# "ISOLATION AND CHARACTERIZATION OF OXALATE DEGRADING BACTERIA"

## A RESEARCH PROJECT

Submitted by

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UNDER THE GUIDANCE OF

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**DEPARTMENT OF MICROBIOLOGY**

**VIVEKANAND COLLEGE, KOLHAPUR**

**(AN EMPOWERED AUTONOMOUS INSTITUTE)**

**YEAR 2024-2025**

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This is to certify that **Mr. SHALOM V. NAIK** studying in M. Sc. part II Microbiology at Vivekanand College, Kolhapur (An Empowered Autonomous Institute) has sincerely completed research project work entitled **"ISOLATION AND CHARACTERIZATION OF OXALATE DEGRADING BACTERIA"** during academic year 2024-25.

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**Research Project Guide**

29/12/24

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# INDEX

# 1.0- INTRODUCTION

# 1.0 INTRODUCTION

*Lactobacillus* is classified within the order *Lactobacillales,* which comprises Gram-positive, low-GC, acid-tolerant bacteria that are generally non-sporulating and non-respiring. These bacteria can be either rod-shaped (bacilli) or spherical (cocci) and exhibit a range of common metabolic and physiological characteristics.

Lactic acid bacteria (LAB) are a diverse group of microorganisms predominantly found in decomposing plant matter and dairy products. They are characterized by their ability to produce lactic acid as the primary metabolic end product of carbohydrate fermentation, which is why they are commonly referred to as lactic acid bacteria. This production of lactic acid is crucial as it facilitates food fermentation processes, effectively inhibiting the growth of spoilage organisms and pathogens through acidification (Saez, Lara et al., 2015).

In addition to lactic acid, several LAB strains synthesize proteinaceous compounds known as bacteriocins, which serve as an additional barrier against spoilage and pathogenic microorganisms. The metabolic byproducts of LAB, including lactic acid, significantly enhance the organoleptic properties such as flavor and texture of various food items. The industrial relevance of LAB is underscored by their Generally Recognized as Safe (GRAS) status, attributed to their widespread presence in food products and their positive contributions to the microbiota of both animal and human mucosal surfaces (Gomez-Llorente et al., 2015).

The core genera comprising LAB include *Lactobacillus, Leuconostoc, Lactococcus*, and *Streptococcus,* along with peripheral genera such as *Aerococcus, Enterococcus, Oenococcus, Sporolactobacillus, Tetragenococcus, Vagococcus,* and *Weissella*. All these genera except for *Sporolactobacillus*—belong to the *Lactobacillales* order and are classified under the *Bacillota* phylum. While LAB are primarily associated with the *Lactobacillales* order, it is noteworthy that bacteria from the genus *Bifidobacterium* (within the *Actinomycetota* phylum) also produce lactic acid as a significant product of carbohydrate metabolism (Sonomtok Yokot, 2012).

Overall, LAB plays a critical role in food fermentation and preservation, contributing not only to food safety but also to the sensory qualities and nutritional value of fermented products.

Lactic acid bacteria (LAB) are typically either rod-shaped (bacilli) or spherical (cocci) and are distinguished by their remarkable tolerance to acidic environments, allowing them to thrive in low pH conditions. This characteristic enables LAB to effectively outcompete other bacterial species during natural fermentation processes, as they can endure the increased acidity resulting from organic acid production, such as lactic acid. Laboratory media designed for cultivating LAB generally include carbohydrate sources, given that most LAB species are unable to respire. Additionally, LAB are catalase-negative, which further defines their metabolic profile.

LAB represents one of the most significant groups of microorganisms utilized in the food industry. Their relatively simple metabolic pathways have made them ideal candidates for use as microbial cell factories, facilitating the production of various commodities across both food and non-food sectors (Ganzle, 2015). The ability of LAB to ferment carbohydrates efficiently not only contributes to food preservation but also enhances flavor and texture, making them invaluable in food processing applications.

Lactic acid bacteria (LAB) have a wide range of applications in both medical and industrial fields. One of their primary uses is probiotics, which are products designed to deliver live, potentially beneficial bacterial cells to the gut ecosystem of humans and other animals. Most probiotic strains belong to the genus *Lactobacillus*, with several key species utilized for probiotic production. Notable genera of *Lactobacillus* that are commonly used include *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus brevis* and *Lactobaciluss gasseri.* The significance of LAB as probiotics stems from their ability to confer various health benefits. These microorganisms can enhance gut health by improving digestion, modulating the immune system, and preventing gastrointestinal disorders. LAB are recognized for their potential to inhibit pathogenic bacteria, thereby promoting a balanced gut microbiota.

Lactic acid bacteria (LAB) have emerged as promising therapeutic agents for the management of kidney stones, particularly those composed of calcium oxalate. Calcium oxalate stones are mineral deposits that form in the renal calyces and pelvis, either free-floating or attached to the renal papillae. These stones consist of crystalline and organic components

and typically develops when urine becomes supersaturated with certain minerals. The primary constituent of many kidney stones is calcium oxalate, which forms when oxalated a compound commonly found in various plant-based foods that is absorbed through the intestines and subsequently precipitates with calcium in the kidneys. Research indicates that specific LAB can effectively degrade oxalate, thereby potentially reducing the risk of kidney stone formation. For instance, a study involving six patients with idiopathic calcium-oxalate urolithiasis demonstrated that a daily intake of freeze-dried LAB significantly reduced 24-hour urinary oxalate excretion. The treatment resulted in a mean reduction from baseline values, indicating the potential of LAB to mitigate hyperoxaluria, a major risk factor for renal stone development (PubMed, 2001).

Over 80% of diagnosed kidney stones are identified as calcium oxalate stones. Individuals who form these stones often experience a high recurrence rate, and treatment options remain limited despite decades of focused research. Recently, the intestinal microbiome has emerged as a potential target for novel therapies. Studies have indicated that specific species of Lactobacillus, the most included genus in modern probiotic supplements, can degrade oxalate in vitro and even reduce urinary oxalate levels in animal models of primary hyperoxaluria. Although the purported health benefits of Lactobacillus probiotics vary significantly among species, there is supporting evidence for their potential use in managing oxalate-related diseases. Probiotics, defined as "living microorganisms that, when administered in adequate amounts, confer a health benefit to the host, have gained widespread popularity in the global health market. These products are often marketed as food or dietary supplements and come in various formulations designed to provide specific health benefits, including immune support, gastrointestinal regularity, serum cholesterol control, management of allergic diseases, and relief from mental health issues such as anxiety and depression. Among the more than 200 species of these gram-positive, rod-shaped microorganisms known to exist and utilized for probiotic production, Lactobacillus is the most frequently included genus.

Oxalate is a toxic compound that enters the human body exogenously through dietary sources and endogenously through natural metabolism in the liver. Humans do not metabolize oxalate; instead, it is absorbed across the intestinal epithelium and precipitated with calcium in urine, forming calcium oxalate kidney stones. These stones account for over 80% of all urinary calculi, resulting in an annual economic burden exceeding $10 billion for treatment. The intestinal absorption of oxalate significantly contributes to urinary oxalate levels, which is a primary risk factor for nephrolithiasis. Consequently, there has been increased interest in

intestinal bacteria capable of degrading dietary oxalate as a potential future probiotic therapy for urinary stone formation and other oxalate-related conditions, such as the rare genetic disorder primary hyperoxaluria (Khan et al., 2016). Probiotics, particularly those consisting of various Lactobacillus species, are utilized in the treatment of nephrolithiasis, a condition characterized by the accumulation of calcium oxalate in the kidneys during filtration (Kwak et al., 2006). Since humans lack the enzymes necessary to metabolize both endogenous and dietary oxalate, certain Lactobacillus species can effectively degrade this toxic compound.

A kidney stone is a hard object formed from chemicals present in the urine. There are four primary types of kidney stones: calcium oxalate, uric acid, struvite, and cystine. Treatment options for kidney stones include shockwave lithotripsy, ureteroscopy, percutaneous nephrolithotomy, or nephrolithotripsy.

Urine contains various waste products dissolved within it; when there is an excess of waste and insufficient liquid, crystals begin to form, these crystals attract other elements and aggregate to create a solid that can grow larger unless expelled from the body through urine. Typically, these chemicals are eliminated by the kidneys, which serve as the body's primary filtration system. In most individuals, adequate fluid intake helps flush out these substances or other components in the urine prevent stone formation. The chemicals that contribute to stone formation include calcium, oxalate, urate, cystine, xanthine, and phosphate.

**Symptoms**

Kidney stones can vary in size, ranging from as small as a grain of sand to as large as a golf ball. Notable symptoms include:

1. **Severe pain** in the lower back, often on either side.
2. **Vague pain** on either side or a persistent stomachache.
3. **Blood in urine**(hematuria).
4. **Nausea or vomiting.**
5. **Fever and chills.**
6. **Urine that smells foul or appears cloudy**.

**Causes**

Possible causes of kidney stones include inadequate water intake, excessive or insufficient exercise, obesity, weight-loss surgery, and consumption of foods high in salt or sugar. Additionally, infections and family history may play a significant role for some individuals. A high intake of fructose is also correlated with an increased risk of developing kidney stones.

There are four main types of kidney stone:

1. **Calcium Oxalate** (F.L. Coe, A. Evan, 2005)

This is the most common type of kidney stone, formed when calcium combines with oxalate in the urine (Baggio et al., 1993).

2. **Uric Acid Stones** (L. Giannossi & V. Summa, 2012)

Uric acid stones are prevalent and are often associated with the consumption of foods high in purines, such as organ meats and shellfish.

3. **Struvite Stones** (F.L. Coe & A. Evcen, 2005)

These stones are less common and typically result from infections in the upper urinary tract.

4. **Cystine Stones** (F.L. Coe & A. Evan, 2005)

Cystine stones are rare and tend to run in families; they account for 1% to 2% of stone formers.

**Treatment**

The treatment for kidney stones is similar in both children and adults and includes the following options:

1. **Shock-wave Lithotripsy**

2. **Ureteroscopy**

3. **Percutaneous Nephrolithotomy**

4. **Nephrolithotomy / Nephrolithotripsy**

5. **Biological Treatment (Probiotics)**

**Disadvantages of Treatment** (A. Slayer, 2008)

Kidney stone surgery is generally considered safe; however, it can have side effects and complications, which may include:

1. **Infection**

   This may manifest as a urinary tract infection (UTI) or sepsis, a severe infection that spreads through the bloodstream.

2. **Bleeding**

   There is a risk of bleeding during surgery or other bleeding complications.

3. **Damage to Organs**

   Potential damage to the kidney, ureter, bladder, bowel, or liver.

4. **Scarring**

   Scarring or narrowing of the ureter may occur.

5. **Recurrence of Kidney Stones**

   There is a possibility that kidney stones may recur.

6. **Pain**

   Patients may experience pain following surgery.

To mitigate such side effects, the use of probiotics is considered a beneficial option, particularly those containing *Lactobacillus spp.*, which can degrade kidney stones without causing side effects. Consequently, a research project was undertaken to isolate, screen, and identify oxalate-degrading lactic acid bacteria.

# Review of Literature

Calcium oxalate (CaOx) crystals, the principal component of kidney stones, are a major health concern worldwide. The development of microbial-based therapies, particularly the use of Lactobacillus strains for the degradation of calcium oxalate, has gained significant attention in recent years. Lactobacilli are probiotic bacteria that play a crucial role in human health, particularly in the gut, where they assist in digestion and modulation microbiota.
Several studies have explored the potential of Lactobacillus species in degrading calcium oxalate, offering an alternative to traditional treatment methods. This review synthesizes the literature on the isolation, characterization, and mechanisms involved in the calcium oxalate degrading activity of Lactobacillus. Additionally, it evaluates the therapeutic potential of these bacteria in the management of kidney stone disease (nephrolithiasis) and explores the future directions for clinical applications.

Kidney stones, also known as nephrolithiasis, are solid deposits of minerals and salts that form in the kidneys and can cause severe pain, urinary tract obstruction, and other complications. Calcium oxalate (CaOx) stones are the most common type, accounting for approximately 70-80% of all kidney stones (Pfleger et al., 2020). The formation of these crystals involves the supersaturation of urine with calcium and oxalate ions, which then precipitate and form stones. Several risk factors, including dietary habits, dehydration, genetic predisposition, and metabolic abnormalities, contribute to the formation of calcium oxalate crystals.

Current treatment options for kidney stones include surgical removal, shock wave lithotripsy, and pharmacological therapies that reduce calcium or oxalate levels. However, these treatments can be invasive, expensive, and sometimes ineffective. As a result, alternative therapeutic strategies, including the use of probiotics and microorganisms to degrade or dissolve calcium oxalate crystals, have been explored. Among the microorganisms investigated, Lactobacillus species, commonly known for their probiotic properties, have shown promise in breaking down calcium oxalate.

- **Calcium Oxalate Crystals and Nephrolithiasis**

Calcium oxalate crystals form when calcium ions bind with oxalated ions in an environment of supersaturation. The precipitation of calcium oxalate in the kidneys leads to the formation of stones, which may vary in size, shape, and number. These crystals can cause severe symptoms, including hematuria (blood in urine), flank pain, and renal colic (Painful

urinary spasms). The crystals may also obstruct the urinary tract, leading to infections or renal damage if left untreated (Erdem et al., 2018).

The process of calcium oxalate crystallization involves several steps, including nucleation, growth, aggregation, and retention in the renal tubules (Boudh-Houri et al., 2020). Hypercalciuria, hyperoxaluria, dehydration, and changes in urine pH are some of the factors that contribute to the crystallization of calcium oxalate. The treatment strategies for kidney stones often aim to reduce calcium and oxalate levels, increase hydration, or dissolve the stones through invasive techniques (Jung et al., 2019). However, these treatments are not always effective, and the recurrence of stones is common.

- **The Role of Lactobacillus in Calcium Oxalate Degradation**

*Lactobacillus* is a genus of bacteria that is commonly found in the gastrointestinal tract of humans and other animals. Known for their probiotic properties, Lactobacillus strains play essential roles in digestion, the regulation of the intestinal microbiota, and immune modulation.

Over recent years, certain Lactobacillus species have shown the ability to degrade calcium oxalate crystals, making them a potential candidate for alternative treatments in kidney stone management.

Several studies have demonstrated the ability of Lactobacillus strains to degrade calcium oxalate both in vitro and in vivo. This capability is largely attributed to the secretion of enzymes such as oxalate decarboxylase and oxalate oxidase, which break down oxalate ions into less harmful products. These enzymes, along with organic acids like lactic acid produced by Lactobacillus, create an acidic environment that enhances calcium oxalate dissolution (Vila et al., 2022). Additionally, Lactobacillus may also alter the intestinal absorption of oxalate, thereby reducing the amount of oxalate available for crystal formation.

- **Isolation of Calcium Oxalate Degrading Lactobacillus Strains**

The isolation of calcium oxalate-degrading Lactobacillus strains typically involves the collection of fecal or urinary samples from human or animal subjects, followed by culturing the samples under selective conditions that promote the growth of lactobacilli. Once isolated, the bacterial strains are screened for their ability to degrade calcium oxalate. Several isolation techniques have been employed, including solid agar plates containing calcium oxalate as the sole carbon source and liquid media with added calcium oxalate to assess degradation (Kumar et al., 2019).

In one study by Kumar et al. (2020), a Lactobacillus strain was isolated from the feces of healthy individuals and found to degrade calcium oxalate at a rate of 85% within 48 hours of incubation. Similarly, *Lactobacillus rhamnosus* and *Lactobacillus plantarum* have been isolated and shown to possess high oxalate-degrading capabilities (Alves et al., 2021).

To ensure the efficacy of the isolated strains, a variety of biochemical and molecular techniques are employed to confirm the degradation activity. These include the measurement of oxalate concentration in the growth medium, the analysis of oxalate degradation products, and the sequencing of 16S rRNA genes for species identification.

- **Mechanisms of Calcium Oxalate Degradation**

The mechanisms by which Lactobacillus species degrade calcium oxalate are diverse and not entirely understood. However, several key pathways have been identified in the literature.

- **Oxalate Decarboxylase (OxDc)**

Oxalate decarboxylase (OxDc) is one of the primary enzymes involved in oxalate degradation by Lactobacillus. This enzyme catalyzes the decarboxylation of oxalate to form formate and carbon dioxide. The activity of OxDc has been observed in multiple Lactobacillus species, including *Lactobacillus rhamnosus, Lactobacillus plantarum*, and *Lactobacillus fermentum* (Chew et al., 2020). The activity of OxDc helps in reducing the concentration of oxalate in the gut or urinary tract, thereby preventing the formation of calcium oxalate stones.

- **Oxalate Oxidase (OxOx)**

In addition to OxDc, oxalate oxidase (OxOx) is another enzyme produced by some Lactobacillus species. This enzyme oxidizes oxalate to produce carbon dioxide and hydrogen peroxide. The production of hydrogen peroxide can also contribute to the dissolution of calcium oxalate crystals by creating an oxidative environment that destabilizes the crystal structure (Zhang et al., 2020).

- **Lactic Acid Production**

Lactic acid production by Lactobacillus strains also plays a role in calcium oxalate degradation. Lactic acid creates an acidic environment that promotes the dissolution of calcium oxalate. The ability of Lactobacillus to produce lactic acid from fermentable sugars can lower the pH of the surrounding environment, thereby enhancing the solubility of calcium oxalate (Aslam et al., 2020).

- **Competition for Oxalate in the Intestinal Tract**

Lactobacillus species may also influence oxalate metabolism indirectly by competing with other microorganisms for oxalate in the gut.

Some Lactobacillus strains have been shown to inhibit the growth of oxalate-producing bacteria, such as Oxalobacter formigenes, thereby reducing oxalate levels in the gut and preventing its absorption (Kang et al., 2021).

- **Clinical Applications and Future Directions**

The therapeutic potential of Lactobacillus strains in the treatment of calcium oxalate nephrolithiasis is a promising area of research. Probiotic interventions using Lactobacillus species that degrade oxalate could be a non-invasive, cost-effective treatment to prevent or reduce the recurrence of kidney stones. Clinical trials are necessary to validate the efficacy of these probiotics in human patients.

In addition to traditional probiotics, genetic engineering techniques could be employed to enhance the oxalate-degrading capabilities of Lactobacillus strains. The use of genetically modified Lactobacillus strains that overexpress oxalate-degrading enzymes or lactic acid production could improve the efficacy of this approach (Kang et al., 2022).

Furthermore, the use of Lactobacillus strains in combination with other probiotics or dietary modifications may provide a synergistic effect in reducing kidney stone formation.

For example, increasing the intake of dietary fiber or calcium could reduce oxalate absorption and crystal formation in conjunction with Lactobacillus supplementation.

The isolation and characterization of calcium oxalate-degrading Lactobacillus strains represent an exciting new frontier in the management of kidney stone disease. These probiotic bacteria offer a potential alternative to traditional treatments, with the ability to degrade oxalate and prevent crystal formation. While the results from in vitro and animal studies are promising, more clinical trials are needed to assess the feasibility and effectiveness of Lactobacillus-based therapies in humans. Future research should also focus on optimizing the enzymatic pathways involved in oxalate degradation and exploring the potential for synergistic treatments that combine probiotics with dietary interventions.

# 3.0- MATERIAL AND METHODS

# 3.0 MATERIAL AND METHODS

## 3.1 Collection of Samples

Various types of natural and artificial samples were collected for the isolation of Lactic Acid Bacteria (LAB). For instance, **Spurthi dahi**, **Kapil dahi**, **Warna dahi** were obtained from local sweet markets. Additionally, **tomatoes** and **spinach** were sourced from the market yard in Kolhapur. LAB-containing preparations, such as **Sporlac powders** and **New Nutrolin B Plus**, were collected from medical stores. Furthermore, **idli batter** was included in the sample collection.


Photograph 1. Gokul Dahi


Photograph 2. Krushna Dahi


Photograph 3. Homemade Dahi


Photograph 4. Chitale Dahi


Photograph no. 5 Sporlac Powder

## 3.2 Enrichment of LAB from the Samples

### 3.2.1 Enrichment of LAB Using Tomato and Spinach

Tomato and spinach samples were initially surface sterilized to minimize contamination. The samples were washed with 75% ethanol for 2 minutes to reduce microbial load effectively. After this, the ethanol was decanted, and the samples were rinsed thoroughly with distilled water (d/w) to remove any residual ethanol. Subsequently, the tomato and spinach samples were aseptically ground into a fine paste using a sterile mortar and pestle. This grinding process ensured uniform cell disruption.

After homogenization, each sample was combined in a 1:1 proportion within a conical flask to serve as the enrichment medium. The sample mixture underwent sterilization by autoclaving at 121°C for 30 minutes to ensure the complete elimination of any existing microorganisms. Once autoclaved, the sterilized samples were allowed to cool to room temperature before being utilized as the enrichment medium. Sterilized media were inoculated with 1% **Kapil dahi** (market dahi). Subsequently, the flasks were properly labelled and incubated at 37°C in an anaerobic jar for a duration of 3 days.

### 3.2.2 Enrichment of LAB using MRS broth

Firstly, 4 flasks of MRS broth were prepared composition of MRS broth, The composition of MRS (deMan, Rogosa, Sharpe) broth for 100 mL is as follows:

| Ingredient | Amount (gm) |
|---|---|
| **Peptone** | 1.0 |
| **Meat Extract** | 1.0 |
| **Yeast Extract** | 0.5 |
| **Dextrose (Glucose)** | 2 |
| **Sodium Acetate** | 0.5 |
| **Magnesium Sulfate** | 0.01 |
| **Manganese Sulfate** | 0.005 |
| **Dipotassium Phosphate** | 0.2 |
| **Tween 80** | 1ml |
| **Tri ammonium Citrate** | 0.2 |

Then each curd sample (Spurthi dahi, Warna dahi, Kapil daki, homemade dahi) was inoculated in each flask. The flasks were labelled properly & incubated". at 37°C in anaerobic jar for 3 days.

### 3.2.3 Activation of Lactobacillus from Commercial Preparations

Lactic Acid Bacteria (LAB) were activated using commercial Lactobacillus preparations, including **Sporlac powders** and **New Nutrolin B Plus**. MRS broth was prepared in two conical flasks and subsequently autoclaved. After cooling, each flask was inoculated with either **New Nutrolin B Plus** and **Sporlac powder**. The flasks were then labeled appropriately and sealed with parafilm tape to prevent contamination. Following this, the flasks were incubated at room temperature in an anaerobic jar for a duration of 3 days to facilitate the activation of the LAB.

## 3.3 Isolation of Lactobacilli

The isolation of Lactic Acid Bacteria (LAB) from the enriched samples was conducted using MRS agar medium and NRCL medium. The enriched sample cultures were isolated utilizing the four-quadrant streaking method.

### 3.3.1 Isolation from Dahi Sample Enriched in Spinach and Tomato

MRS agar medium was prepared according to the manufacturer's instructions to facilitate the isolation process. Following the preparation, the agar was allowed to solidify before proceeding with the isolation of LAB from the dahi samples that had been enriched with spinach and tomato. This method is widely recognized for its effectiveness in isolating lactobacilli due to its rich nutrient composition, which supports the growth of these beneficial bacteria while inhibiting the growth of unwanted microorganisms.

**Composition of MRS**

| Ingredient | Amount (gm) |
|---|---|
| **Peptone** | 1.0 |
| **Meat Extract** | 1.0 |
| **Yeast Extract** | 0.5 |
| **Dextrose (Glucose)** | 2 |
| **Sodium Acetate** | 0.5 |
| **Magnesium Sulfate** | 0.01 |
| **Manganese Sulfate** | 0.005 |
| **Dipotassium Phosphate** | 0.2 |
| **Tween 80** | 1ml |
| **Tri ammonium Citrate** | 0.2 |

**Composition of NRCL**

| Ingredient | Amount(gm) |
|---|---|
| **Peptone** | 0.3 |
| **Meat extract** | 0.3 |
| **Yeast Extract** | 0.3 |
| **Lactose** | 1 |
| **CaCO3** | 1.5 |
| **Neutral red** | 0.5ml |
| **Agar** | 2.5 |
| **Distil water** | 100ml |
| **pH** | 6.8 |

**Composition of ATCC**

| Ingredient | Amount(gm) |
|---|---|
| **K2HPO4** | 0.025 |
| **KH2PO4** | 0.025 |
| **(NH4)2SO4** | 0.5 |
| **MgSo4.7H2O** | 0.0025 |
| **Sodium acetate** | 0.032 |
| **Yeast extract** | 0.1 |
| **Trypticale peptone** | 0.1 |
| **Sodium oxalate** | 0.5 |
| **Na2CO3** | 0.4 |
| **L-cysteine. HCl** | 0.45 |
| **Distilled water** | 100ml |

From the MRS plates, the isolated colonies were further subculture onto freshly prepared MRS plates using the zigzag streaking technique to minimize cross-contamination. The inoculated plates were then placed in an anaerobic jar, which was sealed with parafilm tape to maintain an anaerobic environment. These plates were incubated at room temperature for 3 days to facilitate the growth of anaerobic microorganisms. This careful subculturing process ensured the purity and viability of the isolated LAB colonies for subsequent analysis.

### 3.3.2 Isolation from Dahi Sample Enriched in MRS

The enriched sample was streaked onto MRS agar medium using the four-quadrant streaking method. The plates were labelled appropriately and sealed with parafilm tape to prevent contamination. Following this, the plates were incubated at room temperature in an anaerobic jar for a duration of 3 days to promote the growth of Lactobacilli.

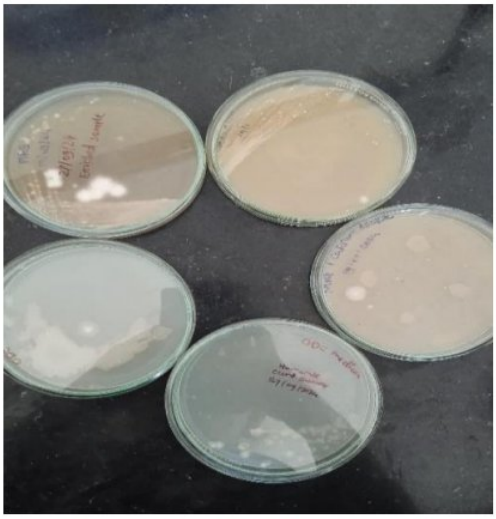
### 3.3.3 Isolation of Lactobacilli from Activated Samples

The MRS broth containing active Lactobacilli was streaked onto both MRS agar and NRCI medium using the four-quadrant streaking method. The plates were labeled correctlyand sealed with parafilm tape to maintain an anaerobic environment. These plates were then incubated at room temperature in an anaerobic jar for 3 days to facilitate the growth of the isolated bacteria.

### 3.3.4 Direct Isolation from Idli Batter

A loopful of idli batter was streaked onto MRS agar and NRCI medium using the four-quadrant streaking method. The plates were properly labelled and sealed with parafilm tape to prevent contamination. Subsequently, they were incubated in an anaerobic jar at room temperature for 3 days to allow the growth of Lactobacilli from the idli batter samples.

Photograph no. 8 Incubation in anaerobic jar.



Photograph no.9 Stricking of sample

### 3.4 Identification of Lactobacilli

#### 3.4.1Catalase Test

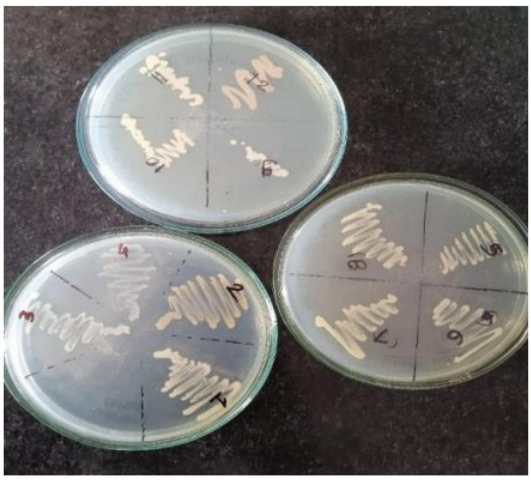
The colonies grown on MRS and NRCI plates were identified as Lactobacilli based on the results of the catalase test. This test was conducted by inserting an isolated colony using a sterile glass rod into a saline tube containing a 3% hydrogen peroxide ($H_2O_2$) solution.

All catalase-negative isolates were subjected to Gram staining to assess the purity of the isolates. Impure isolates were purified through serial transfers onto sterile MRS plates.

#### 3.4.2 Purification of Impure Cultures by Serial Transfer on MRS Medium

To ensure the accuracy of downstream analyses, it was essential to obtain pure cultures of the isolated microorganisms. Initially, the cultures obtained from the isolation process were impure, containing a mixed microbial population. To purify these cultures, serial transfer on MRS medium was employed.

The serial transfer process involved repeatedly subculturing the impure cultures onto fresh MRS plates, where the cells were spread to isolate individual colonies. This procedure was repeated multiple times to ensure the elimination of contaminants and the successful attainment of pure cultures. Through this meticulous process, the impure cultures were effectively purified, yielding single colony isolates that were subsequently used for further characterization and analysis. All purified isolates were then tested for catalase activity. The final isolates, which were confirmed as catalase-negative, were designated as Isolate 1, Isolate 2, Isolate 3, and Isolate 4.



Photograph no. 10 Purification of sample.

### 3.4.3 Morphological Study

All catalase-negative isolates were streaked on sterile MRS plates using the four-quadrant method. The plates were labelled appropriately, sealed with parafilm tape, and incubated at room temperature in an anaerobic jar for 3 days to promote the growth of *Lactobacilli*. The gram characteristics and other morphological characteristics of isolates were studied.

## 3.5 Detection of Calcium Oxalate Degradation Ability of Isolated Strains

The ability of microorganisms to degrade calcium oxalate is of significant interest due to its implications in various fields. The formation of a clear zone around the colonies on media containing oxalate indicates successful calcium oxalate degradation.

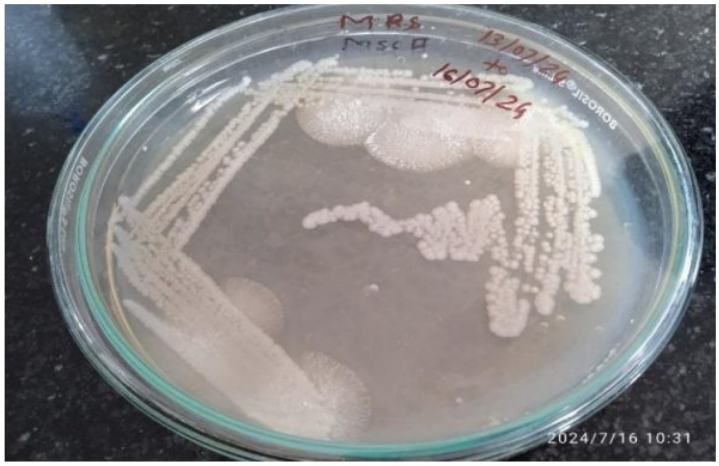
### 3.5.1 Method 1: Four-Quadrant Streaking

To enhance the growth and isolation of microorganisms capable of degrading calcium oxalate, enrichment cultures were established using calcium oxalate-containing media. The enrichment medium consisted of MRS broth and calcium oxalate, which were separately wrapped in paper and placed in a Petri plate. Both the MRS broth and calcium oxalate medium were autoclaved at 120°C for 30 minutes.

Following sterilization, the calcium oxalate was aseptically added to the sterilized MRS broth. A loopful of isolates 1, 2, 3, and 4 was aseptically added separately using a sterilized wire loop into the calcium oxalate-enriched MRS broth. The cultures were labelled appropriately, sealed with parafilm tape, and incubated at room temperature for 3 days in an anaerobic jar.

A loopful culture from the above enrichment was then streaked onto calcium oxalate-containing MRS and NRCL media, which included oxalate decarboxylase (ODC) and ODC + pH indicator, using the four-quadrant streaking method. The plates were labelled correctly, sealed with parafilm tape, and incubated at room temperature for 3 days in an anaerobic jar.

Simultaneously, the four isolates were also streaked on sterile ODC medium containing calcium oxalate and a pH indicator. Oxalate-degrading bacteria were identified based on the presence of a clear zone around the colonies on this medium, indicating successful degradation of calcium oxalate.




Photograph no. 11 Four Quadrant Stricking on ODC

**Composition of ODC medium**

| Ingredient | Amount(gm) |
|---|---|
| **Peptone** | 0.5 |
| **Tryptone** | 0.5 |
| **Calcium oxalate** | 0.2 |
| **Na2CO3** | 0.1 |
| **Agar** | 2.5 |
| **pH indicator** | 0.001 |
| **Distilled water** | 100ml |

### 3.5.2 Method 2: Agar well method

MRS agar plates containing calcium oxalate were prepared for the assessment of degradation ability. Using a sterile cork borer, three wells were created in each agar plate. A fresh suspension of the four isolates was prepared, and 0.1 ml of the suspension of each isolate was added into the respective wells. The plates were labelled appropriately, sealed with parafilm tape, and incubated at room temperature in an anaerobic jar for 3 days to facilitate the growth of the isolates and observe any potential calcium oxalate degradation

# 4.0 RESULT AND DISCUSSION

# 4.0 RESULT AND DISCUSSION

## 4.1 Enrichment of LAB

**4.1.1 Enrichment of LAB from various samples was done in tomato & spinach containing enrichment medium.**


Photograph no. 12 Enrichment in Spinach and Tomato.

**4.1.2 Enrichment of LAB using MRS broth.**

Photograph no. 13 Enrichment in MRS Broth.

**4.2 Activation of LAB from commercial preparations.**

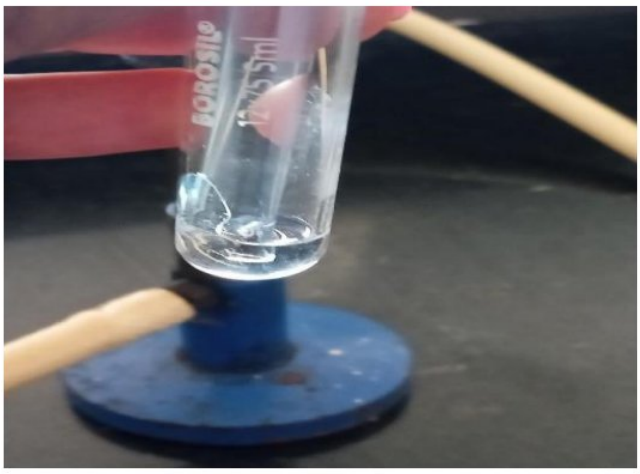

Photograph no. 14 Activation of sporolac sample.

**4.3 LAB from commercial preparations were successfully activated in MRS broth.**

More than 100 different isolates were isolated on various isolation media used in study

**4.4 Identification of LAB based on catalase activity.**

Above 100 different isolates from various media when tested for catalase activity, 4 colonies were showed catalase test negative. All these four isolates were from 4 days old homemade dahi. These isolates were labelled as isolate 1, isolate2, isolate 3 and isolate 4.






Photograph no. 15 Catalase activity.

**4.5 Identification of LAB based on colony characteristics.**

Table No: 1

Colony characters of **isolate 1** colony grown on MRS plate incubated at $37^0$C for 3 days in anaerobic jar.

| Size | Shape | Color | Margin |
|---|---|---|---|
| **2mm** | **Circular** | **Creamy** | **Entire** |

| Surface | Elevation | opacity | Consistency |
|---|---|---|---|
| **Smooth** | **Convex** | **opaque** | **Moist** |

Table No: 2

Colony characters of **isolate 2** colony grown on MRS plate incubated at $37^0$C for 3 days in anaerobic jar.

| Size | Shape | Color | Margin |
|---|---|---|---|
| **2mm** | **Circular** | **Creamy** | **Entire** |

| Surface | Elevation | opacity | Consistency |
|---|---|---|---|
| **Smooth** | **Convex** | **opaque** | **Moist** |

Table No: 3

Colony characters of **isolate 3** colony grown on MRS plate incubated at $37^0$C for 3 days in anaerobic jar.

| Size | Shape | Color | Margin |
|---|---|---|---|
| **2mm** | **Circular** | **Creamy** | **Entire** |

| Surface | Elevation | opacity | Consistency |
|---|---|---|---|
| **Smooth** | **Convex** | **opaque** | **Moist** |

Table No: 4

Colony characters of **isolate 4** colony grown on MRS plate incubated at $37^0$C for 3 days in anaerobic jar.

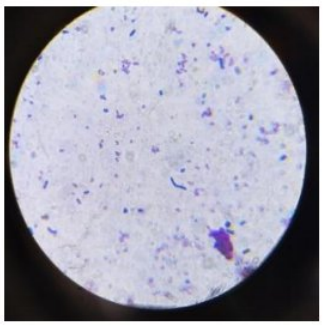
| Size | Shape | Color | Margin |
|---|---|---|---|
| **2mm** | **Circular** | **Creamy** | **Entire** |

| Surface | Elevation | Opacity | Consistency |
|---|---|---|---|
| **Smooth** | **Convex** | **Opaque** | **Moist** |

### 4.6 Identification on the basis of Gram Characteristics:

Table no:

| Sr.no | Isolate name | Gram nature |
|---|---|---|
| 1. | Isolate 1 | **Gram +ve, short rods, arranged signally** |
| 2. | Isolate 2 | **Gram +ve, short rods, arranged signally** |
| 3. | Isolate 3 | **Gram +ve, long rods, arranged signally** |
| 4. | Isolate 4 | **Gram +ve, long rods, arranged signally** |

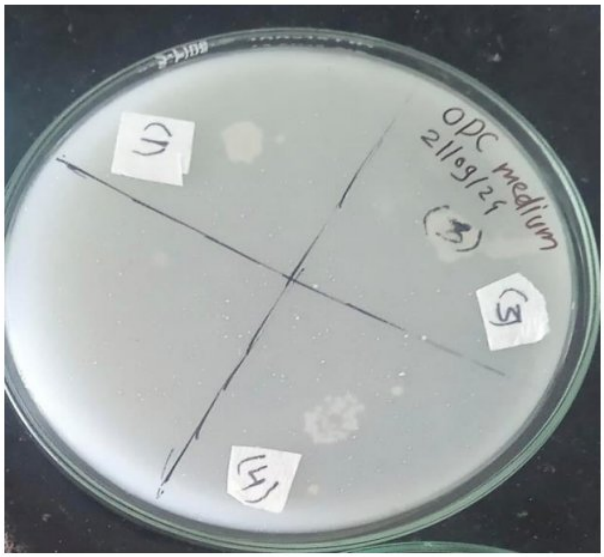


Photograph no. 16 Gram staining of isolated colonies.

### 4.7 Detection of oxalate degradation ability of isolated strains

Method 1: -

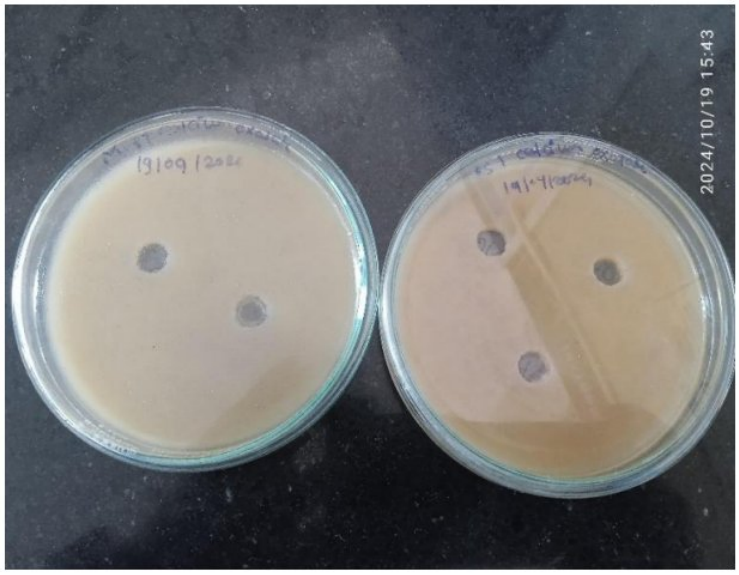
All four isolates have shown growth on MRS, NRCLA, ATCC medium containing calcium oxalate. However, no clear zone was seen by any isolates around growth.




Photograph no. 17 Spot inoculation.

Method 2: -

All 4 isolates when tested for calcium oxalate degradation ability using agar well method no clear zone was obtained around the well.




Photograph no. 18 Well Diffusion method.

# 5.0 SUMMARY AND CONCLUSION

# 5.0 SUMMARY AND CONCLUSION

Lactic acid bacteria (LAB), particularly species like *Lactobacillus acidophilus* exhibit significant potential for degrading oxalates which are the compound linked to Kidney stone formation. Studies have shown that *L. acidophilus* can degrade oxalate up to 48% on artificial gastric condition while other strains like *Lactobacillus fermentum* and *Lactobacillus salivarius* also demonstrate notable oxalate degrading capabilities, which off efficiency ranging from 40% to 62%

The exploration of oxalate-degrading lactic acid bacteria reveals promising avenues for managing dietary oxalate levels and preventing Kidney stone formation. Strains like *L. fermentum, L. acidophilus, L. salwiring* show significant potential due to their high degradation efficiencies and favourable probiotic properties. Continued research is needed for understanding the full scope of their metabolic capabilities and optimizing them.

In present research various samples like market dahi, Homemade dahi, idli batter etc were used for isolation of oxalate degrading bacteria. From homemade curd sample colonies were isolated on MRS & NRCL medium. From commercial dahi sample Kapil dahi, Spurti dahi and tablet less than 40 colonies were obtained from MRS and NRCL medium. After testing of all colonies for catalase test nearly 4 isolates were showing catalase test negative.

All 4 catalase negative isolates when tested for oxalate degradation ability, no oxalate degradation was observed.

Thus, further study and repetition of experiment are essential for successful isolation of oxalate degrading *Lactobacilli.*

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