Probiotics) were inoculated on Sabouraud's Dextrose Agar medium, yeast like fungi was isolated after an incubation of 48 hours. Its colonies on SDA medium were cream coloured, smooth, small and lens shaped. The yeast like fungus was stained with Safarnin and Lactophenol blue. Its cells were oval shaped to elongated, always seen in groups. A single cell with a bud was also observed. The yeast showed a slight growth at 15^0C but maximum growth at 28^0C. It fermented glucose, dextrose, and maltose and GA lactose with the production of gas. Other carbohydrates were not fermented. On these results yeast like fungus was identified as *Saccharomyces boulradii*. The identified strain of *S. boulradii* was lyophilized at Veterinary Research Institute Lahore, Pakistan.

Preparation of Probiotics

Determination of dry cell weight

As the dose of probiotic bacteria is in billions so it was necessary to determine the dry cell weight to find out the quantity of dry cell mass of bacteria to be filled in each capsule. See the Table 5 for the calculated dry weight.

Measurement of the Density of the Culture

Opacity tube method

Opacity tube method was used to determine cell density of suspension before the freeze drying. The test tube matched with the 4th McFarland's Nephalometer opacity tube. The approximate density was 1.2×10^9 approximately before freeze drying. See Table 2 for results. The microbiological evaluation declared that there was a discrepancy between the quantitative viable count CFU/ capsule and viable count stated on the label of the commercial probiotics, Probiotic DDS-1 and *acidophilus* and *bifidus* i.e. the viable count is less than the count stated on the label of the products. However, the isolated strains were identified in pure cultures and the suspensions were freeze dried at the Veterinary Research Institute Lahore, Pakistan. The identity of the culture was based on the characteristics of the bacteria as described in Bergey's Manual of Determinative Bacteriology, fermentation of different carbohydrates, gas production from glucose and growth at various temperatures. The suspensions of the isolated strains were freeze dried in 5 ml glass ampoules and those were designated as laboratory prepared Probiotics. The viable count after the freeze drying indicated that *Bifidumbacteriam* bifidus (anaerobic) survived more than *Lactobacillus acidophilus* (microaerophilic) and yeast survive more than the both bacterial strains due to size.

CONCLUSION

From the above study it can be concluded that it is possible to isolate and identify different types of probiotic microbes and the microbes in pure cultures can be subjected to freeze drying not only at laboratory scale but also at pilot plant scale without the severe damage to microbes during freeze drying i.e. according to the viable count after freeze drying which according to the standards required for typical daily dosing. A typical daily dose should supply about 3 to 5 billion live microorganisms. The typical dose of *Saccharomyces boulardii* yeast is 250 mg twice daily. But the discrepancy found in the viable count stated on the label of commercial probiotics and actual viable count in the laboratory which is indicating instability of the product. Owing to this reason instead of being effective in various illnesses probiotics are not finding a good way as an alternative treatment in the prescription drug pattern and other probiotic containing retail products like ice cream and yogurt etc. are being more popular among consumer.

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