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ISOLATION AND IDENTIFICATION OF LACTOBACILLUS FROM MILK PRODUCTS

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ABSTRACT

From four different samples of milk and milk products, 25 lactic acid bacterial strains were isolated. The strains were termed as lactobacillus by performing various biochemical tests. Further antibacterial analysis of the secondary metabolites produced from these isolates was performed and found that culture C25 shows potent activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli*. The effect of pH and temperature on the growth of lactobacillus isolates was carried out and effective results were obtained at 37^0C and pH 7.

Key words: Secondary Metabolites, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*.

INTRODUCTION

For more than 2000 years, fermented milk products have been prepared. Allowing milk that creates an acidic substance that does not purify to ferment naturally. Fermented milk products are quickly digestible and wholesome. Yoghurt, kefir, koumiss and acidophilus milk are examples of such products.

These essence of fermented dairy products, depending on the local indigenous microflora, varies from region to region **[1].** "Ergo" is traditional Ethiopian fermented milk produced in a non hygienic setting by spontaneous fermentation using traditional utensils **[2].**

Several lactic acid bacteria are present in Kefir, namely *Lactococcus lactis, Lactococcus cremoris, Lactobacillus kefir, L. casei, L acidophilus and Leuconostoc species*. It also requires yeast. A very important part of the everyday diet is indeed dairy items made from locally raised raw milk. These products have one common feature; fermentation **[3,4,5].**

Lactic Acid Bacteria (LAB) including Lactobacillus spp. are Generally Recognized as Safe (GRSA) bacteria that have been used in the processing of fermented food for centuries. They occur naturally as indigenous microflora in fermented milk products such as Yoghurt. There is a growing interest in the use of Lactobacillus spp. As probiotic due to the increasing emergence of antibiotic resistance **[6].** Long before the term „probiotic" was coined **Eli Metchnikoff**, the Nobel laureate immunologist suggested in 1908 that the reason Balkan peasants lived long lives was that they drank milk fermented with *Lactobacillus bulgaricus* and *Streptococcus thermophilus* . These bacteria would suppress "putrefactive fermentation" leading to better health and longevity. Today, many potential health benefits of probiotic bacteria are under investigation, from improving the microbial balance in the intestine to enhancing immune system function.

Lactic acid bacteria play a significant role in food fermentation and they have antagonistic effects against food-borne pathogenic microorganisms and help to improve biochemical features of fermented milk products **[7,8]**.

They have an enzyme system to utilize carbohydrates for the production of organic acid such as lactic acid or acetic acid. Most foodborne contaminants and pathogenic organisms are sensitive to these acids since they have the potential to reduce the pH value of fermented milk products **[9,10]**. They also have the potential to produce antibacterial substances such as hydrogen peroxide, Diacetyle bacteriocins, and CO2 which can play part in the antagonism foodborne and pathogenic microorganisms. Lactic acid bacteria also produce different types of compounds that impart characteristic aroma, color, flavor, and test of fermented milk products **[11,12,13]**. The application of antagonistic compounds by lactobacilli is not limited to food preservation, antimicrobials of LAB have been employed successfully to prevent the formation of biogenic amines to inhibit pathogen causing mastitis and to inhibit enteropathogens in the small intestines of animals **[14].** The intense and different antagonistic activity induced by *lactobacilli* against various foodborne pathogens thus demonstrating that using selected lactic acid bacteria strains as adjunct cultures could be an effective strategy to prevent the development of foodborne pathogens in artisanal raw milk cheeses **[15].**

MATERIALS AND METHODOLOGY

Samples collection:

The different milk products were collected from the local shops of different location of Lucknow.

Isolation of bacteria from samples:

The milk and milk product were serially diluted in 0.85% NaCl solutions. Further, diluted samples were spread on nutrient agar plates and then pure culture plates were prepared by selecting the cultures on the basis of different morphological parameters **[16]**.

Strain identification of isolates:

Various biochecmical tests suchgrams staining, endospores staining, glucose fermentation test, mannitol test etc. as were performed and strains were identified by using Bergy's manual **[17]**.

Assessment of antimicrobial activity of extracted crude metabolites from isolated cultures:

The pure culture broths were prepared and then the antibacterial activity was analysed against gram positive and gram negative strains. *Escheriachia coli, Pseudomonas aeruginosa, Staphylococcus aureus.* The tests were carried out by using agar well diffusion method **[18]**.

Study of growth parameters of isolates:

The cultures were inoculated in sterilized MRS medium and then the absorbance were taken at 620 nm in UV-Vis spectrophotometer at different time intervals **[19]**.

Effect of pH & temperature on growth of isolates:

The cultures were treated at different temperatures and different pH to observe the effect of these parameters on the growth of isolates **[20]**.

RESULTS

Collection of milk product samples:

The milk and milk products were collected from different locations of Lucknow.

Table 1: Collected milk products

S.No	Sample Name
1.	 Curd
2.	 Milk
3.	 Yogurt
4.	 Cheese

Bacterial isolation by serial dilution:

Total 25 bacterial cultures were isolated from all four collected milk & milk product samples using serial dilution and spread plate method. These cultures were selected based on their different morphological characteristics. Six cultures were obtained from each sample 1, 2 & 3, seven from sample 4, and four cultures from sample 4. These cultures are named as C1, C2….. C25.

Figure 1: Bacterial cultures on agar plates after the serial dilution and spreading.

Bacterial purification:

Bacterial purification was done using the streak plate method by streaking the selected cultures in Petri plates.

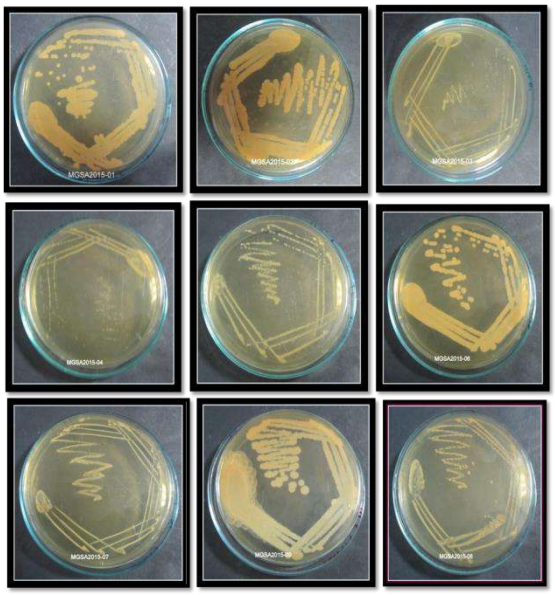


Figure 2: Few pure bacterial culture in agar plates after streaking.

Colony morphology

The cultures obtained from different samples were differentiated based on their morphology. The morphology of all the selected colonies is given in the table below.

Table 2: The morphology of all the selected colonies.

Culture name	Shape	Margin	Elevation	Pigmentation	Surface	Texture	Opacity
C1	Circular	Entire	Flat	White	Rough	Hard	Opaque
C2	Spindle	Discrete	Raised	Off-white	Smooth	Soft	Translucent
C3	Filamentous	Curled	Convex	Green	Smooth	Soft	Opaque
C4	Punctiform	Lobate	Pulmonate	Yellowish	Rough	Hard	Translucent
C5	Circular	Discrete	Flat	Off-white	Smooth	Soft	Opaque
C6	Rhizoidal	Curled	Convex	White	Rough	Hard	Opaque
C7	Circular	Discrete	Flat	Off white	Rough	Gummy	Opaque
C8	Circular	Entire	Raised	Yellowish	Smooth	Hard	Translucent
C9	Irregular	Lobate	Convex	Off white	Smooth	Soft	Opaque
C10	Spindle	Discrete	Raised	White	Rough	Hard	Opaque
C11	Filamentous	Curled	Convex	Off-white	Smooth	Soft	Translucent
C12	Punctiform	Lobate	Pulmonate	Green	Smooth	Soft	Opaque
C13	Circular	Discrete	Flat	White	Rough	Hard	Opaque
C14	Irregular	Entire	Flat	Off white	Rough	Soft	Opaque
C15	Circular	Lobate	Convex	Yellowish	Convex	Soft	Opaque
C16	Circular	Lobate	Flat	Off white	Smooth	Gummy	Opaque
C17	Irregular	Lobate	Raised	Off white	Convex	Hard	Opaque
C18	Circular	Curled	Raised	Off white	Convex	Hard	Opaque
C19	Circular	Lobate	Flat	Yellowish	Smooth	Soft	Opaque
C20	Circular	Curled	Raised	White	Smooth	Soft	Opaque
C21	Irregular	Entire	Convex	Off-white	Convex	Soft	Opaque
C22	Circular	Lobate	Pulmonate	Green	Convex	Hard	Opaque
C23	Irregular	Lobate	Flat	Off-white	Rough	Gummy	Opaque
C24	Circular	Entire	Convex	Yellowish	Convex	Soft	Opaque
C25	Circular	Lobate	Flat	Off white	Smooth	Gummy	Opaque

Table 3: Showing results of Gram's Staining, Endospores staining.

Culture	Gram's staining	Shape	Endospores staining
C1	Gram Negative	Rod	Negative
C2	Gram Positive	Rod	Negative
C3	Gram Positive	Rod	Positive
C4	Gram Positive	Coccus	Negative
C5	Gram Negative	Rod	Negative
C6	Gram Negative	Rod	Negative
C7	Gram Negative	Coccus	Negative
C8	Gram Negative	Rod	Negative
C9	Gram Positive	Rod	Positive
C10	Gram Positive	Rod	Positive
C11	Gram Positive	Coccus	Negative
C12	Gram Negative	Rod	Negative
C13	Gram Negative	Rod	Negative
C14	Gram Negative	Rod	Negative
C15	Gram Positive	Rod	Positive
C16	Gram Positive	Coccus	Negative
C17	Gram Positive	Coccus	Negative
C18	Gram Positive	Coccus	Negative
C19	Gram Positive	Coccus	Negative
C20	Gram Negative	Rod	Negative
C21	Gram-Positive	Rod	Negative
C22	Gram-Positive	Coccus	Positive
C23	Gram-Positive	Coccus	Positive
C24	Gram-Positive	Rod	Positive
C25	**Gram-Positive**	**Rod**	**Negative**

Table 4: Showing results of Catalase test, Mannitol Test, Starch hydrolysis test.

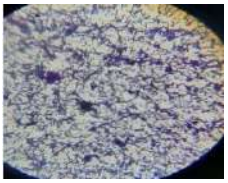
Culture	Mannitol test	Catalase test	Starch hydrolysis test
C1	Negative	Negative	Negative
C2	Positive	Positive	Positive
C3	Positive	Positive	Positive
C4	Positive	Negative	Negative
C5	Positive	Negative	Negative
C6	Negative	Negative	Positive
C7	Positive	Positive	Negative
C8	Positive	Positive	Negative
C9	Negative	Negative	Negative
C10	Positive	Negative	Negative
C11	Positive	Positive	Negative
C12	Positive	Negative	Negative
C13	Positive	Positive	Negative
C14	Positive	Negative	Negative
C15	Positive	Negative	Negative
C16	Positive	Positive	Negative
C17	Positive	Negative	Negative
C18	Negative	Negative	Positive

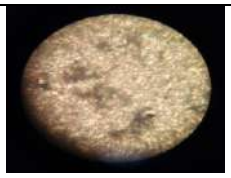
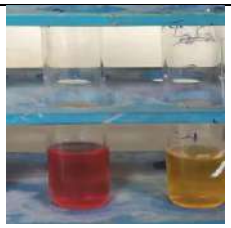
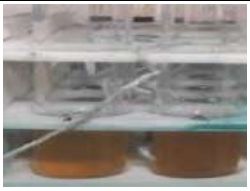
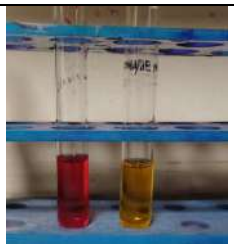
Culture	Mannitol test	Catalase test	Starch hydrolysis test
C19	Positive	Positive	Negative
C20	Positive	Negative	Negative
C21	Positive	Positive	Positive
C22	Positive	Negative	Negative
C23	Positive	Negative	Negative
C24	Positive	Negative	Negative
C25	**Positive**	**Negative**	**Positive**

Table 5: Showing results of Methyl red, Voges Prausker, Glucose Fermentation test, Citrate test.

Culture	MR Test	VP test	GF test	Citrate test
C1	Negative	Negative	Negative	Negative
C2	Positive	Negative	Positive	Negative
C3	Positive	Positive	Positive	Negative
C4	Positive	Negative	Negative	Negative
C5	Negative	Negative	Negative	Negative
C6	Negative	Negative	Negative	Negative
C7	Negative	Negative	Negative	Negative
C8	Negative	Negative	Negative	Negative
C9	Positive	Negative	Positive	Positive
C10	Positive	Positive	Positive	Positive
C11	Positive	Positive	Negative	Positive
C12C	Negative	Negative	Negative	Negative
C13	Negative	Positive	Negative	Negative
C14	Negative	Negative	Negative	Negative
C15	Positive	Negative	Positive	Positive
C16	Positive	Positive	Negative	Positive
C17	Positive	Positive	Negative	Negative
C18	Positive	Negative	Negative	Negative
C19	Positive	Negative	Negative	Negative
C20	Positive	Positive	Negative	Negative
C21	Positive	Negative	Positive	Positive
C22	Positive	Positive	Negative	Positive
C23	Positive	Positive	Negative	Negative
C24	Positive	Negative	Negative	Negative
C25	**Positive**	**Negative**	**Positive**	**Negative**

Table 6: Biochemical properties of *Lactobacillus spp. given by* C25

S no.	Tests	Results
1	Gram's staining	 **Positive**
2	Shape	Rod, *Bacillus*
3	Catalase test	 Negative

4	Spore forming test	 Negative
5	Methyl red test	 Positive
6	Voges Prauskeur test	 Negative
7	Citrate test	Negative
8	Starch hydrolysis test	 Positive
9	Glucose fermentation test	 Positive

Antibiotic sensitivity test of crude secondary metabolites from isolates:

Antibiogram analysis test of all the 18 cultures was performed by Agar well diffusion method against test pathogens which were present in the lab viz. Escherichia coli, Staphylococcus aureus, & *Pseudomonas aeruginosa*. The supernatant (Crude 2° Metabolites) of all culture broths were taken after centrifugation and loaded in the wells of NA plates on which pathogens were spread. After an incubation period of 24 hrs. at 37°C, results were obtained. Cultures number was observed with a clear and larger zone of inhibition against all the pathogens and was selected for further production and purification.

Figure 3: Graphical analysis of antibacterial sensitivity test of crude secondary metabolites extracted from C1, C2… C9 cultures.

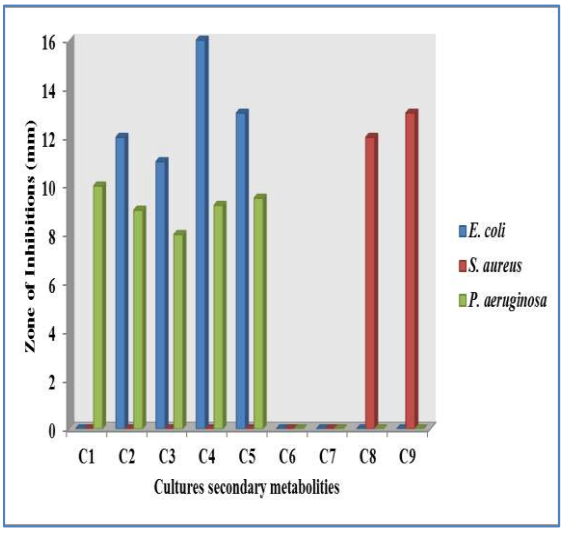


Figure 4: Graphical analysis of antibacterial sensitivity test of crude secondary metabolites extracted from C10, C11… C18 cultures.

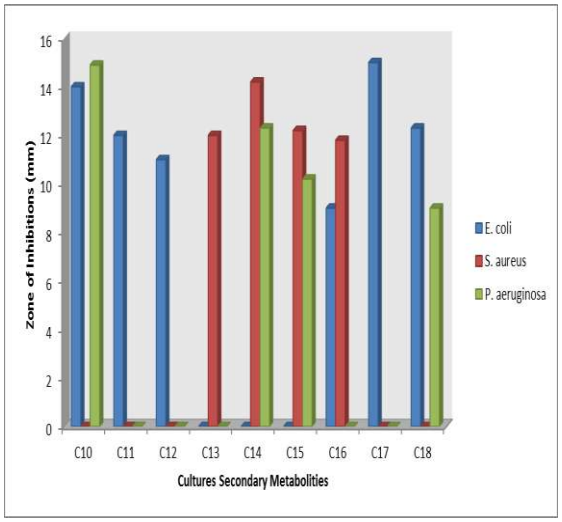
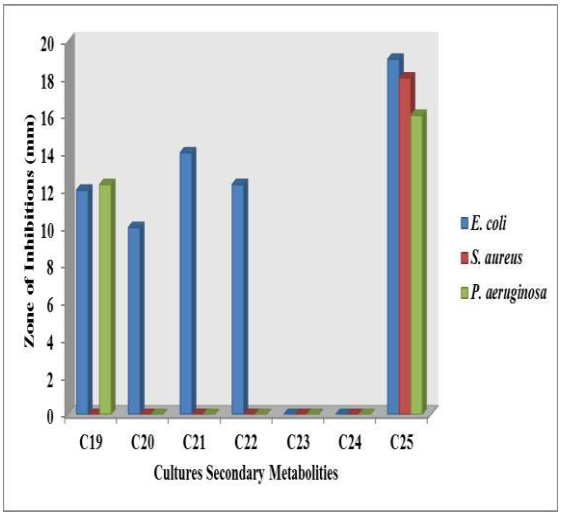


Figure 5: Graphical analysis of antibacterial sensitivity test of crude secondary metabolites extracted from C19, C20… C25 cultures.



Growth curve study of isolates

Growth kinetics of Bacterial strains was studied by taking the absorbance reading of the culture broth at 600nm after every 24 hrs. The stationary phase was observed on the third day of the reading.

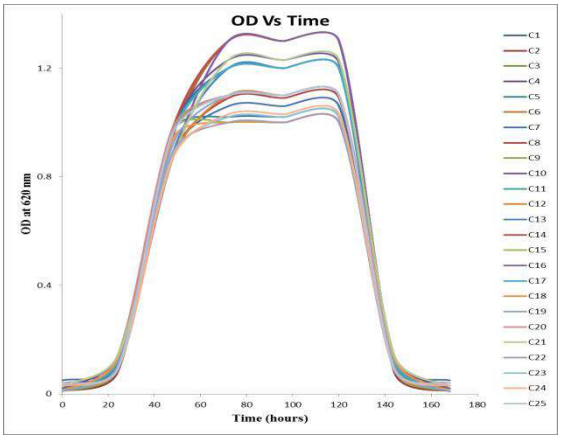


Figure 6: Growth kinetics analysis of isolates

Effect of temperature on isolates

The isolates growth at different temperature range on solid media plates to check the effect of temperature on the growth. The selected temperatures were 4°C, 37°C and 60°C. The maximum growth was found at 37°C. The results are shown below.

Figure 7: Effect of temperature on isolates

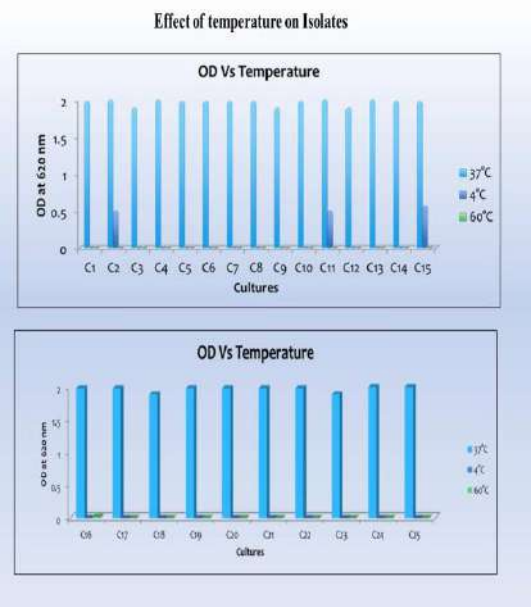
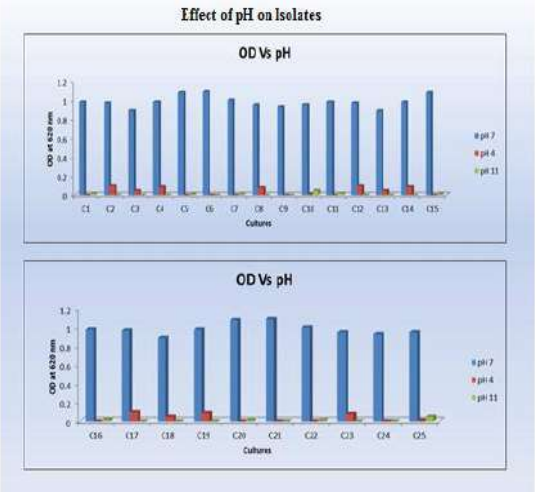


Figure 8: Effect of pH on isolates



DISCUSSION & CONCLUSION

The present study was carried out to isolate and identify the milk product microflora and to study their ability for the production of antimicrobial metabolites active against pathogens. Milk product sample was collected from different locations. 25 bacterial isolation was done by serial dilution method on MRS media and further purification was done by continuous quadrant streaking.

The pure culture broths were prepared from the streak plates, and various biochemical tests were carried out. Biochemical tests such as Gram's staining, endospore staining, catalase test, etc. were done for strain identification given by Bergey's manual.

Further, these isolated Lactobacillus strains were analyzed for their antimicrobial activity against the Gram-positive and Gram-negative bacteria (*E. coli, S. aureus, P. aeruginosa.)* by performing antibiogram analysis of crude metabolites by Agar Well Diffusion method of **Kerby Buer.** After performing the antibacterial test it was found that all strains do not show effective results, but culture 25 has shown the best potential antibacterial screening against all three pathogens.

The growth curve study of all 25 strains was also performed. The effect of pH and Temperature on the growth of all Lactobacillus strains were also performed and found the pH 7 and temperature 37^0C is best for the maximum growth.

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