

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, Lyophilization, 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 .

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 .

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 .

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 10⁶ 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 .

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 .

Lactobacilli, *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 10⁶ cfu/gm at the time of consumption of *Lactobacillus acidophilus*, *Bifidobacteria* and other probiotic bacteria

Traditional yoghurt is produced from milk, fermented by strains of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *Bulgaricus*.

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 these dilutions 10⁻⁴, are selected.

Spread plate technique further with streak plate technique is done on MRS medium. They are incubated in incubator 37°C which is optimum temperature for *Lactobacillus* broth.

Incubation at 37°C 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 these 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°C for long term storage.

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 *Salmonella typhi* (MTCC 890) were provided by National Cholera Institute, Kolkata.

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.

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°C for 48 hours in incubator.

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 liter distilled water, controlling PH 7, kept it 35°C, for 48 hours in rotary incubator.

Change of colour, red from yellow, concluded as positive result.

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°C and observed the zone of inhibition.

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.

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*.

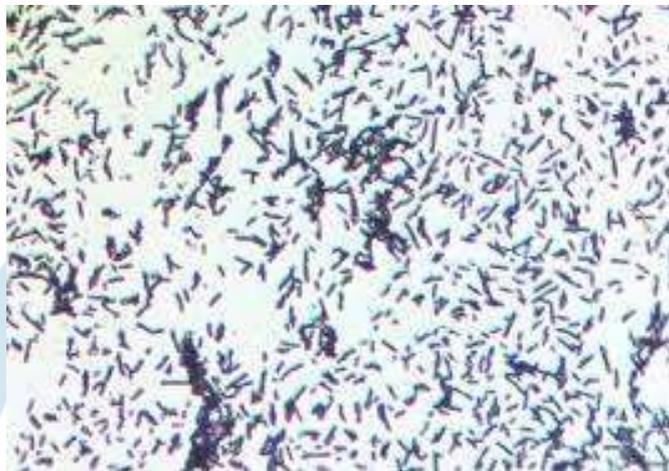
The catalase test is one of the most useful diagnostic tests for the recognition of bacteria due to their simplicity.

In performing catalase test, no bubble was observed indicating that the isolated bacterium is catalase negative and could not mediate the decomposition of H₂O₂ to produce O₂.

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



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

Morphological and physiological characterization of the isolated bacterial strain

Configuration	Round
Margin	Wavy
Elevation	Flat
Surface	Muroid
Texture	Dry
Gram's reaction	+ve
Spores	-ve
Capsule	-ve
Motility	Non motile

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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 which reveals that isolates were sensitive to all the three selected antibiotics.

Antibiotic sensitivity test

Antibiotics	concentration	Sensitivity
Azithromysin	30ug	+
Ampicillin	30ug	+
Tetracycline	30ug	+

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.

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

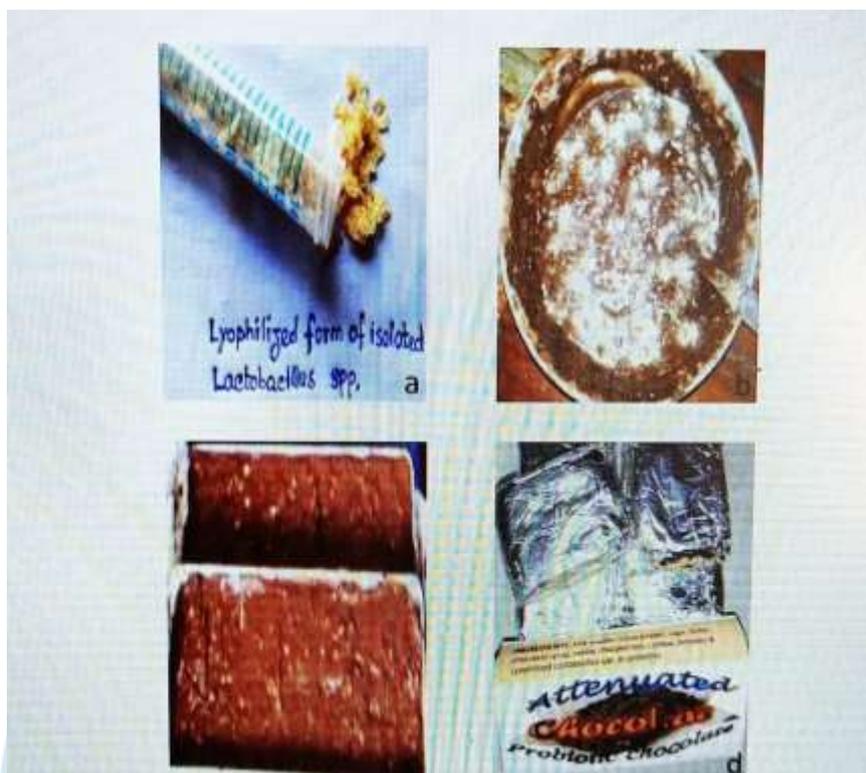
Test organisms	Zone of inhibition (mm)
Salmonela typhi(MTCC 890)	19
Escherichia coli (MTCC 443)	17
Staphylococcus aureus (MTCC 3160)	14
Klebsiella pneumonia (MTCC 109)	17



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).



: 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.

CONCLUSION

Chocolate with lyophilized 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 (10⁵ - 10⁶ 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.

Acknowledgments

Authors are thankful to Panskura Banamali College, Karry Mullis Molecular Biology Laboratory (Sponsored by Boost, Government of West-Bengal), India for providing facilities and financial support for the completion of this research work.

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