

ISOLATION AND CHARACTERIZATION OF LACTIC ACID BACTERIA FROM CURD

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Abstract: Background and Objective: Curd is commonly consumed fermented dairy product in Punjab region. Majority of the dairy products that have undergone fermentation often include lactic acid bacteria. This investigation set out to separate *Lactobacillus* from several curd samples.

Methods: Total six samples of curd were gathered from different resources and isolates were produced by cultivating the samples on MRS (De Man, Rogosa and Sharpe) agar medium. The characterization of isolates was based on their phenotypic traits, biochemical activities and antibiotic sensitivity testing.

Results: The study revealed the presence of *Lactobacillus* bacteria in the isolates. The colonies were creamish white and opaque on MRS agar plating and all bacteria were Gram- positive, rod shaped and non-motile in nature. Different biochemical test results were found to be negative i.e. catalase, methyl red, oxidase etc. The isolates named C1 and C2 were underwent more testing to check their susceptibility towards different antibiotics. Many of the broad- spectrum antibiotics were found to be isolate sensitive. The isolate C1 showed resistance to ampicillin while C2 was resistant towards ceftriaxone and ciprofloxacin. Moreover, amoxiclav and vancomycin were found intermediate in both samples.

Conclusion: The research came to the conclusion that the antagonistic effects of substances by bacteria, such as lactic acid bacteria, are extremely important in enhancing human health and food quality since they can be employed in both pharmaceutical preparations and dairy supplementations.

Keywords: Antibiotic sensitivity, Curd, *Lactobacillus*, Probiotics.

1. INTRODUCTION

Curd is one of the major fermented food items used on daily basis. The main probiotic bacteria found in curd is lactic acid bacteria (LAB), which is having antioxidant, immunotherapeutic and antibiotic properties [1]. LAB are the microbes that occur naturally and do not cause any disease to humans and animals, thus known as GRAS (Generally Recognised As Safe) [2]. They are Gram positive in nature, do not form spores and can be of cocci or rod shapes. Their main product of fermentation from carbohydrate is lactic acid. They are used in numerous food supplements such as formation of cheese, yoghurt and pickles [3]. LAB are non-motile in nature and can be commonly found in soil, water, fruits, plants and also in gastrointestinal and respiratory tracts of humans and animals. Aerobic and anaerobic environments are favourable for their growth. They utilize lactose in order to produce a byproduct known as lactic acid, which makes the pH of milk acidic and denature the casein protein [4]. For the preparation of fermented foods, the food service sector uses LAB as a preferred starter culture such as dairy, meat, fish, cereals, fruits and vegetables. They are vital because of their ability to improve taste, flavour and texture of food products [5]. Probiotics are living microbes and if administered in accurate ratios, can provide health facilities to the host. LAB specially *Lactobacilli* are the probiotics used as health promoting agents because of their ability to provide protection against pathological invasion. Other common probiotic organisms are *lactococci*,

bifidobacteria and *saccharomyces*. The chemicals released by these bacteria also inhibit the invasion of harmful pathogens [6]. Probiotics ought to be utilized on regular time intervals but their fermentation has to be done properly. These can live in harsh environments because of their adaptative properties; thus, they can easily survive in gastrointestinal tracts. Because of their numerous health benefits they can be consumed in various forms such as capsules, syrups and many food products [7]. They are also having another beneficial property of relieving lactose intolerance, reduction of allergy in hypersensitive individuals [8,9]. In addition to this, probiotics are well known for their antimutagenic, anticarcinogenic, immunomodulatory, hypocholesterolaemia, antihypertensive and antiosteoporosis properties[8]. They also reduce the symptoms of colitis, inflammatory bowel disease, alcoholic liver disease, irritable bowel syndrome, risk of colon cancer, constipation, breast cancer and hepatic cancer [10]. This study set out to analyse various commonly available curds for the purpose of differentiating of lactic acid bacteria and evaluating their probiotic properties.

2. MATERIALS AND METHODS

2.1 Sample Collection

Total six specimens of homemade curd were collected while maintaining aseptic conditions. After that the samples were transported to the laboratory with maintained temperature conditions. Then the curd samples were homogenized using a sterile blender/mixer in order to achieve a uniform mixture.

2.2 Isolation of Lactic acid bacteria

Using streak plate method, the samples were cultured on MRS agar. Then at 37°C plates were incubated for 24 hours. After the growth of colonies, they were analysed morphologically as well as microscopically and stored at -4°C for further examinations [11].

2.3 Phenotypic characterization

All isolates underwent phenotypical characterization based on their physical and biochemical properties.

Gram stain. Morphological and cultural examination was done by using the Gram stain procedure. The bacteria specimens were analysed using gram staining method. On the fixed culture crystal violet stain was poured for 1 minute and rinsed with water. Then again after applying Gram's iodine solution, rinsed with water. Then it was followed by the decolourizer. After rinsing with water, safranin was applied and again rinsed with water, air dried and visualized at different magnifications such as 10x, 40x and 100x (oil immersion)[12].

Biochemical Examination. Various biochemical tests were performed to find out the metabolic characteristics of isolated Lactic acid bacterial strains. These tests include – Catalase, Indole, Methyl red, Voges- Proskauer and Oxidase.

Catalase Test. For this test a sterilized inoculating loop was used to smear a small amount of selected isolated colony. One drop of hydrogen peroxide was poured on the slide to check enzyme activity. If the catalase enzyme breakdown H_2O_2 into H_2O and oxygen showing bubble formation due to O_2 release, that indicates a positive reaction [13].

Indole test. Four to five drops of Kovac's reagent were added to 24-48 hrs growth. The results were examined after gentle shaking of the tube. A negative result shows no colour (original colour of Ehrlich's reagent or Kovac's reagent) or lighter than the medium and a positive result has a red ring on the medium's surface [14].

Methyl red. The MR-VP broth containing test organism was incubated at 37°C for 24 hrs, after incubation it was transferred into two sterilized test tubes. Then 5 ml of MR indicator was added to both tubes and shaken well. Production of yellow colour indicates negative reaction, whereas red colour shows positive [15].

Voges Proskauer. The MR-VP broth containing test organism was incubated for 24 hrs, after incubation it was transferred into two sterilized test tubes. After this the Barrett's reagent A and B added in the two test tubes respectively. If the broth turns into pink colour, it is the indication of positive test [15].

Oxidase test. The prepared culture was applied upon the oxidase test strip placed on the clean microscopic slide and then observed for variations in colour [16].

Antibiotic sensitivity. The susceptibility of LAB is assessed by antibiotic disk diffusion method. Bacterial inoculum was prepared in PBS (Phosphate Buffered Saline) and then culture was streaked on MHA (Mueller Hinton Agar) plate and antibiotic discs were placed aseptically on inoculated plates. Following incubation for 18-24 hrs the zones were measured around each disc [17].

3. RESULTS

3.1 Phenotypical investigation

The obtained isolates were observed for their structural properties. The colonies of all isolates found creamish white and opaque in nature and majority of them were oval in shape while some were large in size and some were small [figure 1]. The number of colonies observed were $>10^5$ CFU/ml. The interpretations observed from microscopic examinations of various isolates showed signs of Gram positive, rod shaped [figure 2] and non-motile characteristics of bacteria as presented in Table 1.

Table 1. Microscopical, cultural and Gram characteristics of Lactic acid bacteria

Serial no.	Isolates name	Colony Form	Colony Colour	Opacity	Gram nature	Morphology
1	C1	Circular	Creamish white	Opaque	Positive	Rod
2	C2	Circular	Creamish white	Opaque	Positive	Rod
3	C3	Circular	Creamish white	Opaque	Positive	Rod
4	C4	Circular	Creamish white	Opaque	Positive	Rod
5	C5	Circular	Creamish white	Opaque	Positive	Rod
6	C6	Circular	Creamish white	Opaque	Positive	Rod



Figure 1. Lactic acid bacteria colonies on MRS agar

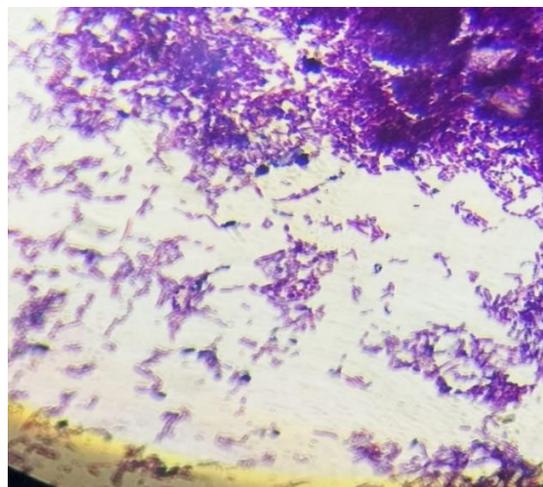


Figure 2. Rod shaped Lactic acid bacteria under 100x magnification

3.2 Biochemical characterization of isolates

Various isolates were examined for different biochemical tests such as catalase activity, oxidase, Methyl red, VP, indole production. Results from all the isolates were observed negative as shown under Table 2. The biochemical analysis and Bergey's manual of bacteriology indicates the presence of *Lactobacilli* species.

Table 2. Biochemical test results of isolates

Isolates name	Catalase test	Indole Test	Methyl red test	Voges Proskauer	Oxidase test
C1	-	-	-	-	-
C2	-	-	-	-	-
C3	-	-	-	-	-
C4	-	-	-	-	-
C5	-	-	-	-	-
C6	-	-	-	-	-

Note: *-* = Negative

3.3 Antibiotic sensitivity results

Isolates such as C1 and C2 were further tested for antibiotic activity against various drugs [fig.3 & 4] such as Amikacin, Amoxyclav, Ampicillin, Cefepime, Cefixime, Cefoperazone, Tetracycline, Ceftazidime, Trimethoprim-Sulfamethoxazole, Ceftriaxone, Ciprofloxacin, Gentamicin, Levofloxacin, Meropenem, Nitrofurantoin, Netilmicin, Piperacillin/ Tazobactam. The majority of the broad-spectrum antibiotics were found to be sensitive to the isolates. The isolate C1 showed resistance to Ampicillin while C2 was resistant against Ceftriaxone and Ciprofloxacin. Adding to this, Amoxiclav and Vancomycin were found intermediate in both samples. Table 3 and 4 displays each isolate's response to several antibiotics in terms of sensitivity and resistance.

Table 3. Antibiotic sensitivity results of C1 and C2 isolates

Antibiotic	Antibiogram (C1 isolate)		Antibiogram (C2 isolate)	
	Zone size (mm)	Interpretation	Zone size (mm)	Interpretation
Amikacin	25	Sensitive	24	Sensitive
Amoxyclav	12	Intermediate	12	Intermediate
Ampicillin	02	Resistance	12	Intermediate
Cefepime	22	Sensitive	32	Sensitive
Cefixime	26	Sensitive	20	Sensitive
Cefoperazone	20	Sensitive	25	Sensitive
Ceftazidime	25	Sensitive	22	Sensitive
Ceftriaxone	32	Sensitive	02	Resistance
Ciprofloxacin	31	Sensitive	02	Resistance
Gentamicin	18	Sensitive	15	Sensitive
Levofloxacin	36	Sensitive	14	Intermediate
Meropenem	23	Sensitive	23	Sensitive
Nitrofurantoin	20	Sensitive	26	Sensitive
Netilmicin	23	Sensitive	25	Sensitive
Piperacillin/ Tazobactam	28	Sensitive	20	Sensitive
Tetracycline	20	Sensitive	20	Sensitive
Trimethoprim- Sulfamethoxazole	25	Sensitive	22	Sensitive
Vancomycin	10	Intermediate	10	Intermediate

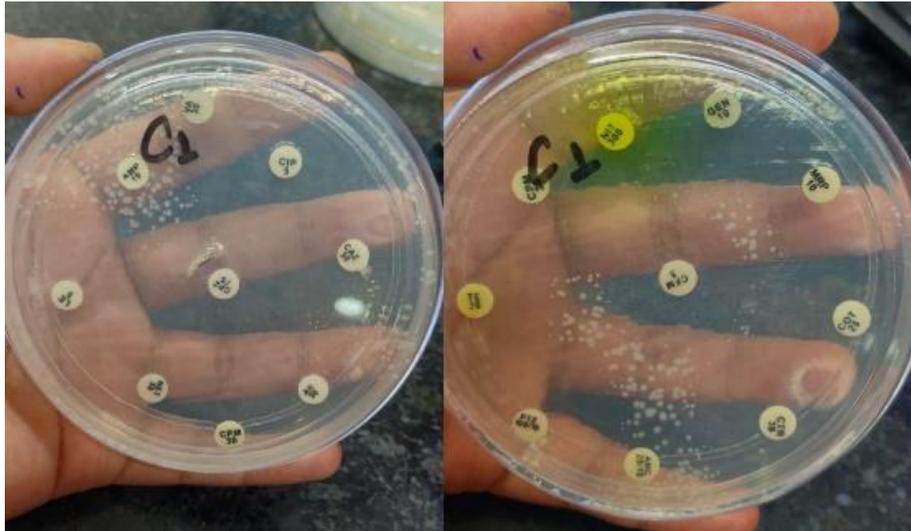


Figure 3. Antibiotic sensitivity of isolate C1

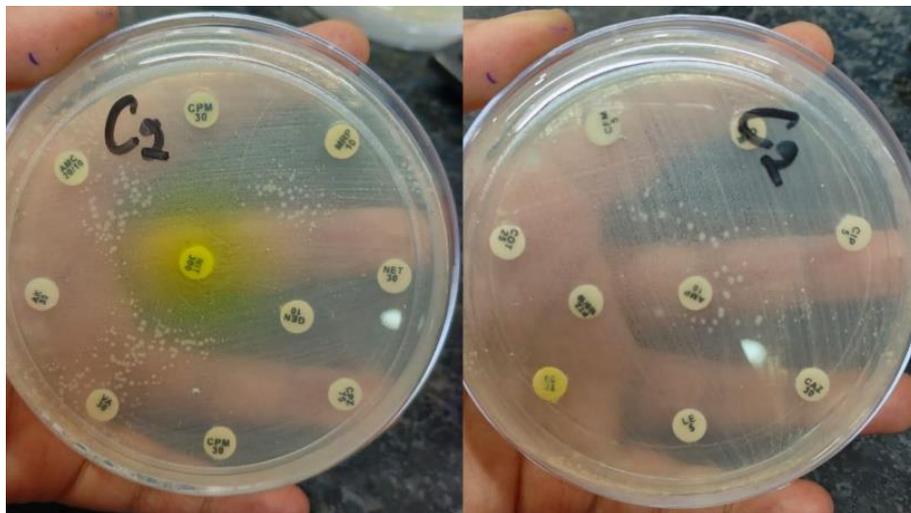


Figure 4. Antibiotic sensitivity of isolate C2

4. DISCUSSION

Goyal et al. in 2012 identified 28 LAB strains from 14 curd samples. The colonies were creamish white, including small and large sizes moreover, majority of the isolates were Gram positive, rods and cocci shaped, these results were similar with our analysis, which demonstrated the Gram-positive nature of isolated strains along with the appearance of colonies [18]. The findings of present research were closely related with the study of Sonwane et al., 2022, who stated negative reaction for catalase and other biochemical tests [19]. The research conducted by Suganya et al. in 2013, showed that Vancomycin was resistant for all the isolates, whereas other antibiotics such as Gentamycin, Chloramphenicol and Ciprofloxacin were sensitive and resistant for various isolates [20]. We investigated the resistance of C1 isolate to Ampicillin and C2 isolate against Ceftriaxone and Ciprofloxacin. Other drugs were intermediate and sensitive.

5. CONCLUSION

In this study six isolates were collected from homemade curd, which were then analysed for their antagonistic properties. The colonies were found to be creamish white and opaque on MRS agar plating. In addition to this, all bacteria were rod shaped, Gram positive and non-motile. Various biochemical test results were negative such as Catalase, Voges Proskauer, Methyl red, Indole and Oxidase. Antibiotic sensitivity of isolate C1 showed resistant behaviour towards the Ampicillin and C2 against Ceftriaxone and Ciprofloxacin. This study revealed that antagonistic effects of compounds made from bacteria such as lactic acid bacteria, are very crucial in improving quality of food and health of human beings because they can be used for both medicinal preparations as well as for dietary supplementations. Understanding LAB in curd is essential for improving its quality, safety and fermentation process.

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HK and DK designed the experiments for the isolation and characterization of Lactic acid bacteria. HK performed the sample collection, bacterial isolation, detailed identification and characterization of isolated strains of LAB. Both the HK and DK analyzed the data and interpreted the results. HK drafted the initial manuscript and DK provided critical revisions. All authors reviewed and approved the final manuscript.

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