Isolation of Lactobacillus from Different Samples And Its Cryopreservation

Inception Report

Submitted by

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1. ABSTRACT :

The objective of this internship was to isolate *Lactobacillus* species from various natural and fermented samples, including milk, yogurt, cheese, and olives. *Lactobacillus* is a genus of lactic acid bacteria known for its probiotic properties and significant role in food fermentation and gut health. The isolation process involved enrichment of samples in MRS (de Man, Rogosa, and Sharpe) broth, followed by serial dilution, culturing on MRS agar, and incubation under anaerobic conditions. Colonies with typical *Lactobacillus* morphology were selected for microscopic examination and biochemical characterization. This study successfully identified and isolated potential *Lactobacillus* strains from all tested samples, demonstrating the diversity and abundance of these bacteria in common food sources. The findings contribute to understanding the natural distribution of *Lactobacillus* and their potential applications in food biotechnology and probiotic development.

2. INTRODUCTION :

Lactobacillus species exhibit significant antagonistic effects against pathogens such as *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*, making them suitable candidates for use in health-promoting food products and biopreservation strategies. Probiotics, particularly species from the *Lactobacillus* genus, are beneficial microorganisms widely recognized for their role in maintaining gut health and inhibiting pathogenic bacteria. They are commonly isolated from fermented dairy products such as curd, yogurt, cheese, and milk, which serve as natural reservoirs of lactic acid bacteria (LAB) due to their nutrient-rich composition and microbial diversity. The increasing demand for functional foods and natural alternatives to antibiotics has led to a growing interest in isolating probiotic strains with high antimicrobial activity and tolerance to gastrointestinal conditions. [1,2].

3. REFERENCES:

1. De, S., Pramanik, A., Das, A. K. R., Paul, S., Jana, S., & Pramanik, P. (2017). Isolation and characterization of *Lactobacillus* spp. from curd and its pharmacological application in probiotic chocolate. *The Journal of Phytopharmacology, 6*(6), 335–339.
2. Karami, S., Roayaei, M., Hamzavi, H., Bahmani, M., Hassanzad-Azar, H., Mahmoodnia, L., & Rafieian-Kopaei, M. (2017). Isolation and identification of probiotic *Lactobacillus* from local dairy and evaluating their antagonistic effect on pathogens. *International Journal of Pharmaceutical Investigation, 7*(3), 137–141. https://doi.org/10.4103/jphi.JPHI_8_17

EXPERIMENT 01

1. OBJECTIVE :

To isolate Lactobacillus from milk and its cryopreservation.

2. MATERIALS:

- Samples (milk, yogurt, cheese, pickle, fermented rice water, fermented lentil water)

- Petri Plates
- MRS Broth
- Agar
- Flasks
- Test tubes filled with 9 ml distilled water
- Blender
- Spatula
- Graduated cylinder
- Distilled Water
- Autoclaved Tips
- Jester
- Spreaders
- Slides with Cover slips
- Dyes(crystal violet, iodine, decolorizer, safranin)
- Wireloop
- Spirit Lamp

3. METHOD :

1. Sterilization of Lab Equipment

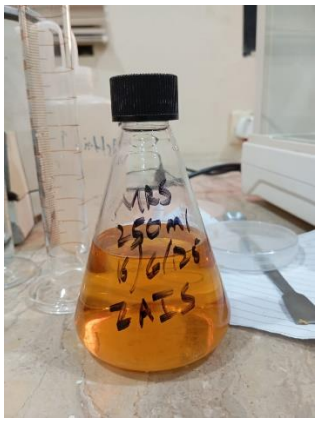
All glassware and materials used in the experiment were sterilized prior to use. This included conical flasks, test tubes, pipettes, petri plates, measuring cylinders, and other necessary instruments. Sterilization was carried out using an autoclave at 121°C for 15–20 minutes to eliminate any potential contaminants.

2. Preparation of MRS Broth and MRS Agar

MRS (de Man, Rogosa, and Sharpe) medium was prepared in two forms:

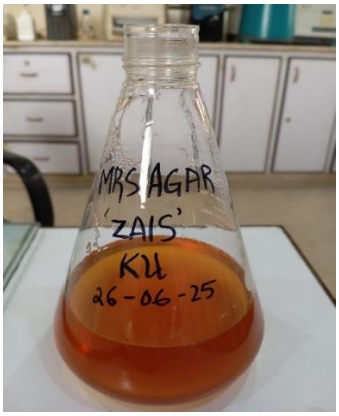
a. MRS Broth

A volume of 250 mL MRS broth was prepared by weighing the required amount of MRS powder.
After mixing with distilled water, the solution was volume made up to 250 mL using a volumetric flask. The broth was then gently heated until it reached boiling point to ensure complete dissolution and autoclaved at 121°C for 15–20 minutes.



b. MRS Agar

To prepare MRS agar, a specific amount of agar powder was added to the MRS broth solution. **(500 ml)**
The medium was then volume made up and heated until it reached a boil. Both MRS broth and MRS agar were transferred to appropriate containers, sealed, and autoclaved at 121°C for 15–20 minutes.



3. Sample Collection

Milk was selected as the primary sample for the isolation of *Lactobacillus*. A volume of 10 mL of raw milk was collected under sterile conditions for further processing.

4. Sample Enrichment in MRS Broth

To enrich lactic acid bacteria, 10 mL of the milk sample was added to 90 mL of sterile MRS (de Man, Rogosa, and Sharpe) broth. The mixture was gently shaken to ensure proper mixing. Following this, the sample was blended using a laboratory blender for approximately 5 minutes to achieve homogeneity.

5. Incubation for Enrichment

After blending, the enriched sample was transferred to a sterile conical flask and incubated at 37°C for 48 hours under anaerobic conditions to promote the growth of *Lactobacillus* species.

6. Serial Dilution

After incubation, a serial dilution of the enriched sample was performed. Sterile test tubes containing 9 mL of autoclaved distilled water were prepared.

- 1 mL of the enriched sample was added to the first tube (10^{-1} dilution), mixed thoroughly, and subsequently, 1 mL was transferred to the next tube (10^{-2} dilution).

- This process continued up to the 10^{-6} dilution.
  All steps were conducted using sterile pipettes to avoid contamination.

7. Preparation of MRS Agar Plates

MRS agar was prepared, autoclaved, and poured into sterile Petri dishes. The plates were allowed to solidify under aseptic conditions.

8. Inoculation of Diluted Samples on Agar Plates

From the 10^{-4}, 10^{-5}, and 10^{-6} serial dilutions, 100 µL of each dilution was transferred onto separate solidified MRS agar plates using a sterile micropipette. The sample was then spread evenly using a sterile glass spreader.

9. Incubation of Plates

After inoculation, the plates were incubated at 37°C for 24 hours under anaerobic conditions to allow the growth of *Lactobacillus* colonies.

10. Microscopy and Gram Staining

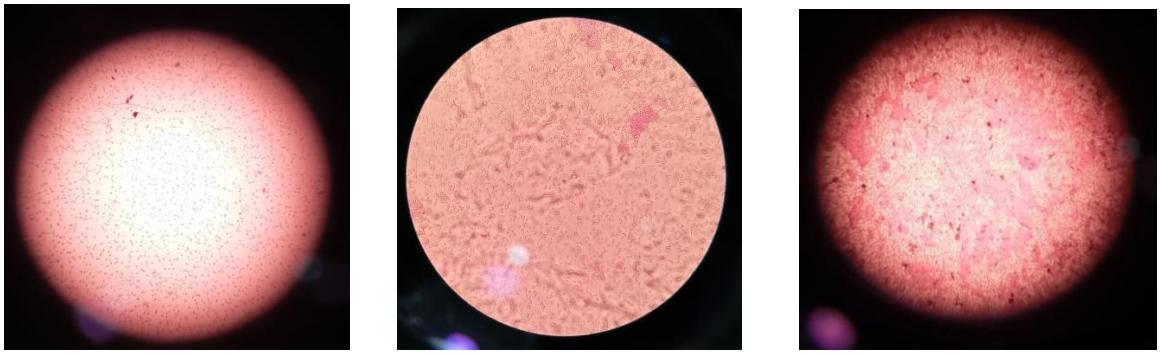
A colony from the agar plate was picked using a sterile loop and mixed with a drop of distilled water on a clean glass slide. The smear was air-dried and heat-fixed. Gram staining was performed using crystal violet (1 min), iodine (1 min), decolorizer (1 min), and safranin (1 min), with rinsing between each step. A cover slip was placed, and the slide was observed under a microscope for bacterial morphology.

4. RESULT :

After 24 hours of incubation, the MRS agar plates were observed for bacterial growth. Contrary to expectations, no typical *Lactobacillus* colonies were detected. Instead, visible contamination was present on the plates, indicating the growth of undesired or non-target microorganisms.

To further investigate the contamination, a sample from the suspected colonies was picked and subjected to microscopic examination. The colony was smeared onto a clean glass slide, heat-fixed, and stained using appropriate dyes. Upon microscopic observation, no morphological characteristics typical of *Lactobacillus* (such as rod-shaped, Gram-positive bacilli) were observed. This confirmed that the colonies grown were not *Lactobacillus*, and the isolation attempt in this trial was unsuccessful due to contamination.

Contamination on Plates of milk sample



Observation Under Microscope

5. DISCUSSION:

The isolation attempt for *Lactobacillus* was unsuccessful due to contamination on the agar plates. One possible reason for contamination could be the over-boiling of MRS agar during preparation, which may have caused nutrient degradation or altered the medium composition, thereby affecting the selective growth of *Lactobacillus*. Additionally, during incubation, other microbial plates, including fungal cultures, were present in the same incubator. This could have facilitated cross-contamination through airborne spores or accidental contact. Such factors highlight the importance of precise media preparation and maintaining strict aseptic conditions, including dedicated incubation spaces for selective cultures, to ensure successful isolation.

6. CONCLUSION :

This study aimed to isolate *Lactobacillus* from milk samples using MRS broth and agar under anaerobic incubation. However, the results showed no *Lactobacillus* growth, and contamination was observed instead. Microscopic examination confirmed the absence of *Lactobacillus* morphological characteristics. The contamination was likely due to media preparation issues and possible cross-contamination during incubation.

EXPERIMENT 02

1. OBJECTIVE :

To isolate *Lactobacillus* from cheese using improved sample handling and contamination prevention methods.

2. MATERIALS AND METHODS:

In this trial, four samples (milk, yogurt, cheese, and olives) were processed, but my work focused specifically on the **cheese sample**. The general methodology followed Experiment No. 1, with the following modifications for cheese:

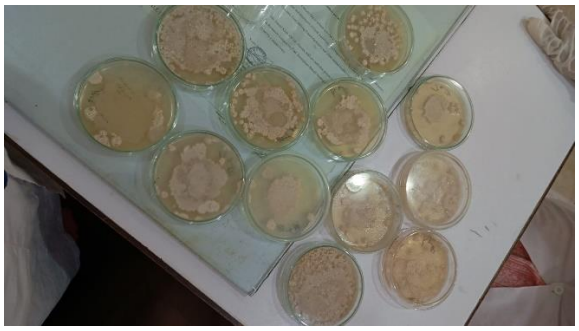
1. **Sample Preparation**
   The cheese sample was ground using a sterile mortar and pestle instead of blending. This approach was chosen to avoid excessive heat generation and maintain bacterial viability. The ground cheese was added directly to sterile MRS broth in a conical flask and shaken thoroughly to achieve uniform mixing.

2. **Incubation and Contamination Prevention**
   Following enrichment, serial dilution, and plating on MRS agar as in Experiment No. 1, the inoculated agar plates were **wrapped in clean paper** before incubation. This measure was implemented to reduce the risk of airborne or cross-contamination from other microbial plates present in the incubator.

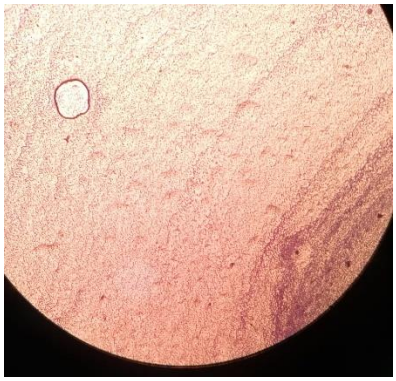
The remainder of the process, including anaerobic incubation, microscopy, and Gram staining, was conducted following the same procedures as described in Experiment No. 1.

3. RESULT :

After 24 hours of incubation, the MRS agar plates containing cheese sample inoculum were observed. No colonies with typical *Lactobacillus* morphology were present. Instead, contamination from non-target microorganisms was again observed. Microscopic examination of selected colonies confirmed the absence of *Lactobacillus* characteristics (Gram-positive rods), suggesting that the isolation attempt for the cheese sample was also unsuccessful.



Contamination



Observation Under Microscope

4. DISCUSSION :

The repeated occurrence of contamination in the second experiment suggests that additional factors beyond those addressed in the modified method may be contributing to the problem. While the risk of airborne contamination was reduced by wrapping the plates, contamination could still have originated from the initial cheese sample, handling steps, or pre-existing

organisms in the working environment. Furthermore, the grinding of cheese using a mortar and pestle, although gentler than blending, may have introduced environmental microbes if absolute sterility was not maintained. Another possible factor could be the use of **plastic spreaders** instead of glass spreaders. Plastic spreaders are more difficult to sterilize effectively, and even after flaming or disinfecting, they may retain microbial contaminants, which could transfer to the agar surface.

5. CONCLUSION :

The attempt to isolate *Lactobacillus* from cheese using modified handling and contamination prevention techniques was unsuccessful. The experiment again resulted in contamination by non-target organisms, with no evidence of *Lactobacillus* growth.

EXPERIMENT 03

1. OBJECTIVE:

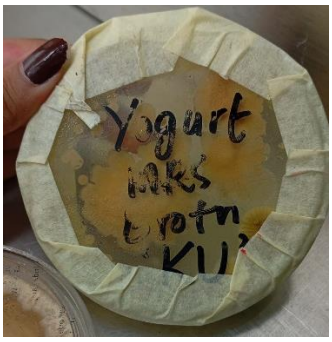
To attempt *Lactobacillus* isolation using direct streaking instead of serial dilution, and to include control plates for contamination monitoring.

2. MATERIALS AND METHODS:

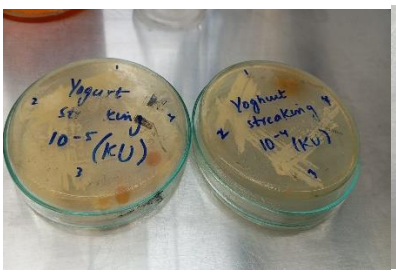
Two samples, **buffalo milk** and **yogurt**, were used. Both were processed as in previous experiments, mixed with MRS broth, and incubated. The following modifications were made:

1. **Control Plates**
   A control plate containing only sterile MRS agar was prepared to check for contamination in the medium and environment.

2. **Direct Streaking**
   Instead of spreading diluted samples, a sterile wire loop was used to streak inoculum directly from the enriched flasks (buffalo milk and yogurt in MRS broth) onto MRS agar plates without serial dilution.



3. **Use of Pre-existing Plates**
   Some pre-existing plates with presumed *Lactobacillus* colonies were present in the laboratory. Colonies from these plates were streaked onto fresh MRS agar plates for microscopic comparison.

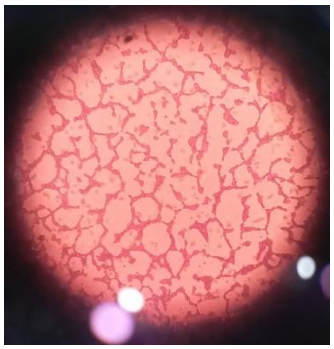



4. **Microscopy**
   After incubation, selected colonies were examined under a microscope following Gram staining.

3. RESULT:

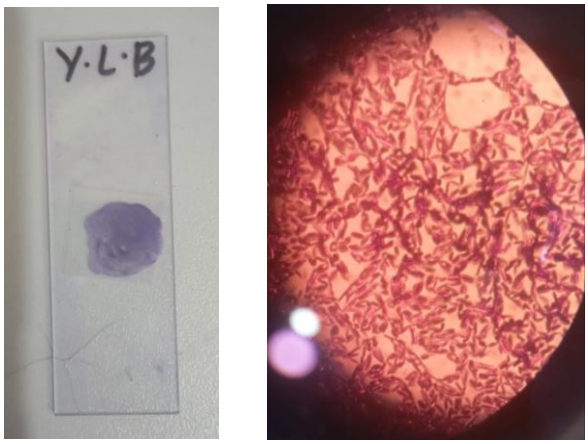
- **Control Plates:** Showed significant contamination, indicating the presence of unwanted microorganisms in the environment or during handling.

- **Buffalo Milk Plates:** No *Lactobacillus* growth was observed.



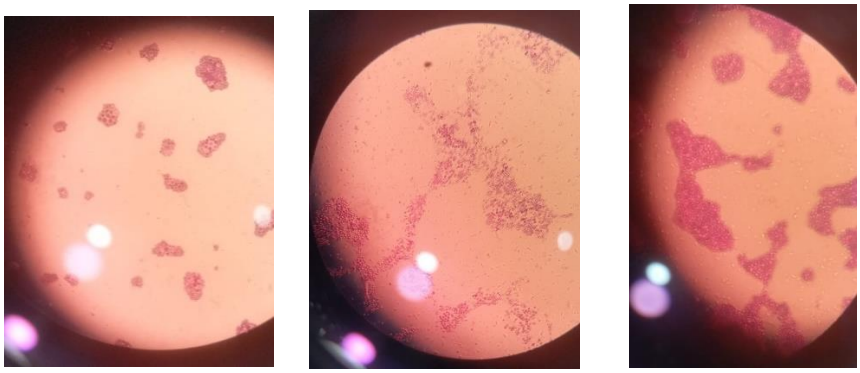
Observation Under Microscope From Buffalo Milk Sample

- **Yogurt Plates:** Microscopy confirmed the presence of *Lactobacillus*, showing typical rod-shaped Gram-positive bacilli.



Scattered Appearance Of Lactobacillus From Yogurt sample

- **Pre-existing Plates:** Microscopy revealed spherical-shaped bacteria rather than rod-shaped *Lactobacillus*, indicating that these cultures were not pure.



Observation Of Spherical Shaped Organism

4. DISCUSSION:

This experiment successfully detected *Lactobacillus* in yogurt but not in buffalo milk, suggesting that yogurt remains a reliable source due to its high lactic acid bacteria content. The heavy contamination on control plates highlights lapses in aseptic technique or environmental sterility during plate handling. The *Lactobacillus* colonies observed on yogurt plates were **scattered and closely spaced**, indicating active growth but limited isolation from surrounding colonies. The finding of spherical-shaped bacteria on pre-existing plates suggests misidentification or culture contamination over time.

5. CONCLUSION:

Direct streaking allowed the detection of *Lactobacillus* in yogurt samples, where colonies appeared scattered and closely spaced, but failed to yield *Lactobacillus* from buffalo milk. Significant contamination was observed on control plates, and spherical-shaped bacteria were seen in pre-existing cultures.

EXPERIMENT 04

1. OBJECTIVE

To perform serial dilution on the yogurt sample that previously showed *Lactobacillus* growth, in order to achieve better colony separation on MRS agar plates, and to confirm *Lactobacillus* identity using the catalase test.

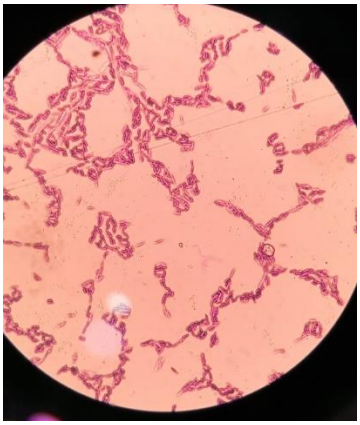
2. MATERIALS AND METHODS

The yogurt sample from Experiment No. 3, which had confirmed *Lactobacillus* growth, was processed again. The same general procedure was followed, but this time **serial dilution** was performed before plating:

1. **Serial Dilution**
   The enriched yogurt culture was serially diluted in sterile distilled water up to 10^{-6}.
2. **Plating**
   Aliquots from selected dilutions were spread on sterile MRS agar plates under aseptic conditions.
3. **Microscopy**
   After incubation, colonies were examined under a microscope following Gram staining to observe bacterial morphology and colony separation.
4. **Catalase Test**
   A fresh colony was picked and placed on a clean glass slide. A few drops of 3% hydrogen peroxide (H_2O_2) were added. The absence of bubbling indicated a **negative catalase reaction**, consistent with *Lactobacillus* species.

3. RESULT

After incubation, plates from the yogurt sample showed **well-separated colonies**, overcoming the close clustering observed in Experiment No. 3. Microscopy confirmed *Lactobacillus* morphology. In the catalase test, no bubbling was observed, confirming that the culture was catalase-negative, a key characteristic of *Lactobacillus*.



Well-separated Colonies Of Lactobacillus


Catalase Test

4. DISCUSSION

The introduction of serial dilution improved colony isolation by reducing overcrowding on agar plates, enabling clearer identification and easier potential isolation of pure colonies. The catalase test further supported the identification of the isolates as *Lactobacillus*, since these bacteria are catalase-negative. This combined approach improved both the physical separation of colonies and the reliability of bacterial identification.

5. CONCLUSION

Serial dilution of the yogurt sample resulted in well-separated *Lactobacillus* colonies, and the catalase test confirmed their identity as catalase-negative organisms, consistent with *Lactobacillus* species.

CRYOPRESERVATION

1. OBJECTIVE

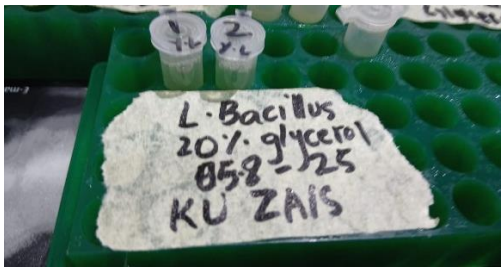
To preserve the *Lactobacillus* culture obtained from yogurt for long-term storage while maintaining its viability.

2. PROCEDURE

1. **Optical Density (OD) Measurement**
   Two colonies from different locations on the yogurt MRS agar plate were picked and inoculated into 100 mL of fresh MRS broth. The cultures were mixed thoroughly and incubated at 37°C for 24 hours under anaerobic conditions.
   After incubation, 3 mL of each culture was transferred into a cuvette, and the OD was measured at 600 nm using a spectrophotometer. The OD readings were **0.56** and **0.46**, indicating optimal bacterial growth for preservation.

2. **Preparation for Cryopreservation**
   For each culture, **75 µL of MRS broth** was mixed with **25 µL of glycerol** in sterile **Eppendorf tubes** to act as a cryoprotectant. The mixture was gently mixed to ensure even distribution.

3. **Storage**
   The prepared Eppendorf tubes were frozen at the required temperature for long-term cryopreservation.



Final Remarks

Over the course of four experimental trials, *Lactobacillus* isolation was attempted from various samples including milk, yogurt, cheese, and olives. Initial trials faced contamination issues, overcrowded colonies, and possible procedural errors. Step-by-step improvements—such as modifying sample preparation, wrapping plates to reduce airborne contamination, using mortar and pestle instead of blending, avoiding plastic spreaders, and incorporating serial dilution—gradually enhanced results.

Yogurt proved to be the most reliable source, consistently yielding *Lactobacillus* with characteristic rod-shaped morphology and a negative catalase reaction. Serial dilution provided well-separated colonies suitable for further use. Final cultures were grown to optimal density and cryopreserved in Eppendorf tubes with glycerol, creating a viable long-term stock for future applications in probiotic research or food biotechnology.