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Isolation, Identification and Characterization of Lactobacillus bacteria from soy yoghurt

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ABSTRACT

The aim of this study was isolation, identification and characterisation of lactic acid bacterial strains from soy yoghurt. Four soy yoghurt samples were prepared. Sample A consisted of 100% soy milk, sample B consisted soymilk and 1.5% skim milk, sample C consisted soymilk and 3% skim milk and sample D consisted soy milk and 4.5% skim milk using starter culture Lactobacillus bulgaricus and Streptococcus thermophilus. The sample A had pH 5.37 ± 0.1 which was statistically insignificant with sample C (5.38±0.1) and sample D (5.4±0.02). The pH of sample B (5.43±0.1) was statistically significant with sample A but statistically insignificant with sample C and D. The water holding capacity of sample A (28.53±0.4), sample B (24.51±0.01), sample C (27.39± 0.20) and sample D (30.11± 0.01) were statistically significant. It was observed that bacteria isolated from sample A, sample B and sample C were creamish white in colour while as bacteria isolated from sample D were yellowish in colour. Catalase test was negative whereas oxidase test was positive. Gram staining detected that isolated bacteria were Gram positive. The glucose fermentation test was positive. NaCl tolerance test showed that isolated lactic acid bacteria were able to tolerate 10% and 15% NaCl solution.

Key Words: Biochemical Test, Characterization, Identification, Lactobacilli, Soy Yoghurt.

I INTRODUCTION

Yogurt is produced by adding two starter cultures, *Lactobacillus delbrueckii* subsp. bulgaricus and *Streptococcus thermophilus* to milk [1]. During the fermentation, hydrolysis of the milk proteins occurs, the pH drops, the viscosity increases, and bacterial metabolites are produced that contribute to the taste and possibly to the health promoting properties of yogurt. Several health benefits have been reported for traditional yogurt [2, 3, 4], and this healthy image is enhanced by supplementation with probiotic bacteria. Probiotic bacteria are defined as "live microorganisms that when administered in adequate amounts confer a health benefit on the host" [5]. Fermented foods that have potential probiotic properties are produced worldwide from a variety of food substrates [6]. Lactic acid bacteria including lactobacilli and bifidobacteria are the most common bacterial species considered as potential probiotics [7]. Yogurt produced from cows' milk is consumed in both developing and industrialized countries. However, the demand for alternatives to cows' milk is growing due to problems with allergenicity, desire for vegetarian alternatives, etc., and therefore interest in a soy-based yogurt has developed. Probiotic milk-based

yogurts are now being marketed, and consequently it would be desirable to know if probiotic bacteria can also be incorporated into soy-based yogurt-type fermentations. Probiotic bacteria generally do not grow rapidly in cows' milk. Thus, in yoghurt manufacture, they do not attain as high numbers as the starter cultures [8]. However, many studies indicate that soy is a good substrate for probiotic bacteria [9, 10, 11], but not for the traditional yoghurt starter *L. delbrueckii* subsp. bulgaricus [12, 13]. These findings suggest that some probiotic bacteria could better compete with yoghurt cultures in a soy-based substrate.

Soy milk-based yogurts have emerged as a popular alternative to traditional dairy-based yogurt due to their reduced level of cholesterol, saturated fat and lactose. Soybean (*Glycin max*) is the most widely grown and utilized legumes in the world and the soy products have immense health benefits because soy has good amino acid profile, contain higher levels of essential fatty acids, soluble fiber, vitamins, and minerals. Soy also contains phytochemicals which include isoflavones (genistein, daidzein and glycitein), phytic acid and saponins which have strong antioxidant properties and have capability of lowering the cholesterol level. Furthermore, the incorporation of probiotic bacteria as dietary adjuncts has given rise to increased consumption of fermented soy milk which promotes the ecosystem of intestinal tract. Thus soymilk based yogurts have gained significant consideration for their many nutritional health benefits including reducing cardiovascular disease, weight loss, arthritis and brain function. Enrichment of soy yogurts with health promoting ingredients such as omega 3 and omega 6 PUFAs enhance functional attribute of soy yogurt. Omega 3 PUFA (Poly Unsaturated Fatty Acid) family consists of ALA (C18:3) and its longer chain metabolites EPA (C20:5) and DHA (C22:6) whereas omega 6 PUFA consists of GLA (C18:3).

Unfortunately, little information is available on the growth of probiotic bacteria in mixed cultures with yoghurt strains in soy substrates, because most studies on the growth of probiotics in soy extracts have been carried out using pure cultures [10, 15, 16, 17, 18]. Although some data are available on mixed cultures of probiotics and S. thermophilus [9, 13, 19, 20], little is known of more complex mixtures involving both traditional yoghurt strains. Bifidobacterium [15] can indeed grow more extensively in soy than in cows' milk under comparable conditions. However, very wide variations have been noted in the growth abilities of strains within a given species [15, 11] and more data are needed to better characterize the potential of soy as a substrate to support good growth of bifidobacteria in combination with yoghurt strains. Lactobacilli are also extensively used as probiotics. Soy has been examined as a substrate for the Lactobacillus species *L. casei* [15, 21], *L. helveticus* [14, 15], *L. fermenti* [9, 22], *L. fermentum* [23, 24], *L. reuteri* [25] and *L. acidophilus* [9, 15, 26, 13, 19, 6].

The aim of this study was isolation, identification and characterisation of lactic acid bacterial strains from soy yoghurt.

II MATERIALS AND METHODS

2.1 Sample preparation

The soybean seeds were obtained from the local market, Hazratbal Srinagar. The starter culture (*Lactobacillus bulgaricus* and *Streptococcus thermophillus*) was procured from a local factory, Khyber Milk and Milk products,

lethpora Pulwama. Plastic cups were purchased from retailers in the local market. Fresh skim milk was prepared in the departmental laboratory from a reputed brand of spray dried skim milk powder obtained from the local market.

2.2 Chemicals

The chemicals used for processing and quality evaluation of soy yogurt were obtained from Hi-media and Merck.

2.3 Preparation of soymilk

Soy milk was made from whole soybeans or full fat soy flour. Soymilk was prepared as per the procedure suggested [27] and included following steps. Soyabean kernels after cleaning of stones and broken beans were soaked in 0.5% sodium bicarbonate solution overnight (16-18 hours). Soaking in sodium bicarbonate and for long time results in production of soymilk with less beany flavour. When the beans split open easily and were flat on inside, they were ready to be drained. The soaking water was discarded and beans were washed well repeatedly in clean water, drained, washed with tap water, grinded, steeped for 4-5 h in tap water (100g soybean mixed with 100ml tap water) and filtered. The washed beans were ground in a grinder with enough water to give the desired solid content to the final product. The ratio of water to beans on a weight basis was kept about 3:1.The resulting slurry was kept in a clean pot for some time and then filtered through a cheese cloth to remove the insoluble residue (soy pulp fibre or okra) and obtain the soymilk. The soymilk obtained was brought to boil in order to improve its nutritional value by heat inactivating soybean trypsin inhibitor, improve its flavour and to sterilise the product. Heating at or near the boiling point is continued for a period of time 15-20 minutes. Bringing filtered soymilk to boil avoids the problem of foaming. It is generally opaque, white or off white in colour and approximately of the same consistency as cow`s milk. The flow diagram for the preparation of soymilk is given in Fig.1.

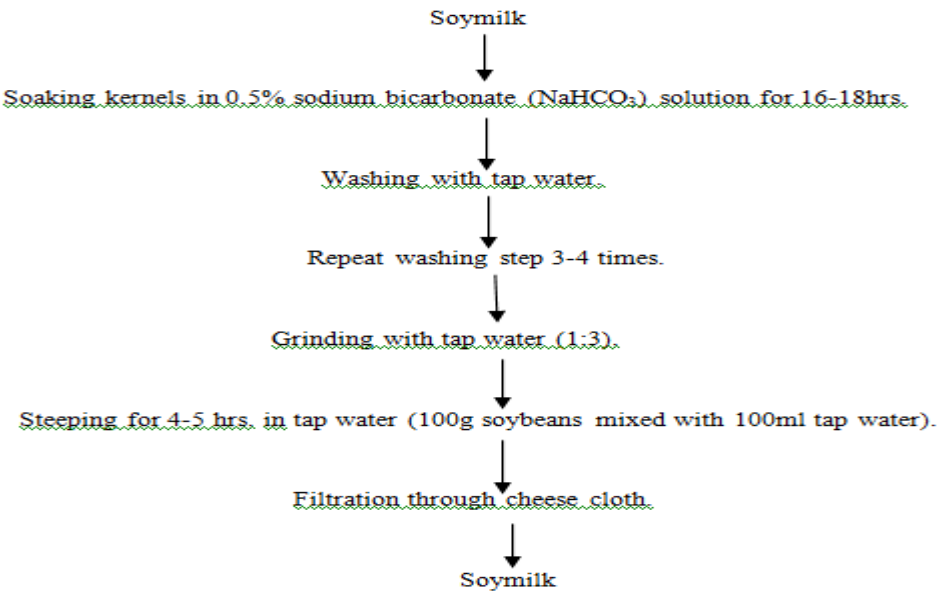

Figure 1. The flow diagram for the preparation of soymilk

2.4 Preparation of soy yoghurt

Four samples were prepared. Sample A consisted of 100% soy milk, sample B consisted of soymilk and 1.5% skim milk, sample C consisted soymilk and 3% skim milk and sample D consisted of soy milk and 4.5% skim milk. Soy yogurt was prepared following the procedure given below:

All the samples were pasteurised at 85^{o}C for 10 minutes in order to improve its nutritional value by heat inactivating soybean trypsin inhibitor, improve the flavour and sterilise the product. All the samples were then cooled to a temperature of 45^{0}C. Starter culture (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) at the rate of 1% of milk volume was added. The starter culture was mixed well with milk samples. The milk mixture was placed in plastic cups and kept in an incubator at 45^{o}C for 3-6 h for yoghurt formation. The cups filled with yoghurt were cooled and then transferred to refrigerator and stored under refrigeration temperature for analysis. The flow chart for the preparation of soy yoghurt is given in Fig.2.

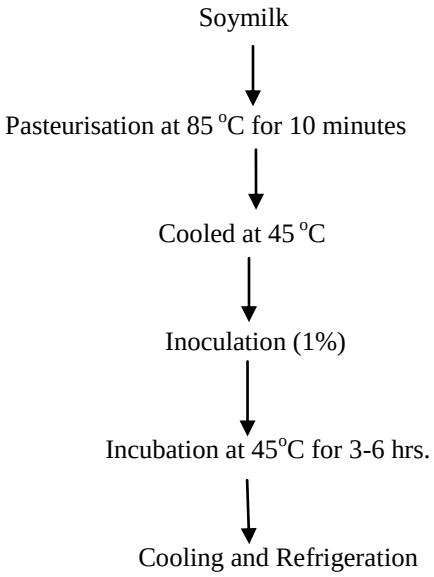

Figure 2. The flow chart for the preparation of soy yoghurt.

2.5 Laboratory analysis

Microbiological analysis of milk was done by plate count test using the methodology of APHA – AWWA-WEF (1998).

2.6 Media

Different media used for culturing of bacteria were Nutrient agar, MRS Agar, Violet Red Bile Glucose Agar (VRBGA), XLD Agar, Centrimide Agar Base (CAB). Nutrient Agar was used for determining total plate count of bacteria and MRS Agar for determining standard plate count of lactobacillus. The media were prepared by dissolving agar powders in distilled water and sterilized in an autoclave at 121^{o}C, 15 lb. /inch^{2} for 15-20 min.

2.6.1 Dispensing of Media

The sterilized medium for bacteria was dispensed into previously sterilized petri plates. About 15-20ml of medium was poured in each petri plates on a laminar flow cabinet. The medium after cooling got solidified. The most important method used for the measurement of microbial community is spread plate technique, which measures the number of viable cells. To ensure that colony counts were within this range, seven dilutions were prepared using serial dilution technique (10-1, 10-2, 10-3, 10-4, 10-5, 10-6 and 10-7).

2.6.2 Serial Dilution Technique

Before inoculation, the sample was diluted to different levels in order to get the approximate number and density of the bacteria easily. The original sample was diluted up to seven dilutions viz, 10-1, 10-2, 10-3, 10-4, 10-5, 10-6 and 10-7 using normal saline solution (NSS).

2.6.3 Inoculation

Spread plate technique was followed by inoculation of sample. The technique involves distribution of 0.1 ml of serially diluted yogurt sample over surface of prepared agar plate with L. shaped bent rod. This technique allows the microbial colonies to grow over the surface of the medium and eventually counting becomes easier. The petri dishes were then kept in inverted position in the incubator at 37 ^{o}C for 24 hrs.

2.6.4 Enumeration of Colonies

Colonies that develop on agar plates were counted with unaided eyes [28]. Only plates with colonies between 30-300 were selected for counting. The counts were expressed as cfu/ml of milk sample. The number of colonies counted were expressed as cfu/ml and were calculated by using the following formula

cfu/ml = n x f N = no. of colonies

f = dilution factor = 1/ dilutions 10-1, 10-2, 10-3.

For standard plate count, same procedure was done as in case of total plate count except in this case selective media was used. MRS Agar was used for lactobacillus.

2.7 Identification

Grams staining technique was used to differentiate the bacteria at group level i.e., Gram +ve and Gram –ve.

2.8 Morphological characteristics of Lactic acid bacteria

Various morphological characteristics like pigmentation, size, elevation, margin, form and optical characters of isolated lactic acid bacteria were observed.

2.9 Biochemical Tests

2.9.1 Catalase test.

For catalase test, a single isolated colony was streaked on a glass slide and one drop of 3% H_2O_2 was added to it. The effervescence of O_2 indicated the positive response of bacteria to catalase test (29, 30).

2.9.2 Oxidase Test

For oxidase test well isolated colony is taken on an inoculating loop and is spread on oxidase discs. The reaction is observed within 2 minutes at 25-30°C. If the area of inoculation turns violet to purple, the test is positive. Absence of colour indicates negative test.

2.9.3 Carbohydrate fermentation Test

Phenol red broth base medium was used as a medium for this test. Sugar substrates namely sucrose was used. 0.1 g (0.1 % w/v) of sugar substrate was added to 100 ml of the medium. 5 ml of each mixture was transferred into each tube. For gas detection, Durham tube was inserted into the test tube containing glucose. All the tubes were sterilized for 15 min at 121 °C. The tubes were inoculated with a single colony of the bacteria under study. The positive reaction of the bacteria was indicated by the changes in the colour of the medium [31].

2.9.4 Salt (NaCl) Tolerance

For the determination of NaCl tolerance of isolated lactobacillus 10 test tube containing MRS broth were adjusted with different concentration (1-15%) of NaCl. After sterilization, each test tube was inoculated with 1% (v/v) fresh over night culture of lactobacillus and incubated at 37°C for 24 h. After 24 h of incubation their growth were determined by observing their turbidity. Maximum growth were indicated as double positive sign (+ +), normal growth as single positive sign (+) and no growth were indicated as negative sign (-).

2.10 Determination of lactic acid content

The titratable acidity in yogurt was estimated by titrating a suspension (20 g yogurt in 20 ml distilled water). The samples was boiled to drive off the carbon dioxide and cooled. The sample then was titrated with 0.1M sodium hydroxide (NaOH) to pink colour in the presence of 1% of phenolphthalein as indicator and expressed as percent lactic acid [36].

% lactic acid is calculated as follow:

% Lactic acid = ml of alkali x Normality of alkali x 9/Weight of sample.

2.11 Determination of pH

The pH values were determined using a pH meter. Five ml of distilled water was added into 25 g of sample. The electrode was immersed in the sample and the pH reading was taken after allowing the meter to stabilize for 1 min [36].

2.12 Water holding capacity or Syneresis

Water-holding capacity of yoghurt was determined using a procedure (36). 20g of yoghurt (Y) was centrifugated for 30 min at 1250xg and 20°C (h = 4.8 cm). The whey expelled (WE) was removed and weighed. The water-holding capacity (WHC) was determined as:

WHC= 100 x(Y-W)/Y.

III RESULTS AND DISCUSSION

3.1 Physical properties of soy yogurt with different concentrations of skim milk

The sample A had pH 5.37 ± 0.1, which was statistically insignificant with sample C (5.38±0.1) and sample D (5.4±0.02). The pH of sample B (5.43±0.1) was statistically significant with sample A, but statistically insignificant with sample C and D. The water holding capacity (WHC) of sample A (28.53±0.4), sample B (24.51±0.01), sample C (27.39± 0.20) and sample D (30.11± 0.01) were statistically significant (TABLE 1).

Table 1. Physical properties of soy yogurt with different concentrations of skim milk

SAMPLE	Percent lactic acid	pH	WHC
A	0.255 ± 0.007^{a}	5.37 ± 0.014^{a}	$28.53 \pm .042^{c}$
B	$0.3 \pm .014^{a}$	$5.43 \pm .014^{b}$	24.51 ± 0.014^{a}
C	0.445 ± 0.063^{b}	$5.38 \pm 0.014^{a,b}$	27.39 ± 0.205^{b}
D	0.47 ± 0.014^{b}	$5.4 \pm 0.028^{a,b}$	30.11 ± 0.014^{d}

Where A,B,C,D refer to the pure soy yogurt, yogurt with 1.5% skim milk, yogurt with 3% skim milk and yogurt with 4.5% skim milk respectively.

3.2 Morphological characterisation of Lactobacillus

Morphological characteristics like pigmentation, size of cell colonies, elevation, margin, cell form and optical characteristics of isolated lactic acid bacteria were observed. It was observed that bacteria isolated from pure

sample, sample with 1.5% skim milk and sample with 3% skim milk were creamish white in colour while as bacteria isolated from sample with 4.5% skim milk were yellowish in colour. Cell colonies of pure sample, sample with 1.5% skim milk and 3% skim milk were medium sized where as large sized colonies were found in sample with 4.5% skim milk. Cell colonies from pure sample, sample with 3% and 4.5% skim milk were convex whereas flat colonies were observed in sample with 4.5% skim milk. Cells of pure sample and sample with 1.5% skim milk were entire whereas undulated cells were observed on samples with 3% and 4.5% skim milk. Circular colonies were grown on pure sample and samples with 1.5% and 3% skim milk. Optical characters were also observed in which opaque colonies were observed in pure sample and sample with 3% skim milk while as colonies on samples with 1.5% and 4.5 % skim milk were translucent (TABLE 2).

Table 2. Morphological characterisation of Lactobacillus.

Characters	**A**	**B**	**C**	**D**
Pigmentation	Creamish white	Creamish white	Creamish white	Yellow
Size	Medium	Medium	Medium	Large
elevation	Convex	Flat	Convex	Convex
Margin	Entire	Entire	Undulate	Undulate
Form	Circular	Circular	Circular	Irregular
Optical characters	Opaque	Translucent	Opaque	Translucent

Where A,B,C,D refer to the pure soy yogurt, yogurt with 1.5% skim milk, yogurt with 3% skim milk and yogurt with 4.5% skim milk respectively.

3.4 Catalase test

Catalase is an enzyme produced by many organisms and therefore the lack of catalase is a significant diagnostic characteristic. The enzyme breaks down hydrogen peroxide into water and oxygen as below and gas bubbles are observed. The formation of gas bubbles therefore indicates the presence of catalase enzyme.

$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$

In order to confirm catalase status of the isolates, catalase test was performed. For this purpose, overnight cultures of isolates grown on MRS agar plates at 30 ºC were used. A drop of 3% hydrogen peroxide was placed on a clean microscopic slide. With a nichrome wire loop cells were picked from the centre of a well isolated colony of the test culture and were transferred into the drop of hydrogen peroxide. Both were mixed and observed for gas bubble production. The results observed showed the occurrence of gas bubble formation indicating that the bacteria were

catalase negative (Table 3). Same results were also reported [32]. They isolated catalase negative bacteria at species level from dairy sludge sample. Also reports that lactobacilli isolated from commercial yogurt were catalase negative [30].

3.5 Oxidase test

The oxidase test is used to determine if the bacteria produce certain cytochrome c oxidases. A loopful of fresh culture is taken and inoculated on the oxidase discs. In our study colour change was observed which indicated positive test (TABLE 3). The results of [33] are contradictory to our results. They produced soy yogurt by using lactobacillus isolated from nunu (a Nigerian indigenous fermented cow milk). Their results showed that these isolated lactobacillus were oxidase negative.

Table 3. Biochemical tests for Lactobacillus

Biochemical test	**A**	**B**	**C**	**D**
Gram staining	+ve	+ve	+ve	+ve
Catalase test	-ve	-ve	-ve	-ve
Oxidase test	+ve	+ve	+ve	+ve
Carbohydrate fermentation test	+ve	+ve	+ve	+ve
NaCl tolerance	+ve	+ve	+ve	+ve

Where A,B,C,D refer to the pure soy yogurt, yogurt with 1.5% skim milk, yogurt with 3% skim milk and yogurt with 4.5% skim milk respectively.

3.6 Gram staining

The Gram status of the isolates was determined by light microscopy after the Gram staining. LAB are known to be Gram positive and the blue-purple colour indicates the Gram positive nature of the bacteria. Cells from fresh cultures have to be used for Gram staining. For this purpose 24 hour old, cultures were grown in MRS broths. Gram staining technique showed that all the isolated bacteria were Gram +ve (Table 3). Similar results have been also obtained [30].They isolated lactobacilli from commercial yogurt and after gram staining they observed that isolated bacteria were gram positive.

3.7 Glucose fermentation test

Glucose fermentation test was conducted by taking 5 ml of MRS broth in culture tubes with inverted Durham tubes. Inoculation with fresh cultures was done and tubes were then incubated for 24 hours at 37^0 C. After incubation colour change was observed which indicated that the test is positive (Table 3). Our results were similar to the result obtained [30, 31].

3.8 NaCl tolerance

NaCl is an inhibitory substance which may inhibit growth of certain types of bacteria. Our results showed that isolated LAB from soy yogurt were able to tolerate 10% and 15% NaCl solution and good growth was observed (Table 3). Same results were found (34) in case of Lactobacilli isolated from gastrointestinal tract of swine that were tolerable to 4-8 % NaCl (34). Similar results were reported [35] and lactobacillus was isolated from selective regional yogurts in Bangladesh. They reported that isolated bacteria were tolerant to 1-9% NaCl solution.

IV CONCLUSION

The dairy products need to be improved in term of processing, packaging, storage, handling and distribution in order to produce safe and quality products for consumption. The products of soy yoghurt with the addition of skim milk at different concentrations was evaluated for lactic acid bacteria. During storage of soy yoghurt at refrigerated temperature, the increase in number of beneficial probiotic lactobacillus bacteria increased which was supposed to improve its storage stability and functionality. The product was also evaluated for its physico-chemical properties in order to standardize the product for commercial production. The product showed good storage behaviour for both microbiological and overall quality. The incorporation of skim milk enhanced the microbiological quality, functional food value and also did value addition to the soy yoghurt product

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