



ISOLATION AND CHARACTERIZATION OF *Lactobacillus spp.* FROM CURD AND ITS APPLICATION IN FOOD INDUSTRY.

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Abstract: *Lactobacillus sp.* were isolated from curd sample and identified. These bacteria are lactic acid producers and are essential in foods as they increase the nutritional value of the product. *Lactobacillus sp.* is probiotic bacteria which when consumed confer a health benefit to the host. Probiotics benefit humans by improving the gut micro biota balance, stimulating immune system and also has antibacterial activities. The lyophilized form of *Lactobacillus sp.* is added to chocolate in two varieties thus probiotic milk chocolate and probiotic dark chocolate are made. Probiotic chocolates are a way to add good bacteria to the body. Probiotics consumed along with chocolate increases good gut bacteria and improves the overall health.

Keywords: *Lactobacillus sp.*, Probiotics, Antibacterial activities, Lyophilization, Probiotic milk chocolate, Probiotic dark chocolate.

INTRODUCTION:

Lactobacillus is a gram positive, facultative anaerobic bacterium. Most of the members of *Lactobacillus* convert lactose and other sugar to lactic acid. Lactic acid bacteria are essential in food and feeds as they considerably increase the nutritional value of the product. Considering some of the metabolic properties of lactic acid bacteria they play an important role in the food industry. They greatly contribute to the flavor and texture and in many cases the nutritional value of the food product is also increased. Some *Lactobacillus species* are used industrially for the production of yoghurt, cheese, pickle, beer, wine, cider, chocolate, and other fermented foods, as well as animal feeds. In human these *Lactobacillus* are mainly present in the vagina and gastrointestinal tract. The production of lactic acid makes its environment acidic, thus inhibiting the growth of some harmful bacteria. There are several health benefits for probiotic bacteria.

Probiotic bacteria benefit human health by improving the gut micro biota balance and the defenses against pathogens. Other health benefits of probiotics are: they stimulate the immune system, reduce blood cholesterol, vitamin synthesis, anti-carcinogenesis and has anti-bacterial activities. *Lactobacilli*, *Bifidobacterium* and several other lactic acid bacteria are regarded as probiotics and the main sources of these organisms are fermented dairy products such as curd. In general, the food industry had applied the recommended level 10 cfu/gm at the time of consumption of *Lactobacillus acidophilus*, *Bifidobacterium* and other probiotic bacteria.

Chocolate consumption has been associated with better physical health, chocolate has also been suggested to have short term benefits on reducing blood pressure and serum cholesterol and on improving insulin sensitivity chocolate consumption can also lower the risk of cardiovascular disease. Chocolate contains high levels of antioxidants. Chocolate also contains large number of calories. Chocolate most commonly are in dark, milk and white varieties. There are several types of chocolate according to the quantity of cocoa and milk used in a particular formulation.

Milk chocolate is one of confectionary products that consist of cocoa mass, cocoa butter, sugar, and milk powder with or without the addition of other food ingredients and food additives. Milk chocolate is the most popular type of chocolate. It contains only 10 to

40% cacao mixed with sugar and milk. Milk chocolate is much, much sweeter than dark or bitter sweet chocolate and has a lighter color than dark chocolate. Milk chocolate is well known for its high antioxidant properties. Dark chocolate is called black chocolate and is produced by adding fat and sugar to cocoa. This chocolate is with no milk or much less milk than milk chocolate. Dark chocolate is semi sweet and extra dark with bitter sweet. The chocolate is the richest source of energy, protein, magnesium, calcium, iron and riboflavin of varying amounts, which are essential for mental health and heart function. The cocoa seeds are rich in copper, sulphur and vitamin C. Dark chocolate is naturally rich in flavonoids. These compounds are thought to lower blood pressure and protect against heart diseases.

Lyophilization is a process of drying in which water is sublimed from the product after it is frozen. The lyophilized form of *Lactobacillus* can be easily added to the chocolate mixture. This does not harm the viability of the bacteria. The present study aims at isolation, characterization and identification of *Lactobacillus spp* and its application in food industry by incorporating the powdery dried form of the probiotic bacteria into two varieties of chocolates.

MATERIALS AND METHODS:

SAMPLE COLLECTION:

Curd sample was collected from the Cheran Maligai store, Nehru nagar, Coimbatore.

COLLECTION OF TEST ORGANISM:

The pathogenic organisms were collected from KMCH, Coimbatore. The collected organisms were *Escherichia coli*, *Staphylococcus aureus*, *Salmonella spp*, *Klebsiella spp*. They were cultured and maintained in nutrient agar butt slants at 4°C.

ISOLATION OF *Lactobacillus spp* FROM CURD:

Curd was taken in a sterilized flask. Under the aseptic conditions curd was serially diluted from 10^{-2} to 10^{-6} . Spread plate technique was done on MRS medium. The plates were incubated at 37°C for 24hours. After incubation growth was observed and they were sub cultured for further analysis.

IDENTIFICATION OF THE ISOLATED BACTERIAL STRAIN:

The isolated colony of bacteria from curd sample was identified by observing its morphology, cultural characteristics and biochemical characteristics. Gram staining, endospore staining, Motility test, catalase test, Oxidase test, Indole test, methyl red test, Voges-Proskauer test, Citrate utilization test, urease test, starch hydrolysis test, carbohydrate fermentation test, NaCl tolerance test, Phenol tolerance test, Milk coagulation assay, Lactose utilization test, antibiotic sensitivity test and antibacterial activity test were performed to identify the bacteria.

CARBOHYDRATE FERMENTATION TEST:

The isolated bacteria were tested for the fermentation of sugars such as glucose, lactose, sucrose and mannitol. The organism was inoculated into carbohydrate fermentation broth and incubated at 37°C for 24 hours.

NACL TOLERANCE TEST:

Fresh overnight culture of *Lactobacillus spp* was prepared. This was then inoculated into MRS broth with 4%Nacl concentration and incubated at 37°C for 24 hours. After incubation the turbidity of the culture was measured at 620nm in spectrophotometer.

PHENOL TOLERANCE TEST:

Fresh overnight culture of *Lactobacillus spp* was inoculated into MRS broth with 0.4%phenol concentration and incubated at 37°C for 24 hours. The turbidity of the culture was measured at 620nm in spectrophotometer after incubation.

MILK COAGULATION ASSAY:

For milk coagulation assay skim milk was taken. Skim milk was prepared by boiling buffalo milk in a nonstick pan for 8-10minutes. Cool it for at least 2-3 hours and kept in refrigerator for at least 10-12hours. The cream was removed from the top. These steps are repeated three more times to obtain skim milk. Fresh overnight culture of bacteria was inoculated into 10%skim milk and incubated at 37°C for 48 hours.

LACTOSE UTILISATION TEST:

Media was prepared using 1gram of peptone, 1.5gram of NaCl, 0.0018gram of phenol red, 0.5gram of lactose in 100ml distilled water and the pH of the media is 7. The organism was inoculated into the media and incubated at 37°C for 48 hours in rotary incubator.

ANTIBIOTIC SENSITIVITY TEST:

The Antibiotic Sensitivity test was performed by Disk diffusion method. The isolated sample of bacteria was swabbed in MRS Agar plate. 30µg concentration Erythromycin, Gentamycin and Amoxicillin were used for this experiment.

ANTIBACTERIAL ACTIVITY:

Antimicrobial activity of isolated bacteria was observed by agar well diffusion method. Fresh overnight broth culture of the pathogen *Escherichia coli*, *Staphylococcus aureus* *Salmonella spp*, *Klebsiella spp* were swabbed onto different MRS agar plates and wells were made in each plate and the wells were filled with 100µl and 50µl of isolated *Lactobacillus* culture respectively. The plates were incubated at 37°C for 24 hours. The zone of inhibition was measured.

LYOPHILIZATION OF THE PROBIOTIC BACTERIA:

The organism was inoculated in 2000ml of MRS broth. It was then freeze dried and made to powdery form using lyophilization technique.

PREPARATION OF MILK AND DARK CHOCOLATE:

For the preparation of milk chocolate double boiler method is used, heat 105 gram of cocoa butter till it melts. After it gets melted add 200 gram of milk powder, 85 gram of honey and 118 gram of cocoa powder and mixed well. Then 1 teaspoon of Vanilla extracted is added and mixed well. It is then allowed to cool. For the preparation of dark chocolate by double boiling method 105 gram of cocoa butter is heated and melted. Then 118gram of cocoa powder and 85 gram of honey is added and mixed well. It is then added with 1 teaspoon of vanilla extract and mixed well and allowed to cool.

APPLICATION OF PROBIOTIC BACTERIA IN FOODS:

When both varieties of chocolate after preparation reaches the normal room temperature the powdery form of *Lactobacillus sp.*, is added mixed well and immediately stored in freezer.

RESULTS AND DISCUSSION:**Identification of the isolated organism:****GRAM STAINING:**

The bacteria was gram positive, rod shaped occurring singly or in chains (Figure 1). The gram staining results indicated that the bacteria could be identified as *Lactobacillus spp.*,

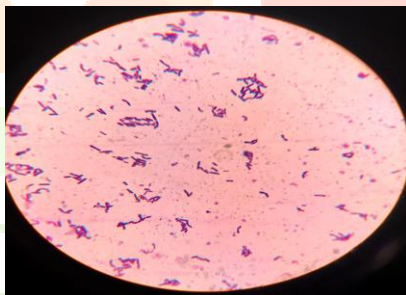


Figure 1: Microscopic examination -Gram positive rod shaped bacteria

ENDOSPORE STAINING:

The isolated bacterium was non spore forming.

MOTILITY TEST (Hanging drop method)

The isolated bacteria were non motile which is characteristic of *Lactobacillus acidophilus*.

BIOCHEMICAL CHARACTERISTICS:

Biochemical characteristics of the bacterial strain were determined by using various biochemical tests like catalase, oxidase, Indole test, Methyl Red test, Voges-Proskauer test, Citrate utilization test, Urease test, starch hydrolysis test etc., and their results were recorded in table 1.

Table 1: Biochemical test for characterization of *Lactobacillus spp.*,

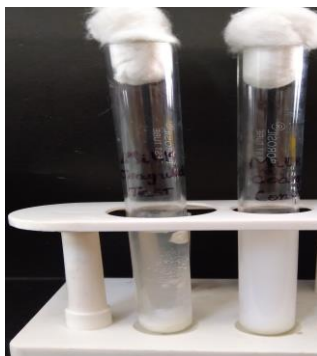
S.No	BIOCHEMICAL TEST	RESULTS
1.	Catalase test	Negative
2.	Oxidase test	Negative
3	Indole test	Negative
4	Methyl Red test	Negative
5	Voges-Proskauer	Negative
6	Citrate utilization test	Negative
7	Urease test	Negative
8	Starch hydrolysis test	No zone of hydrolysis

CARBOHYDRATE FERMENTATION TEST:

The isolated *Lactobacillus spp.*, were able to ferment all the tested sugars (table 2). The colour of the medium changes from red to yellow.

Table 2: Carbohydrate fermentation test

S.No	CARBOHYDRATE FERMENTATION TEST	RESULT
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1	Glucose	Positive
2	Lactose	Positive
3	Sucrose	Positive
4	Mannitol	Positive

NaCl TOLERANCE TEST:

The organism was able to tolerate NaCl. The turbidity of the culture was measured at 620nm (table 3).

Table 3- NaCl tolerance test

NaCl TOLERANCE TEST	OD Value
	2.222

PHENOL TOLERANCE TEST:

The organism was able to tolerate phenol and grow. The OD value of the culture measured at 620nm (table 4).

Table 4 - Phenol tolerance test

PHENOL TOLERANCE TEST	OD VALUE
	2.105

MILK COAGULATION ASSAY:

Coagulation of milk was observed in the tube inoculated with the test organism (figure 2).



Figure 2- coagulation of milk

LACTOSE UTILIZATION TEST:

Lactose utilization test is positive for *Lactobacillus* as the colour is changed from yellow to red.



Figure 3- Lactose utilization test

ANTIBIOTIC SENSITIVITY TEST:

The organism was sensitive to all the three selected antibiotics (table 5).

Table 5- Antibiotic sensitivity test

S.No	ANTIBIOTIC DISC	MEASUREMENT
1	Erythromycin	23mm
2	Gentamycin	17mm
3	Amoxicillin	11mm

ANTIBACTERIAL ACTIVITY:

The isolated *Lactobacillus sp* was able to inhibit the growth of all the four test organisms (table 6). This indicates the probiotic nature of the organism isolated from curd.

Table 6- Antibacterial activity

S.No	ORGANISM	ZONE OF INHIBITION (100µl)	ZONE OF INHIBITION (50µl)
1	<i>Staphylococcus aureus</i>	17mm	12mm
2	<i>E.coli</i>	22mm	13mm
3	<i>Salmonella sp.,</i>	19mm	12mm
4	<i>Klebsiella sp.,</i>	13mm	10mm

From the above characteristics the isolated organism could be identified as *Lactobacillus acidophilus*.

APPLICATION OF PROBIOTIC BACTERIA IN FOODS:

The lyophilized powdery form of bacteria is added to both the varieties of chocolate and mixed at the final stage and stored at freezer. Thus Probiotic milk chocolate and Probiotic dark chocolate is prepared. The images of chocolate preparation are given in figure 4.

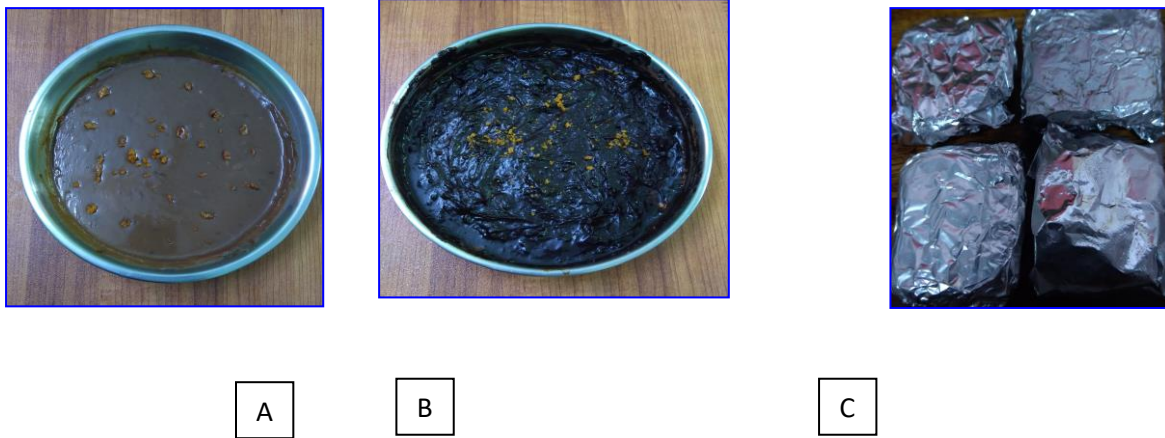


Figure 4-

- a) *Lactobacillus spp.*, In MRS broth
- b) Lyophilization.
- c) Lyophilized form of *lactobacillus spp*
- d) Probiotic milk chocolate
- e) Probiotic dark chocolate
- f) Probiotic dark and milk chocolate wrapped in aluminum foil

DISCUSSION:

The aim of the study was to isolate the probiotic *lactobacillus spp.*, from curd sample and its application in probiotic chocolate in two variants. Chocolate with lyophilized powder contain probiotic *Lactobacilli species* and thus making it probiotic. The organism was isolated and further characterized by biochemical test. It was then freeze dried and made to powder. This lyophilized form of *lactobacillus* was used in the preparation of probiotic chocolate in two variants. Chocolate contains natural antioxidants and the nutritional quality of this was increased by making it probiotic. It has been found that chocolates are suitable for incorporating probiotic bacteria.

SUMMARY AND CONCLUSION:

The chocolate prepared is easily absorbed and it is better than tablets. Dark chocolate is rich in many key nutrients that our body requires; it includes iron, copper, magnesium, zinc, phosphorus, and flavanols. In addition to being known for its rich nutrients, dark chocolate is also helpful in improving brain function, prevents heart disease, and is even a gut healthy food. Polyphenols found in dark chocolate are anti-inflammatory. Antioxidants in dark chocolate are helpful in decreasing inflammation of cardiovascular tissue. Flavones present in dark chocolate encourage arteries to relax, which increases blood flow. Polyphenols boost our immune system. Our brain function could be boosted by polyphenols. When probiotic are consumed along with dark chocolate, the population of good gut bacteria increases this improves the overall health as a result we are doubling benefited.

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REFERENCE

- Renuga Goyal, Harish Dhingri, Pratima, Bajpai and Navneet Joshi. 2012. Characterization of Lactobacillus isolated from different curd sample. African journal of biotechnology, 11(79):14448-14452.
- S. Kale. 2014. Isolation and identification of bacteria from curd and its application in probiotic chocolate. European journal of experimental biology, 4(6):95-97
- Mohsin Shaikh and Gaurav Shah. 2013. Determination of probiotic properties of lactic acid bacteria from curd. Global journal of biology, Agriculture and Health Sciences, 2(2):119-122
- Vidhya Pratishtan's school of Biotechnology, Baramati and ATG lab, Pune. 2014. Isolation and identification of bacteria from curd and it's application in probiotic chocolate. European Journal of Experimental biology, 4(6):95-97
- Estifanos Hawaz. 2014. Isolation and identification of probiotic lactic acid bacteria from curd and in vitro evaluation of its growth inhibition activities against pathogenic bacteria. African journal of Microbiology result, 8(13):1419-1425
- Asmitha khalal and Niranjana Koirala. 2019. Screening and analysis of probiotic properties of Lactobacillus spp. From famous commercial brand of yogurt found in Kathmandu valley. Current Trends in Biomedical Engineering and Biosciences, 18(4):2572-1151
- M.Mizanur Rahman, K.Moazzem Hossain and S.M.Mahbubur Rahman. 2016. Isolation characterization, and properties study of probiotic lactic acid bacteria of selected yogurt from Bangladesh. African Journal Of Microbiology Research, 10(1):23-31.
- LC, Law LC, Suzannah S, Miswani S, Cloke M. 2009. Polyphenols in Cocoa (Theobroma cacao L.). Asian Journal of Food and Agro Industries, 2(4):702-722.
- Somnath De, Atanupramanik, Aditya KR Das, Suchismita Paul, Sourav Jana, Priyanka Pramanik. 2017. Isolation and characterization of Lactobacillus spp. From curd and its pharmacological application in probiotic chocolate. Journal of phytopharmacology, 6(6):335-339.
- Md. Abdullah - Al- Mamun, Saifullah, S.M. Khaledur Rahman. 2017. Assessment of Probiotic properties of isolated Lactic Acid Bacteria from Human Milk sample. African Journal Of Microbiology Research, 8(6):140-146.