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Isolation and characterization of *Lactobacillus spp.* from curd and its pharmacological application in probiotic chocolate

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ABSTRACT

The isolated and identified bacteria from curd sample are of *Lactobacillus* sp. The bacteria of this spp. are lactic acid producers. These sp. resembles to *Lactobacillus acidophilus*. A probiotic is a microorganism known to be friendly and beneficial to its host when consumed. In today's time, probiotics are a very popular subject of research among scientists and pharmaceutical companies. Due to the over consumption of antibiotics, the normal micro flora of body does not survive. The spores obtained from pharmaceuticals are not good in taste, so generally children avoid eating them, hence we have made the probiotic chocolate. It is better to eat chocolate than medicine. The chocolate containing lyophilized *Lactobacillus spp*. may help directly for enhancing resistance against intestinal pathogens and in the prevention of diseases.

Keywords: Probiotics, *Lactobacillus spp*, Lyophillization, Probiotic chocolate.

INTRODUCTION

Chocolate is a suspension of fine solid particles of sugar, cocoa and milk powder in a continuous fat phase. Chocolates are solid at ambient (20°C - 25°C) and melt at body temperature (37°C) giving a smooth suspension of particulate solids with a pleasing cooling sensation in the mouth [1]. The continuous phase influences the sensory characteristics such as mouth feel or melt in mouth.

Despite high fat and sugar contents, chocolate consumption makes a positive contribution to human nutrition through provision of antioxidants, principally poly- phenols including flavonoids such as epicatechin, catechin and notably, the procyanidins [2]. Chocolates also contain minerals, specifically potassium, magnesium, copper and iron. Due to presence of cocoa, it is rich in natural antioxidants having health benefits. Milk solids added as spray-dried skimmed milk powder or full cream milk powder contributes to flavour, texture and liquid flow properties [3].

Numberous functional foods are consumed as part of a normal diet and they provide consumers with well-documented and physiological benefits such as probiotic bacteria. Probiotics are live microorganisms and proliferate in the human bowels that confer a health benefit by altering the enteric microflora. The main sources of these organisms are fermented dairy products, for example, yoghurts. However, the functional dairy product must contain a defined number of live probiotic bacteria (usually at least 10^6 cfu/g). Furthermore, their number at the end of the shelf life is the most important criterion when the health-promoting value of a given foodstuff is evaluated [4-7].

Probiotic bacteria beneficially affect human health by improving the gut micro biota balance and the defences against pathogens. Additional health benefits attributed to probiotics are the stimulation of the immune system, blood cholesterol reduction, vitamin synthesis, anti-carcinogenesis and anti-bacterial activities. Two other important criteria to determine the efficacy and the success of the product containing probiotics are the acceptance of the product by the consumers and the survival of probiotic microorganisms during its production [8]. *Lactobcilii*, *Bifido* bacteria and several other lactic acid bacteria are regarded as probiotics, as they do not induce mucosal inflammation. The main sources of these organisms are fermented dairy products for example yoghurt (curd). In general, the food industry had applied the recommended level of 10^6 cfu/gm at the time of consumption of *Lactobacillus acidiophilus*, *Bifidobacteria* and other probiotic bacteria [9]. Traditional yoghurt is produced from milk, fermented by strains of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* [10].

Today, India is the largest producer of milk in the world and the Indian dairy industry is witnessing rapid changes. Yoghurt/curd is the most popular fermented dairy product in India, prepared by the use of mixed mesophilic cultures that ferment lactose to lactic acid. Products like yoghurt are known more for their therapeutic significance than nutritional value [11]. It has a limited keeping quality of 1 - 2 days at ambient temperature and its quality is not retained for more than 1 week under refrigerated conditions [12]. Dried yoghurt powder has enhanced shelf life and it can also be used as a base for formulation of health foods [13] have reported that chocolate formulated with isomalt and enriched with viable cells of lactic acid bacteria, introduced in the form of powdered yoghurt, is not only a sucrose-free, low-calorie product but additionally displays nutritional and dietetic attributes, and can be regarded as a functional food additional species in the fingerprints. Possemiers *et al*. [14] have reported bacteria and chocolate to be a successful combination for probiotic delivery. They have showed that coating of the probiotics in chocolate is an excellent solution to protect them from environmental stress conditions and for optimal delivery. Lyophillization technique is used for freeze drying of bacteria i.e. in the powdery form so we can easily add them into chocolate mixture. The viability of *Lactobacillus* species is not harmed in lyophilization technique up to some extent. Probiotic microorganisms are often incorporated in food in the form of yoghurt and yoghurt type fermented food. Recently, there are probiotic ice cream, cheese, infant formulas, breakfast cereals, sausages, chocolate and puddings. Non dairy food also has been manufactured with the addition of the same types of microorganisms. In fact, there are also medical probiotics in the form of capsules and tablets [15].

The present study has been carried out the isolation, characterisation and identification of *Lactobacilli spp*. and incorporate dried probiotic powder for the preparation of probiotic chocolate, and study the pharmacological effects of the probiotic chocolates.

MATERIALS AND METHODS

Isolation of Lactobacillus spp.

Curd is the best source for *lactobacillus spp*. Among the other dairy products such as milk, buttermilk etc. Curd is taken in sterilized flask. Under the aseptic conditions curd was serially diluted from 10^{-2} to 10^{-5} from this dilutions 10^{-4}, are selected. Spread plate technique further with streak plate technique is done on MRS medium. They are incubated in incubator 37^0C which is optimum temperature for *Lactobacillus* broth. Incubation at 37^0C for 24 hrs. Broth after 24-48 hrs shown *Lactobacillus* species growth and these species for 24 hours. After the period of incubation the specific isolated colonies were grown. Colony characterization is done for this colonies found to be *Lactobacillus* species. One colony shows 100% resemblance with *Lactobacillus acidophilus*. The isolated colony formed on the MRS agar plates was identified using gram stain, biochemical tests. The identification was performed according to Bergey's manual of determinative of bacteriology. The culture was kept in MRS agar slant and stored at 4 ^{0}C for long term storage [16].

Bacterial strain and culture conditions

Two Gram negative and two Gram positive bacteria used for antibacterial assay respectively, *Escherichia coli* (MTCC 443), *Klebsiella pneumonia* (MTCC 109), *Staphylococcus aureus* (MTCC 3160) and *Salmonela typhi* (MTCC 890) were provided by National Cholera Institute, Kolkata. These Gram positive and Gram negative test organisms were maintained in Brain Heart Infusion Agar butt-slants in screw-capped tubes and kept at 4^{o}C.

Biochemical characterization of the isolated bacterial strain

Identification of the isolated bacteria as *Lactobacillus* species was performed according to their morphological, cultural, and physiological and biochemical characteristics by the procedures as described in Bergey's Manual of Systematic

Bacteriology [17]. The tests carried out were Gram staning, Capsul staning motility test, production of catalase, Indole, Methyl Red, Voges-Proskauer, Citrate, Starch Hydrolysis, endospore test, milk coagulation activities and NaCl and phenol tolerance test.

Tolerance of NACL and Phenol

Observing the tolerance of Nacl of the culture of the 1% fresh one night culture of the bacteria incubate into MRS broth with 4% Nacl Con^c for 24 hours and then observe their turbidity. Similar experiments were performed using 0.4% phenol as inhibitory substance [18].

Milk Coagulation Assay

For milk coagulation test, 100% fresh one night culture of the bacteria was added into 10% sterile skim milk and incubate 37^{o}C for 48 hours in incubator [18].

Lactose Utilization

For this experiment media was prepared using Peptone- 10 gms, Nacl-15 gms, Phenol Red 0.018 gm, Lactose 5 gm in 1 litter distilled water, controlling P^H 7, kept it 35^{o}C, for 48 hours in rotary incubator. Change of colour, red from yellow, concluded as positive result [19].

Antibacterial Activity

For the observation of antimicrobial activity of isolated bacteria species, agar well diffusion method was performed. The overnight culture of pathogen *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumonia*, *Staphylococcus aureus*, were spread into different plates and well were made in each of the plate and the wells were filled by 100 micro litter isolated lactobacillus culture. And the plate was incubated for 24 hours at 37^{o}C and observed the zone of inhibition [20].

Antibiotic Susceptibility Assay

The Antibiotic Susceptibility were performed by Disk diffusion method. The isolated bacterial samples were spread in MRS Agar plate. 30µg concentration Ampicilin, Tetracycline and Azithromycin are used for this experiment [20].

Lyophilization

One colony is taken from the curd streak plated and inoculated in 1000ml of nutrient bacteria were freeze dried (powdery form) using lyophilization technique.

Preparation of Probiotic Chocolate

200gm of milk powder and 2 Chocolate cubes (40gm) heated up to it

melts. Then addition of some volume of milk (for solidification) is done. When it reaches the normal temperature, addition of freeze dried bacteria is done. Mixed it well and stored in freeze at low temperature. When it was solidified then cut into pieces. Wrapped the chocolates in Aluminium foil [15].

RESULTS

Identification of Lactobacillus spp.

The isolated bacteria were observed by phase contrast microscope. It is clear that the bacteria was gram positive, rod shaped occurring single or in chains forms. The gram staining results indicated that the isolated bacteria could be identified as *Lactobacillus acidophilus.* Hanging drop wet method showed that the isolated bacteria were non motile. The non motile behaviour is a characteristic of *L.acidophilus*. Therefore the curd sample `bacterium resembles characters similar to *Lactobacillus acidophilus*. The catalase test is one of the most useful diagnostic tests for the recognition of bacteria due to their simplicity. In performing catalase test, no bubble was observed indicating that the isolated bacterium is catalase negative and could not mediate the decomposition of H_2O_2 to produce O_2. It is well known that *Lactobacillus* is catalase negative. Thus, the results obtained coincided with *L. acidophilus* strain characteristics. Their distinguishing features are shown in (Table 1 and Figure 1). Their biochemical characters are also shown in the (Table 2).

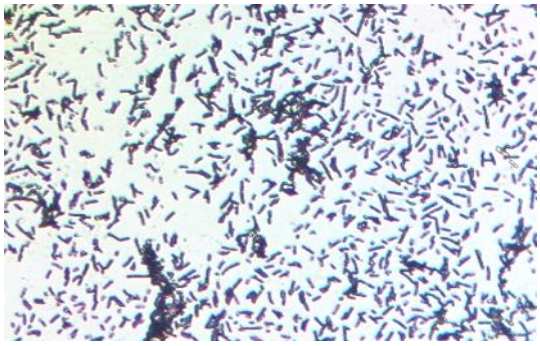

Figure 1: Phase contrast microscopy images of *Lactobacillus sp.* (Magnification 10X)

Table 1: Morphological and physiological characterization of the isolated bacterial strain.

Configuration	Round
Margin	Wavy
Elevation	Flat
Surface	Mucoid
Texture	Dry
Gram's Reaction	+ve
Spore(s)	-Ve
Capsul	-Ve
Motility	Non motile

Table 2: Biochemical characterization of the isolated bacterial strain.

Tests	Results
Catalase test	-ve
Indole	-ve
Methyl red	-ve
Voges-Proskauer	-ve
Starch Hydrolysis	- ve
Milk Coagulation Assay	+ve
Lactose Utilization	+ve
Phenol (0.4%) test	+ ve
4% Nacl test	+ ve

Antibiotic susceptibility test

Results concerning the sensitivity of the isolated *Lactobacillus* species to different antibiotics are shown in (Table 3) which reveals that isolates were sensitive to all the three selected antibiotics.

Table 3: Antibiotic sensitivity test

Antibiotics	Concentration	Sensitivity
Azithromysin	30µg	+
Ampicillin	30µg	+
Tetracycline	30µg	+

Detection of antibacterial activity

Antibacterial activities exhibited by *Lactobacillus* species which indicates that the cell free solution of isolated *Lactobacillus* species were able to inhibit the growth of all the test microorganisms. This experiment clearly indicates that the inhibitory metabolites produced by isolated *Lactobacillus* species were extracellular and diffusible. These results are shown in the (Table4 and Figure2). The experimental results showed that the traditional fermented milk product curd contain *Lactobacilli* which can tolerate inhibitory substances and were able to survive both in acidic and alkaline conditions. They exhibited antibacterial activity against some indicator pathogens. Based on these characteristics the isolates may have potential for natural preservatives and may also be considered for probiotic application.

Table 4 Antibacterial activity of isolated *Lactobacillus spp.* by agar well diffusion method.

Test organisms	Zone of inhibition (mm)
Salmonela typhi(MTCC 890)	19
Escherichia coli (MTCC 443)	17
Staphylococcus aureus (MTCC 3160)	14
Klebsiella pneumonia(MTCC 109)	17

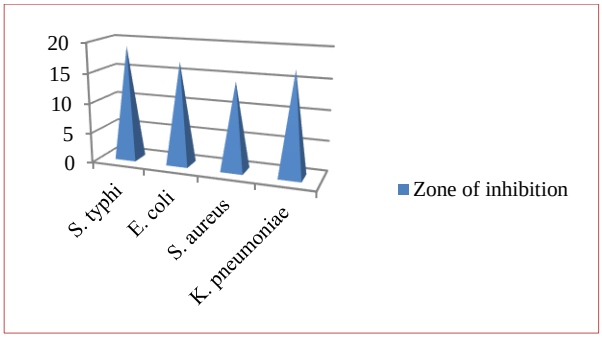

Figure 2: Antibacterial activity of isolated *Lactobacillus spp.* by agar well diffusion method.

Lyophilization and Preparation of Probiotic Chocolate

By using lyophilisation technique the *Lactobacillus spp.* were freeze dried. The freeze dried *Lactobacillus spp.* were in powdery form. This powder was added at the last step of preparation of chocolate. The probiotic chocolate is prepared. The chocolate is easily assimilated and it is better to eat a chocolate than medicinal tablets. The lyophilized freeze dried of *Lactobacillus spp.* the powder addition to the chocolate preparation and after preparation of chocolate all these images are shown in the (Figure 3).

Figure 3: a) The lyophilized freeze dried of *Lactobacillus spp.* b) Powder addition to the chocolate preparation c) Preparation of probiotic chocolate d) After packaging of probiotic chocolate.

CONCLUSSION

Chocolate with lyphilized powder contain probiotic lactobacilli species and thus making it probiotic. Chocolate is reported to contain natural antioxidants and the nutritional quality of this was further enhanced by making it probiotic. It has been found that chocolate masses are suitable matrices for the inclusion of probiotic bacteria. The concentration of viable cells of probiotic bacteria decreased in the course of storage of the chocolate bars, but there remained enough amount of beneficial microflora (10^5 - 10^6 cfu/g), to carry out its inherent preventive role in the consumption of this type of functional food. This probiotic chocolate should have antibacterial activity.

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Conflict of Interest

Authors have no conflict of interest.

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