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RESEARCH ARTICLE

ISOLATION AND IDENTIFICATION OF PROBIOTIC STRAINS FROM DANISCO PREMIX YOGURT CULTURE

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ABSTRACT

Probiotics are alive and nonpathogenic microorganisms that are beneficial to maintain host intestinal balance. It was isolated from YO-MIX, Yogurt Cultures 38360 Sasssenage, DANISCO FRANCE by using Trypticase, Phytone peptone yeast extract (TPY) agar added 0.5% propionoic acid as selective agent and inoculated at 37 ^{o}C for 48 hours in anaerobic condition. Test of the colonies showed Y and V shaped. The Biochemical test for indole, catalase, gelatin, nitrate reduction, gas from glucose and arginine showed negative for all samples. According to identify the LAB probiotic strains milk samples inoculated with MRS agar media and the colonies morphology were observed under the microscope and found Rod shaped. Based on the microbiological and biochemical analysis most of the colonies were probiotic mix culture i.e Y, V shaped bifidobacteria and Rod shaped Lactobacilus Strains.

INTRODUCTION

According to the currently adopted definition by FAO/WHO, probiotics are: "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Health and Nutritional, 2001). Probiotics have been used for many years to aid in restoring and maintaining a health intestinal balance in favor of healthful bacteria which is essential to maintaining good health (Puneeth Kumar *et al*., 2012). The term probiotics was introduced by Lilly and Stillwell in 1965 for growth promoting factors produced by micro organism. The word Probiotic is derived from the Greek meaning for pro- life and has had severel different meaning over the years (Lilly and Stillwell, 1965). Although Probiotic relating to food suppliment only dates from 1974, the history of live microbial feed suppliments goes back thousands of years. These bacteria were found to be the predominant component of the dairy products like yogurt. Probably the first foods that contained living micro organism were the fermented milk that are recorded in the old treatment (Roy Fuller History and development of probiotics, 1992). In the last decade, the use of probiotics in fermented dairy product feed product applications has a noticeable interest and develop. These organisms play significant role in lowering the pH of the large intestine through the release of lactic and acetic acid (Takashi Asahara *et al*., 2004). Lactobacilli, bifidobacteria and non-pathogenic yeasts, such as *Saccharomyces boulardii*, are among the most commonly used and thoroughly evaluated probiotics (Buck and Gilliland, 1994). [l]Probiotic lactobacilli may improve lactose digestion and reduce symptoms of lactose intolerance. The effect of Probiotic on serum cholesterol is still inconclusive. A number of studies have examined the potential of probiotic products to reduce serum cholesterol (Henrik Andersson, *et al*., 2001). Probiotic are also used as a live microbial feed (Fuller, 1989). Probiotics are live microbes that can be formulated into much different type of products. Preparation of probiotic yoghurt is a totally new concept.

In our country a huge number of people have suffering with high Cholesterol problem and ultimate it is converted to the CVD (Cardio Vascular Disease). The main reason is due to the adulterated food. So overcome this type of problem Preparation and consumption of Probiotic yoghurt will have potential impact. The contribution of probiotic bacteria in yoghurt to the improvernent of intestinal micro flora has been widely recognized. For effectiveness, these bacteria should overcome the adverse effects of the low pH of yoghurt, antagonistic action of other fermenting Flora, the hostile gastrointestinal environment and competition with gut micro flora (Kailasapathy and Rybka, 1997). Bifidobacteria are known to inhibit many pathogenic organisms both in vivo and vitro, including Salmonella, Shigella, Clostridium,

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Staphylococcus aureus, Bacilus cereus, candida albicans and Campylobacter jejuni (Anand *et al*., 1985). The main objectives of the present study was to isolate and to identify the suitable probiotic strains of bifidobacteria and lactic acid Bacteria to assess their probiotic potentiality which could be used to enhance the health status of human beings.

MATERIALS AND METHODS

Sampling site and collection of sample

The whole experiment was carried out at the Institue of Food Science and Technology (IFST, BCSIR) Dhaka, Bangladesh. Desired Probiotic bacteria were collected from the YO-MIX, Yogurt Cultures 38360 Sasssenage, DANISCO FRANCE and HOWARU, Premium Probiotic Freeze drying cultures from Germany. According to the guideline as mentioned on the level for making mother cultures, 10 mg samples were measured from both the sachet and mixed into 100 ml of 45^{o}C warm milk and incubated at 42^{o}C for 6 hours.

Preparation of Probiotic diet (Yoghurt)

One liter raw milk was taken into SS pan and boiled up to 90^{0}C for 50 minutes. Ten percent Skim milk powder (SMP) was added into milk and mixed vigorously. When temperature reduced to 50^{o}C then added Probiotic cultures (Danisco Probiotic and Yoghurt culture from Denmark) the samples poured into different cups. The milk samples incubated at 42^{0}C for Eight hours. The sample was taken from the incubator and kept in a Refrigerator for further analysis .This analysis was carried out at Nutrition & Food Engineering Microbiological Lab of Daffodil International University.

Microbiological Reagent and Media

Trypticase Phytone Peptone Yeast Extract (TPY) Medium

The Probiotic sample were plated in to TPY consisting of (g/l): Tryptone , 10.0; Soy Peptone , 5.0; Glucose, 5.0; Yeast Extract, 2.5; Dipotassium Hydrogen Phosphate, 2.0; Cysteine hydrochloride, 0.5; Magnesium Chloride Hexahydrated , 0.5; Zinc Sulphate Heptahydrated , 0.25; Calcium chloride , 0.15; Agar, 10.0; Polysorbate ,80 (also known as Tween 80) 1.0; Ferric Chloride trace, 0.003(µg/l); pH-7 at 37^{0}C. Dissolved media was autoclaved at 15 Ibs pressure (121^{0}C) for 15 minutes .The TPY medium was practically a selective medium for the isolation of Bifidobacteria. Similarly for the identification of LAB MRS Agar (oxoid) was used as selective media for the growth of probiotic lactic acid bacteria. All plates were incubated an aerobically at 37^{0}C for two days in anaerobic gas jars capable of producing hydrogen and carbon dioxide. Bifidobacteria was enumerated using MRS or TPY solid medium and LAB colonies were identified by gram staining. Fifteen colonies from the highest dilution of each sample were picked at random and inoculated into TPY agar medium. At last colonies picked from countable plates were selected for gram reaction, morphology and biochemical test.

Biochemical Test for Identification of Bifidobacteria and Lactobacillus

Morphology based identification

The isolated bacteria were subjected to a range of morphology based tests in order to help in their identification.

Colony Morphology

Bifidobacteria grown on MRS agar and TPY agar. Colonies were observed for morphology.

Gram staining

The method of Gram straining was followed by Burkes (1992): Cruickshank, (1975). A smear of bacteria was prepared on a clean glass slide using a sterile inoculating loop. The smear was fixed with heat and then treated with ammonium oxalate crystal violate solution for 30 seconds. This was gently rinsed off and iodine solution was applied for 30 seconds .This was drained off and 95% ethanol was then applied for 20 seconds as decolorizing agent. Finally a counter strain, safranine was added for 10 seconds. Then the slide was gently rinsed off with water and dried .The slide was viewed fewer than 100 X magnification under microscope. The result was recorded as gram positive or negative.

Biochemical Studies of the Selected Isolates

The following biochemical studies were carried out in order to characterize the isolated bacteria.

Method of Catalase Test

Random colonies of LAB and BB were picked up from the MRS and TPY agar plates and inculcated in the respective broth medium. The sample were incubated overnight anaerobically at 37^{o}C. On the microscopic glass slide on drop of the 3% hydrogen peroxide was placed. A loopful overnight culture of LAB and BB were taken from the universal bottle by using a platinum wire and streaked separately on the Hydrogen peroxide solution .The production of gas bubble from the surface of the broth culture indicates a positive reaction.

Arginine test

The arginine test were determined on TPY broth containing bromocresol purple (0.04g/l) as a pH indicator, and supplemented with 0.5% of L-arginine. After autoclaving the media was inoculated with the strains of bacteria. After 24 hours incubation in anaerobic condition cell growth was determined by measuring the optical density at 650 nm using a spectrophotometer

Method of Nitrate Test

The reduction for LAB and BB were followed according to Cruickshank, (1975). LAB and BB were inoculated inn to MRS and Trypticase Phytone Peptone Yeast extract (TPY) broth and incubated at 37^{o}C anaerobically for overnight .An aliquot of 0.1 ml of test reagent (for nitrate reduction test solution A and B for Sigma, USA) was added into overnight broth b culture of LAB and BB and change in color of broth culture were observed. A red color developing within a few minutes indicated the presence of nitrite and hence the ability of the organism to reduce nitrate.

Method of Indole Test

The test for indole in BB was determined according to Macuddin (1984). TPY broth was modified slightly by adding 20 gm of peptone (Difco) and 5 gm of NaCL into one litre

Table 1. Biochemical Analysis for the Identification of Probiotics Strains in Genus level

No. of Samples	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Gas formation from Glucose	-	-	-	-	-
Indole production	-	-	-	-	-
Gelatin Liquefaction	-	-	-	-	-
Nitrate Reduction	-	-	-	-	-
Catalase production	-	-	-	-	-
Arginine test	-	-	-	-	-
Colony Morphology	+ve	+ve	+ve	+ve	+ve

Table 2.Microbiological analysis for the Identification of Probiotics Strains in Genus level

No of Sample	Shapes	Gram straining	Growth at 37°C	Mortility	Catalase	Nitrate reduction	Oxidase test	Gas form glucose	Fermentation of CHO
S1	Short rods	+ve	+	-	-	-	-	-	+
S2	Short & Long rods	+ve	+	-	-	-	-	-	+
S3	Short & Long rods	+ve	+	-	-	-	-	-	+
S4	Short rods	+ve	+	-	-	-	-	-	+
S5	Short & Long rods	+ve	+	-	-	-	-	-	+

broth and autoclaved at 121°C for 15 minutes. Fresh BB culture was inoculated into above TPY broth and incubated anaerobically at 37°C for 48 hours. After 48 hours 0.5 ml Kovacks reagent (Mixed reagent of isomyl alcohol 150 ml, p-Dimemethyl-amylbenzaidehyde 10g, and conc. HCL 50 ml, Sigma, USA) was added into each TPY bifido broth.

RESULTS AND DISCUSSION

The present study envisages that supplementation of TPY with 0.5% propionoic acid was most selective. Addition of 0.5% propionoic acid with TPY media was more selective and enhanced the growth of probiotic bacteria (Beerens, 1991). Five samples were randomly selected for the identification on the morphological characteristics. Colony morphology was used for the tentative identification of genus bifido bacteria (Mitsuoka, 1992). Probiotic strains could be easily determined by yeast colonial morphology after gram straining by its typical bifido bacteria/morphology.

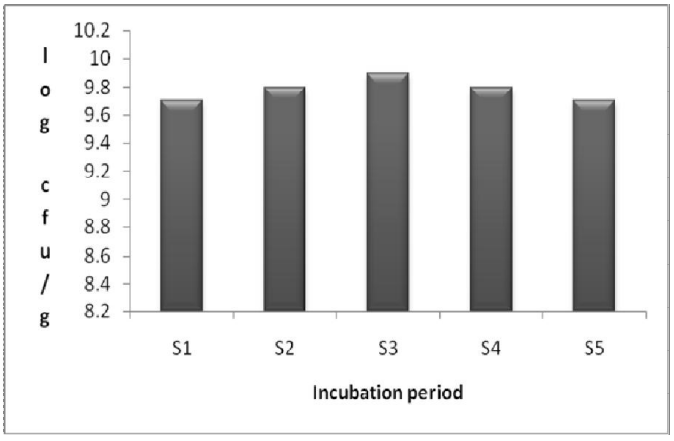

Fig. 1. Log cfu/g of probiotic bacteria in different strains
Figure 1 indicates that bacterial populations S3 was higher 9.9 log cfu/g, whereas for S1, S2, S4, S5 were 9.7, 9.8, 9.8, and 9.7 cfu/g respectively

Definite identifications of the genus bifidobacteria isolated from the yoghurt samples can only be achieved by biochemical test as shown in table 2. It has been shown in table 2 that the biochemical test such as the demonstration of catalase, nitrate reduction, indole production, liquifection of gelatin, gas formation from glucose, alginate all are negative by the genus bifidobacteria. Similar study reported that all the biochemical test such as demonstration of catalase, nitrate reduction, formation of indole, liquifection of gelatin, gas formation from glucose, alginate all are negative by the genus bifidobacteria (Mitsuoka, 1984). Bifidobacteria is commonly used as probiotic bacteria, if the viable cultures could be used as dietary adjunct to human that may have various health benefits. The beneficial effects of bifidobacteria can be expected only by taking viable cells which have an affinity to colonize in the human intestine.

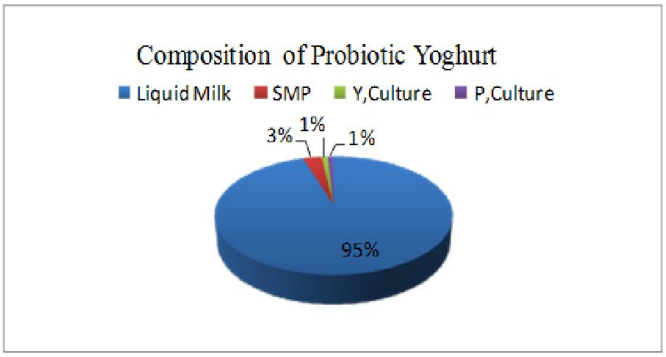

Fig. 2. Composition of Probiotic Yoghurt

Fig 2 shows that the composition of Probiotic yoghurt are liquid milk=95%, SMP=3%, Yoghurt culture=1%, Probiotic culture=1% respectively.

Conclusion

Therefor it can be concluded from this study that the strains isolated from the YO MIX yogurt culture based on the morphological characteristics and biochemical analysis are mainly bifidobacteriam strains and few of them are lactobacillus. After the addition of bifidobacteria and lactobacillus into probiotic yogurt was prepared which have beneficial effect on human health due to production of organic acids i.e. acetic acids and lactic acids that can suppress the pathogenic organisms and increase the immunity systems in the body.

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