Isolation and Characterization of Lactic Acid Bacteria from Yogurt Samples and Evaluation of Their Fermentation Efficiency

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A thesis submitted to the School of Pharmacy in partial fulfillment of the requirements for the degree of
Bachelor of Pharmacy (B.Pharm)

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Declaration

It is hereby declared that

1. The thesis submitted is our own original work while completing degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. We have acknowledged all main sources of help.

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Approval

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Ethics Statement

The study did not use human subjects or animals, so no ethical approval were needed. Research was carried out in a responsible manner and no harm was done to any living organisms, materials or environment.

Abstract

Lactic acid bacteria (LAB) are essential microorganisms in the manufacture of dairy products because they produce lactic acid, a well-known preservative, are used in food fermentation and can have probiotic properties. The present study was designed to isolate and characterise LAB from the local samples of yogurt and compare its fermentation efficiency in milk. Five samples of yogurt (A-E) were taken from various places of Bangladesh and cultured by serial dilution technique on MRS agar. Selected isolates were purified in MRS slants in an anaerobic candle-jar and transferred to pasteurized milk. The pH, liquid volume and dry yogurt weight were used to measure fermentation performance. Sample D was the most successful in colony growth, slant culture development and yogurt formation. The pH of fermented milk samples was in the range 3.98-4.19, showing an effective acidification. The mean dry weight of the yogurt was between 54.68 to 71.11 g and liquid was between 50.25 to 68.25 mL. The optimal performance in fermentation was achieved with the application of 200–300 µL of the starter culture per 150 mL of milk, which corresponds to about 0.13–0.20% (v/v).The results indicate that the yogurt may be a good source of LAB that can be used in the dairy, food preservation, probiotics and pharmaceutical research.

Keywords: Lactic acid bacteria; Yogurt; Dry Weight; Lactobacillus, MRS agar; Probiotics.

Dedication

Abu Horaira Bhuiyan Fahim (22146082)

I thank Allah for His countless blessings, guidance and mercy that has made it possible for me to do this journey.

I am grateful to my parents who have given me unconditional love, prayers and sacrifices. I have always been given the faith of others, and that faith has been my greatest strength. I also have great admiration of my grandparents, particularly my grandmother, Ajifa Khatun who raised me, defined strong woman to me and made me feel safe and loved.

I dedicate this work to the people that have made this journey warm and meaningful. My friend, Sumaiya Hossain Lia, who has always raised my spirits. My beloved sister, Mahita Rahman Noushin, who has always been the source of my support and care. Fahim Shahriar Ahmed, my friend, who never left me alone in any circumstances. Md. Zubayer Ibn Kamal, who always widen the space for me and help to think deeply. My friend Mayeesha Tasnim Aronee, whose honest critiques and contagious energy, pushed me to grow in ways I never expected and Yusuf Ali Naim, whose silent support always means more to me than words can say. This journey has been much more meaningful because of their love, encouragement and presence. I heartily extend my thanks and love to all of them.

Gift Barikder (22146022)

I want to dedicate this work to my parents for their support and patience throughout my bachelor's degree journey.

Farjana Jahan (22146044)

I would like to dedicate this work to my parents for their endless love, support, patience, and encouragement throughout my bachelor’s degree journey. I am also sincerely grateful to my Khala and Mama for always being there for me and believing in me. A special thanks to my beloved Nanu, whose constant faith in me and encouragement gave me the confidence to keep going. I would also like to thank my friends for their support, motivation, and the many memories we shared along the way. This achievement would not have been possible without all of them, and I am truly grateful for their love and support.

Mehnaz Tamanna Bintay Akter (22346070)

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Ahanafe Shahriar Abir (22146050)

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List of Acronyms

LAB	Lactic Acid Bacteria
MRS Agar	de Man, Rogosa, and Sharpe Agar
PBS	Phosphate Buffer Solution
BSL-1	Biosafety Level 1
spp.	Species plural
subsp.	Subspecies
S. thermophilus	Streptococcus thermophilus
L. delbrueckii	Lactobacillus delbrueckii
L. bulgaricus	Lactobacillus delbrueckii subsp. bulgaricus
L. lactis	Lactococcus lactis
A–E	Yogurt Sample Groups A to E
H_2O_2	Hydrogen Peroxide
CO_2	Carbon Dioxide
mL	Milliliter
µL	Microliter
g	Gram
°C	Degree Celsius

Chapter 1
Introduction

1.1 Overview of Lactic Acid Bacteria (LAB)

Lactic acid bacteria (LAB) are microorganisms that play a central role in food fermentation, functioning as starter cultures that initiate rapid acidification of raw materials such as milk (Leroy & De Vuyst, 2004). In recent years, new starter cultures of LAB with industrially important functionality have been developed, offering microbial safety as well as organoleptic, technological, nutritional, and health advantages (Leroy & De Vuyst, 2004). LAB produces several natural antimicrobials during fermentation, including organic acids such as lactic acid and acetic acid, as well as carbon dioxide and hydrogen peroxide, which contribute significantly to food preservation and safety (Leroy & De Vuyst, 2004). Beyond their role in fermentation, many LAB strains also produce bacteriocins, bacterially synthesized antimicrobial peptides that can have narrow or broad host ranges against other microorganisms (Cotter et al., 2005) Bacteriocins produced by food-grade LAB offer food scientists the possibility of directing or preventing the development of specific bacterial species in food, which is particularly useful in food preservation and safety applications (Cotter et al., 2005). In this sense, bacteriocinogenic LAB strains can confer a form of innate antimicrobial protection to food products, helping to extend control over the food flora long after manufacture (Cotter et al., 2005). Lactobacillus species during the fermentation of milk produce lactic acid and other available nutrients. Lactic acid decreases the pH of milk. This acidification makes the casein proteins in milk coagulate, creating a gel-like substance that is the body of the yogurt. The process of pH drop in yogurt formation is closely related to the yogurt texture and gel formation where the pH of the milk is reduced from 6.7 to 4.6 (Lee & Lucey, 2010). Lactobacilli are also researched for their potential as probiotic. But not all strains of Lactobacillus are necessarily probiotic. It is important that a strain is correctly identified, tested for safety and demonstrated to be a health benefit when consumed in appropriate quantities. The strain level nature of this is significant because other strains of the same species may ferment differently, survive differently, produce different acid, have different antimicrobial activity, and have different possible health effects.

1.2 Yogurt as a Source of Lactic Acid Bacteria

Yogurt is the product of fermentation of milk using a defined mixed culture of two thermophilic strains of LAB, Streptococcus thermophiles and Lactobacillus delbrueckii subsp. Bulgaricus (Sieuwerts et al., 2008). In some countries, only yogurt products made from yoghurt starter cultures with both of these organisms are allowed (Sieuwerts et al., 2008). It is a relatively easy-to-manage system of microbial interaction, and because of its global economic significance, this mixed-culture fermentation is an attractive model system for research on microbial interactions (Sieuwerts et al., 2008). During the fermentation process, the two starter organisms have been well studied for their mutualistic relationship. For example, the carbon dioxide generated by Streptococcus thermophilus stimulates Lactobacillus delbrueckii subsp. bulgaricus, and the proteolytic activity of L. bulgaricus releases growth stimulating amino acids and peptides from milk proteins that benefit S. thermophiles (Sieuwerts et al., 2008). The manufacture of yogurt has also taken a new functional and health-focused route with many commercial yogurts and drinks being made using probiotics other than the two starter cultures (Sieuwerts et al., 2008).

1.3 Importance of Isolation and Characterization of LAB

Isolation and characterization of LAB from yogurt is basic processes in food microbiology and biotechnology. By screening for the selection of LAB strains with interesting technological properties and developing them as new, functional starter cultures, fermentation processes can be improved and better quality of the end product can be achieved (Leroy & De Vuyst, 2004). The efficacy of functional starter cultures to produce a desired effect in a specific food product is highly dependent on the strain, however, and highlights the need for detailed characterization of the strain level prior to any industrial use (Leroy & De Vuyst, 2004). Since LAB produce broad-spectrum antimicrobials including bacteriocins, their characterization allows identification of strains suited for biopreservation providing a natural alternative to chemical food additives in combating microbial contamination (Cotter et al., 2005). Characterization enables the identification of LAB strains with probiotic potential. Evidence for the role of probiotics in health maintenance and disease prevention is growing and is supported in some cases by controlled human trials (Sanders, 2003). Probiotics may play an important role in protecting the body from infection, especially along the colonized mucosal surfaces of the gastrointestinal tract (Sanders, 2003). Characterized bacteriocinogenic LAB strains are particularly valuable as bio preservatives in food systems, since bacteriocins produced by LAB can be used to direct or prevent the growth of specific unwanted bacterial species in food, with direct implications for food safety (Cotter et al., 2005).

1.4 Health and Probiotic Significance of LAB from Yogurt

In today's era of antibiotic-resistant pathogens and other looming microbial threats, the value of preventing infection has gained renewed recognition, and probiotics, many of which are LAB may play an important role in helping the body protect itself (Sanders, 2003). The range of probiotic product types containing or based on LAB is now widespread throughout the world, from pills to powders, foods, infant formula and more (Sanders, 2003). The health relevance of LAB is related to probiotics, prebiotics and postbiotics. Probiotics are live microorganisms that, when consumed in sufficient quantities, are beneficial to the health of the host (Hill et al., 2014). A significant number of probiotic organisms are from LAB, particularly Lactobacillus and Bifidobacterium species. Prebiotics are not live microorganisms, unlike probiotics. A prebiotic is a substrate that is selectively metabolised by host bacteria and has a beneficial effect on health, such as dietary fibres and oligosaccharides that act as a substrate for the growth of beneficial gut bacteria (Gibson et al., 2017). Postbiotics are preparations of inactivated microorganisms or parts of microorganisms, with health benefits (Salminen et al., 2021). In contrast to probiotics, the use of postbiotics is not required to be free of living organisms, and thus could be of interest for nutritional and pharmaceutical purposes because of their potential stability and safety.

1.5 Relevance of LAB to the Pharmaceutical Industry

Lactic acid bacteria (LAB) are well known for their safe use in human food and their natural occurrence in mucosal microbial ecosystems and are valued in pharmaceutical and medical research. They are food-grade and mucosal associated and are suitable candidates to deliver therapeutics, anti-infective agents and vaccines at mucosal surfaces (Wells & Mercenier, 2008). Oral delivery of therapeutic proteins like antigens, cytokines and enzymes can also be performed using engineered food-grade LAB strains, particularly strains of *Lactococcus lactis* and *Lactobacillus* spp (Bermúdez-Humarán et al., 2011). Characterization of yogurt derived LAB is therefore of pharmaceutical interest, since safe and functional isolates can be used in future probiotics, in oral drugs and as live mucosal vaccines (Wells & Mercenier, 2008).

1.6 Rationale and Significance of the Present Study

This isolation and characterization of LAB is scientifically important for a number of reasons to the locally produced yogurt samples. For the proper development of new starter cultures and probiotic products, it is necessary to understand the different strains found in yogurt, the interactions that occur between these strains during fermentation and the functional properties they possess. (Sieuwerts et al., 2008). The present study therefore aims to systematically isolate LAB from yogurt samples, identify them using established phenotypic and molecular methods, and evaluate their functional properties relevant to food technology and human health. The practical importance of the study is also related to local dairy production. In many local dairy settings, yogurt quality may vary because of differences in starter culture, hygiene, incubation condition, milk quality, and contamination. By isolating colonies, preparing slant cultures, re-inoculating milk, and measuring pH, liquid volume, and dry yogurt weight, this research attempts to connect microbiological isolation with actual fermentation performance.

1.7 Aim and Objectives of the Study

The aim of this study was to isolate and characterize lactic acid bacteria (LAB) from yogurt samples and evaluate their fermentation efficiency in milk. The objectives of this study are to isolate LAB using MRS agar medium, purify selected colonies through sub-culturing and slant preparation, maintain the isolates under reduced oxygen conditions, inoculate selected cultures into milk, and evaluate yogurt formation by measuring pH, liquid volume, and dry yogurt weight. The study also aims to observe bacterial morphology through Gram staining and discuss the possible food, nutritional, and pharmaceutical relevance of the isolated LAB.

Chapter 2

Methodology

2.1 Study Design

The study followed an experimental laboratory design to isolate and characterize lactic acid bacteria (LAB) from yogurt samples and evaluate their fermentation efficiency in milk. The work included sample collection, serial dilution, plating on selective medium, incubation, colony selection, slant culture preparation, milk inoculation, yogurt formation, and basic quality assessment. The main parameters recorded were pH, liquid volume, dry yogurt weight, and Gram staining observation.

2.2 Sample Collection

Five yogurt or yogurt-and-milk sample groups were collected from different local sources in Bangladesh and labeled as Sample A, Sample B, Sample C, Sample D, and Sample E. Sample A was collected from Sirajganj, Sample B from Salam Dairy Farm, Bashabo, Sample C from Mirpur and Bakshibazar, Sample D from Sirajganj with later milk collected from Salam Dairy Farm, and Sample E from Aftabnagar. Among these, Sample D was selected for detailed analysis because it showed the most complete experimental outcome, including colony growth, slant growth, yogurt formation, and measurable fermentation data.

2.3 Reagents, Equipments & Instruments

Reagent	Equipment	Instruments
Commercial yogurt samples, pasteurized whole milk, MRS agar medium, phosphate buffer solution, sterile saline, crystal violet, iodine, decolorizer, and safranin. Distilled water.	Sterile Petri dishes, sterile test tubes, conical flasks, pipettes, pipette tips, inoculation loops, aluminum foil, sterile tape, and labeling markers.	Autoclave, laminar airflow hood, incubator, vortex mixer, water bath, Bunsen burner, airtight jar, and pH meter.

Table 1: Reagents, Equipment & Instruments

2.4 Culture Medium

MRS agar was used as the selective medium for LAB isolation and cultivation. This medium was originally developed for lactobacilli and is widely used for LAB isolation and enumeration (de Man et al., 1960). The medium was prepared using MRS agar powder and distilled water, followed by sterilization through autoclaving at 121°C for 15 minutes. Although MRS agar supports Lactobacillus growth, it is not completely selective, as other LAB such as Pediococcus, Leuconostoc, and Lactococcus may also grow. Therefore, colony morphology, Gram staining, and further molecular tests are necessary for stronger confirmation.

2.5 Sterilization and Aseptic Conditions

All glassware, media, and laboratory materials were sterilized before use. Inoculation, dilution, and plating were performed inside a sterilized laminar airflow hood under aseptic conditions. Maintaining aseptic conditions was essential because yogurt samples may contain mixed microorganisms, and contamination from air, hands, glassware, or laboratory surfaces can affect LAB isolation.

2.6 Serial Dilution

Serial dilution was performed to reduce microbial concentration and obtain isolated colonies. Yogurt samples were diluted in PBS up to 10^{-4} dilution, and each dilution was vortexed properly to ensure even distribution of microorganisms. Diluted samples were then spread onto MRS agar plates and incubated at 37°C for 24 hours. After incubation, plates were examined for bacterial growth and colony morphology. Lactobacillus-like colonies were expected to appear small, white to cream-colored, circular, convex, and smooth-edged.

2.7 Plating and Incubation

Well-isolated colonies were selected and transferred to fresh MRS agar slants for purification and preservation. In Sample D, one isolated colony from each of four plates was transferred into four separate MRS slants. These slants were incubated under reduced oxygen conditions using the candle-jar method. In this method, a candle burns inside a sealed jar until oxygen is reduced and the flame goes out, creating a low-oxygen environment. Although this method is simple and inexpensive, it is less controlled than a commercial anaerobic jar system and should be considered a limitation (Saha et al., 2016).

2.8 Colony Selection and Slant Culture

The purified LAB cultures were then inoculated into pasteurized whole milk to evaluate their yogurt-forming ability. Successful yogurt formation was assessed by observing coagulation, sour aroma, texture, pH, and viscosity. For Sample D, 300 µL from each of four cultures was inoculated into 150 mL of fresh milk and incubated at 37°C. After three days, yogurt formation was observed. One preparation that produced 43 mL of liquid volume was used as a working stock for further experiments.

2.9 Milk Inoculation and Yogurt Production

Using this working stock, different inoculum volumes 100 µL, 200 µL, 300 µL, 400 µL, and 500 µL were added to milk to compare fermentation efficiency. After incubation, pH, liquid volume, and dry yogurt weight were recorded. pH was measured to assess acid production, as successful yogurt fermentation usually lowers pH close to 4.0–4.6. Liquid volume was recorded to estimate whey separation or remaining liquid after fermentation. Dry yogurt weight was calculated by subtracting the blank beaker weight from the weight of the beaker containing yogurt.

2.10 Evaluation of Fermentation Efficiency

Fermentation efficiency was evaluated using three main parameters: pH, liquid volume, and dry yogurt weight.

2.10.1 pH Measurement

pH was measured to assess acid production during yogurt fermentation. Successful fermentation usually lowers milk pH to around 4.0–4.6, while values above 5.0 may indicate incomplete fermentation.

2.10.2 Liquid Volume

Liquid volume was measured after yogurt formation to estimate whey separation or remaining liquid. Variations in volume may indicate differences in gel strength, water-holding capacity, fermentation condition, or handling across batches and inoculum levels.

2.10.3 Dry Yogurt Weight

Dry yogurt weight was calculated by subtracting the blank beaker weight from the weight of the beaker containing yogurt. This measurement estimated the solid yogurt yield and helped compare fermentation performance among different inoculum concentrations.

2.10.4 Gram Staining

Gram staining was performed as a preliminary morphological test. LAB such as Lactobacillus are expected to appear as Gram-positive purple rods, often singly or in chains. However, Gram staining only supports preliminary identification; biochemical or molecular tests are required for species confirmation.

2.11 Data Recording and Organization

Data were recorded in tables based on sample concentration, pH, volume, beaker weight, blank beaker weight, and dry yogurt weight. The thesis data includes pH tables for four batches and volume/dry weight tables for four batches.

2.12 Biosafety and Waste Disposal

Although LAB such as Lactobacillus are generally considered low-risk BSL-1 organisms, standard laboratory safety practices were followed. Contaminated plates, fungal cultures, pipette tips, and used culture materials were autoclaved before disposal to prevent contamination and environmental release.

Chapter 3

Results

3.1 Overview of Experimental Outcome

This study aimed to isolate lactic acid bacteria from yogurt samples and evaluate their milk fermentation ability.

Sample	Source	Main observation	Final status
Sample A	Sirajganj yogurt	Microbial growth observed, but contamination was present	Interrupted and not usable for further work
Sample B	Salam Dairy Farm, Bashabo	Growth observed in slants; slight yogurt formation observed	Discontinued due to spoilage
Sample C	Mirpur,Bakshibazar	Heavy fungal contamination on plates	Discontinued
Sample D	Sirajganj yogurt; later milk from Salam Dairy Farm	Successful colony growth, slant growth, yogurt formation, and data collection	Main completed sample
Sample E	Aftabnagar yogurt	Colony growth and slant growth observed	Stored in deep freezer for future work

Table 2: Sample, Source and outcome

Sample D showed successful colony growth, slant culture growth, yogurt formation, pH change, liquid volume variation, and dry yogurt weight production; therefore, it was selected as the main sample for result analysis.

3.2 Photographic Observation of Sample D

3.2.1 Growth on Agar plates

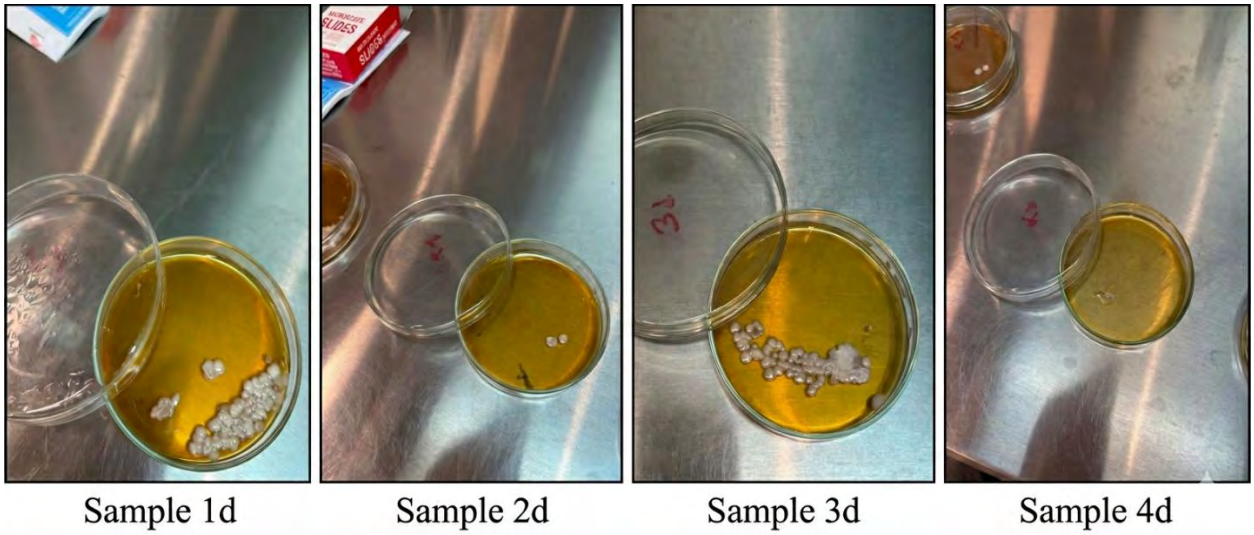


Figure 1: Growth of Lactobacillus spp. from serially diluted yogurt samples on agar plates

3.2.2 Anaerobic Slant culture from agar plate colonies

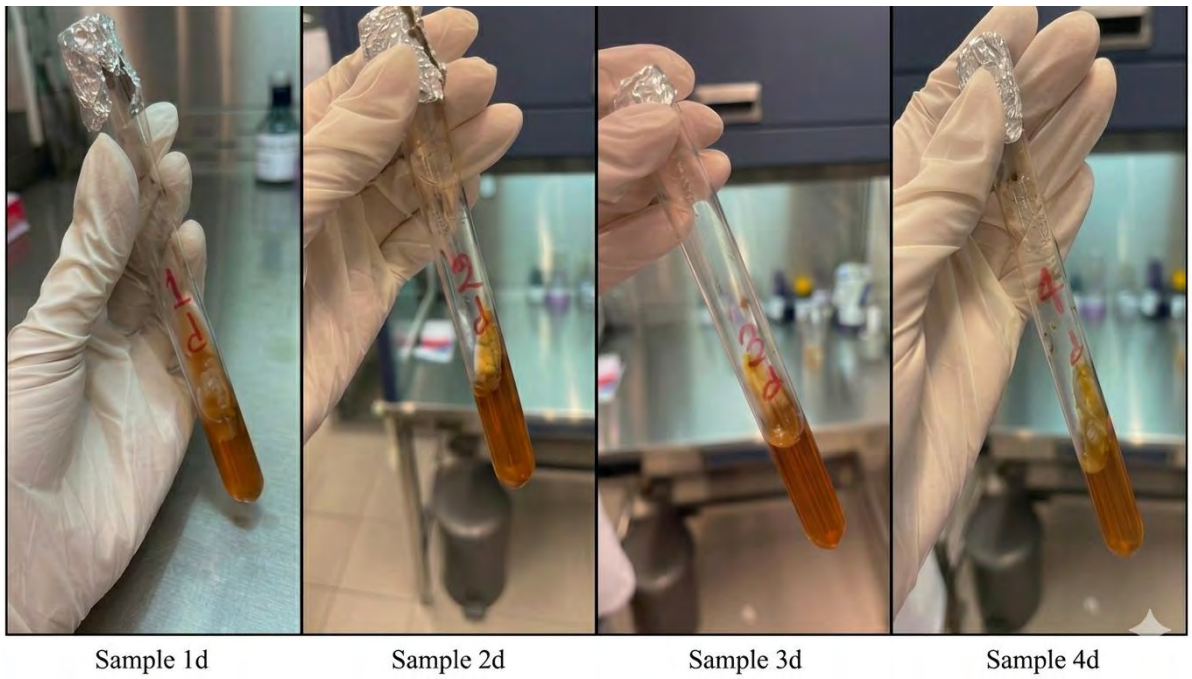


Figure 2: Preparation of anaerobic slant cultures from agar plate colonies

3.2.3 Inoculation of Milk Using a Colony from Slant Culture

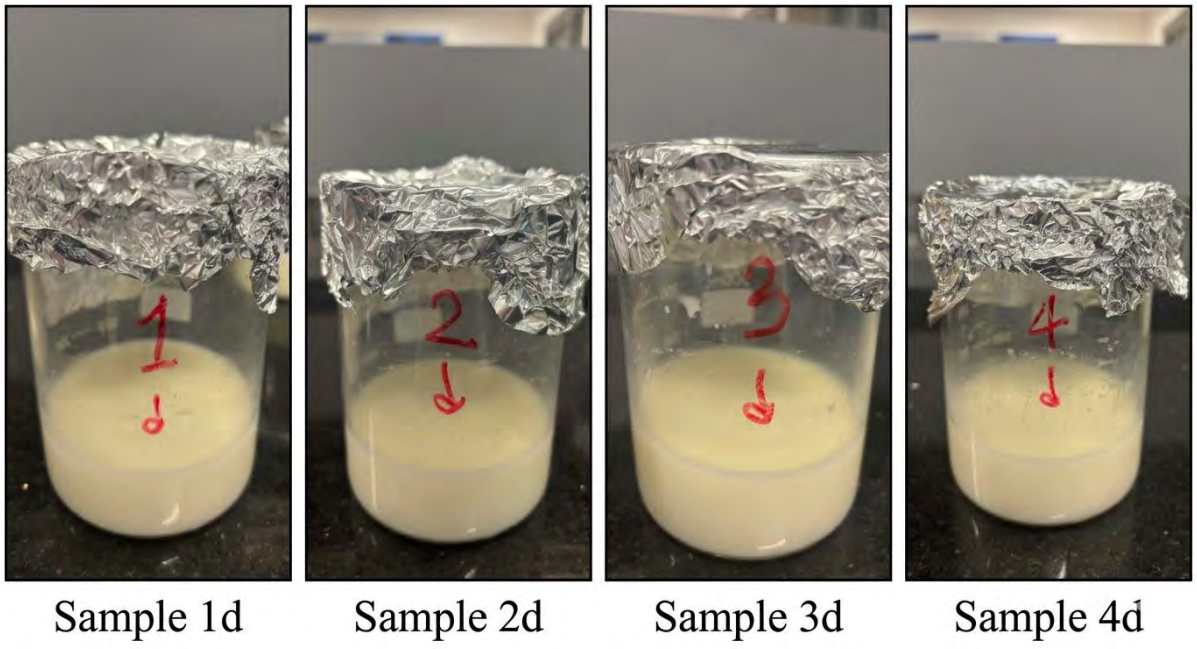


Figure 3: Inoculation of milk with culture obtained from slant culture for yogurt production.

3.2.4 Prepared yogurt samples after removal of excess whey.

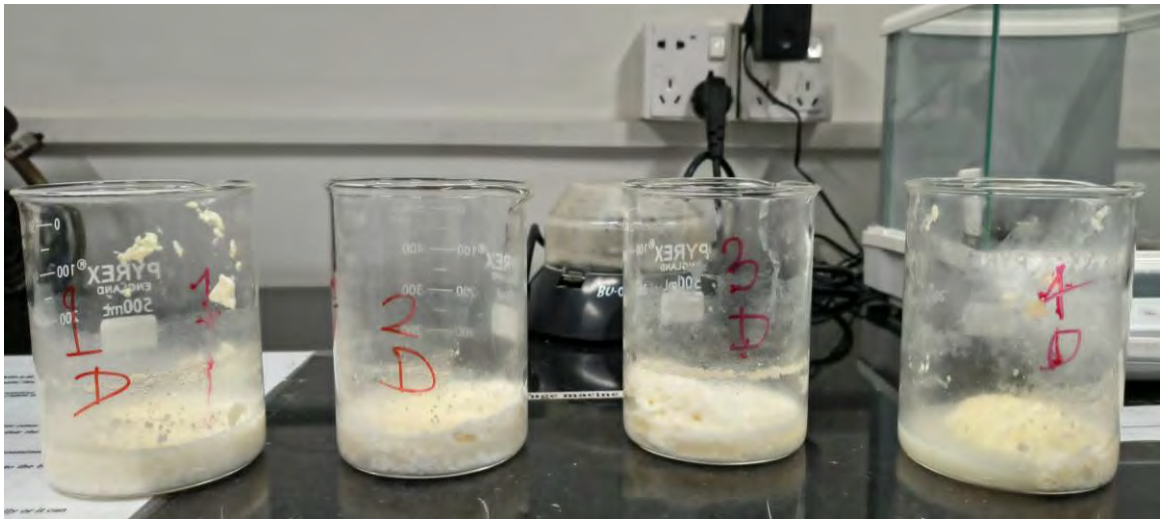


Figure 4: Prepared yogurt after removal of excess whey

3.3 Initial pH Result of Sample D

In the initial Sample D experiment, four different dilution/concentration cultures were prepared and inoculated. The pH values are shown below.

Sample name	Procedure	Total dilution/concentration	pH
1d	1 mL slant culture + 9 mL PBS	10^{-1}	4.47
2d	1 mL from 1d + 9 mL PBS	10^{-2}	4.99
3d	1 mL from 2d + 9 mL PBS	10^{-3}	5.36
4d	1 mL from 3d + 9 mL PBS	10^{-4}	5.42

Table 3: Initial pH of Sample D

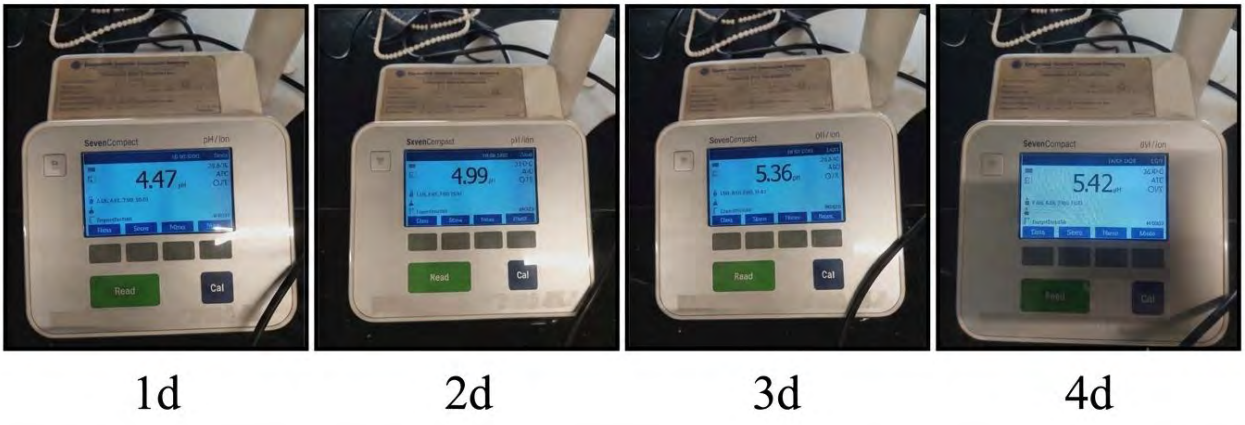


Figure 5: pH values of yogurt sample D at different dilution concentrations

3.3.1 pH Results of Batch Samples

After preparing the working stock from Sample D, four batch groups were tested. These were Batch 1 small, Batch 2 large, Batch 3 small, and Batch 4 large. Each batch contained five inoculum concentrations: 100 µL, 200 µL, 300 µL, 400 µL, and 500 µL.

Sample concentration	Batch 1 small pH	Batch 2 large pH	Batch 3 small pH	Batch 4 large pH	Mean pH
100 µL	3.99	4.08	4.56	4.17	4.20
200 µL	4.00	4.04	4.09	4.06	4.05
300 µL	4.00	4.17	3.98	4.13	4.07
400 µL	4.01	4.15	4.01	3.99	4.04
500 µL	4.07	4.19	4.12	4.16	4.14

Table 4: Mean pH of Sample D

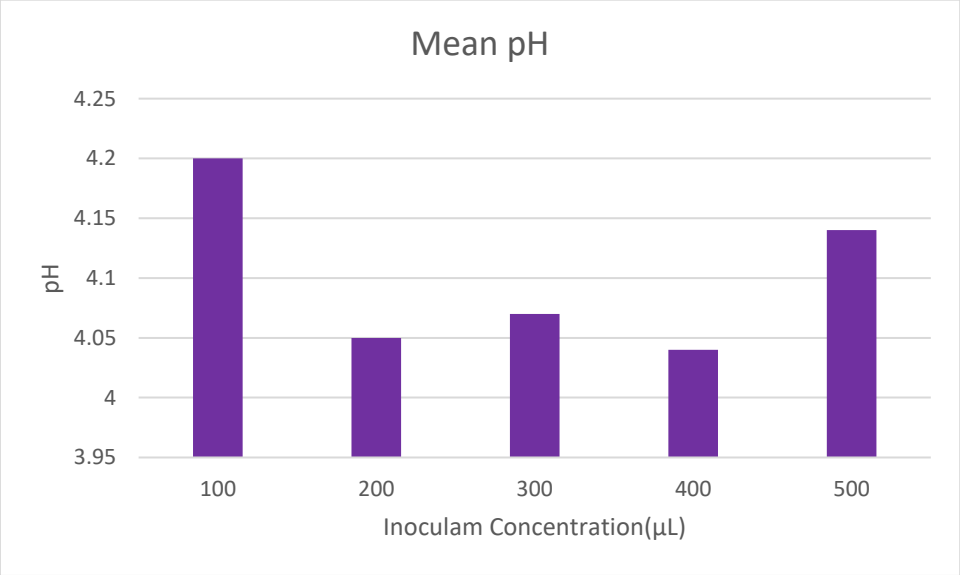


Figure 6*: Inoculum concentration versus Mean pH*

Most pH values were within the expected yogurt range of approximately 4.0–4.6. This indicates that the isolated culture was able to ferment milk and produce acid. Among the concentrations, the 400 µL group had the lowest mean pH of 4.04, followed closely by the 200 µL group with a mean pH of 4.05. The 100 µL group showed the highest mean pH because Batch 3 at 100 µL had a comparatively high pH of 4.56. The pH pattern was not completely linear, indicating that fermentation was influenced by other factors such as inoculum viability, mixing, incubation condition, and handling.

3.4 Volume and Dry Yogurt Weight Results

Liquid volume and dry yogurt weight were measured to evaluate the physical output of fermentation. The volume value represents the remaining liquid portion, while dry yogurt weight represents the solid product obtained after fermentation.

3.4.1 Batch 1

Sample concentration	Volume	Weight of yogurt with beaker	Blank beaker weight	Dry yogurt weight
100 µL	42 mL	149.851 g	93.981 g	55.872 g
200 µL	45 mL	162.197 g	104.410 g	57.787 g
300 µL	32 mL	184.101 g	108.451 g	75.650 g
400 µL	46 mL	204.414 g	109.777 g	94.637 g
500 µL	43 mL	178.695 g	100.697 g	78.018 g

Table 5: Batch 1 Dry yogurt weight and volume

In Batch 1, the highest dry yogurt weight was observed at 400 µL, with 94.637 g. The lowest dry yogurt weight was observed at 100 µL, with 55.872 g. This suggests that in Batch 1, a higher inoculum concentration improved solid yogurt formation up to 400 µL.

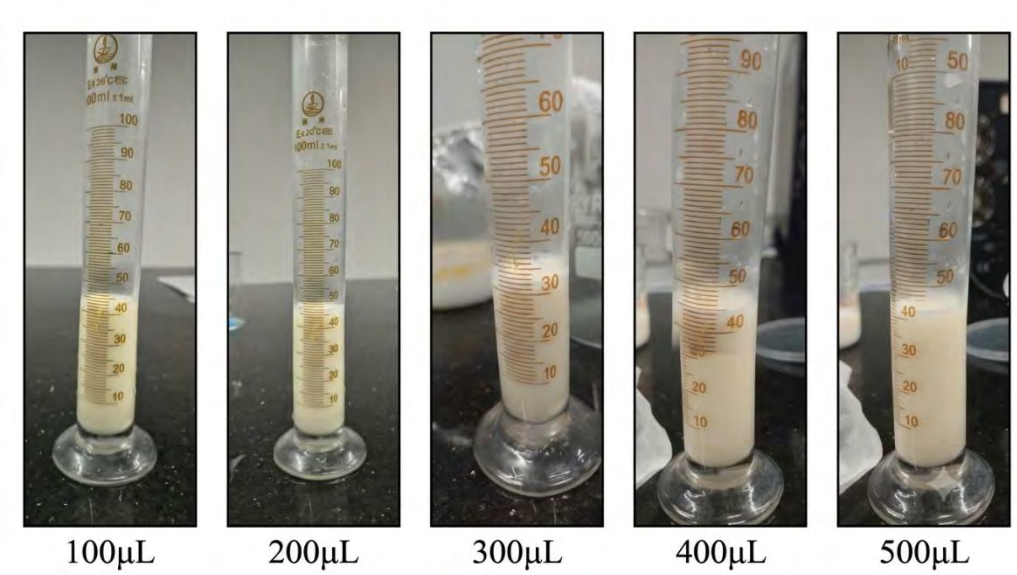


Figure 7: Volume of whey extracted from yogurt from Batch 1

3.4.2 Batch 2

Sample concentration	Volume	Weight of yogurt with beaker	Blank beaker weight	Dry yogurt weight
100 µL	31 mL	278.284 g	156.890 g	121.394 g
200 µL	34 mL	269.171 g	166.006 g	103.165 g
300 µL	41 mL	282.666 g	172.344 g	110.322 g
400 µL	61 mL	229.454 g	172.619 g	56.835 g
500 µL	44 mL	226.288 g	170.556 g	55.732 g

Table 6: Batch 2 Dry yogurt weight and volume

In Batch 2, the highest dry yogurt weight was observed at 100 µL, with 121.394 g. The lowest dry yogurt weight was recorded at 500 µL, with 55.732 g. This result was different from Batch 1 and shows that the relationship between inoculum concentration and dry yogurt weight was not consistent in all batches.

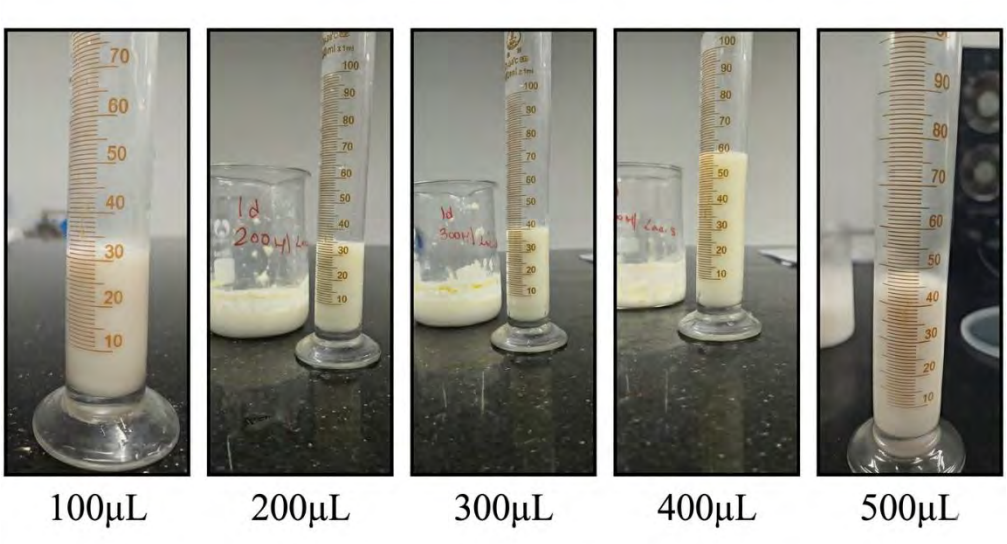


Figure 8: Volume of whey extracted from yogurt from Batch 2

3.4.3 Batch 3

Sample concentration	Volume	Weight of yogurt with beaker	Blank beaker weight	Dry yogurt weight
100 µL	94 mL	133.970 g	93.981 g	39.989 g
200 µL	65 mL	153.379 g	104.410 g	48.969 g
300 µL	57 mL	163.268 g	108.451 g	54.817 g
400 µL	71 mL	132.534 g	100.697 g	31.837 g
500 µL	69 mL	136.578 g	109.777 g	26.801 g

Table 7: Batch 3 Dry yogurt weight and volume

In Batch 3, the highest dry yogurt weight was found at 300 µL, with 54.817 g. The lowest dry yogurt weight was found at 500 µL, with 26.801 g. The volume values were higher in this batch compared with Batch 1, which may indicate more liquid separation or weaker gel formation.

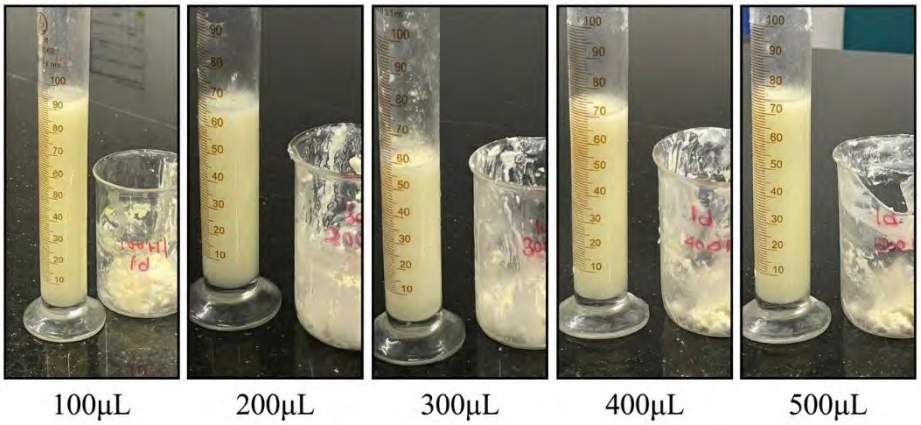


Figure 9: Volume of whey extracted from yogurt from Batch 3

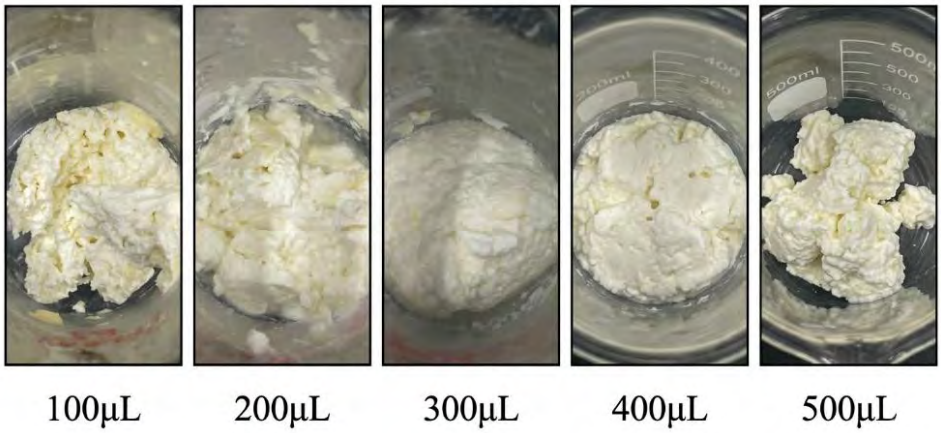


Figure 10: Solid yogurt product prepared from Batch 3

3.4.4 Batch 4

Sample concentration	Volume	Weight of yogurt with beaker	Blank beaker weight	Dry yogurt weight
100 µL	66 mL	224.081 g	156.890 g	67.191 g
200 µL	75 mL	238.596 g	166.006 g	72.590 g
300 µL	71 mL	211.158 g	172.344 g	38.814 g
400 µL	95 mL	232.078 g	172.619 g	59.459 g
500 µL	80 mL	228.739 g	170.556 g	58.183 g

Table 8: Batch 4 Dry yogurt weight and volume

In Batch 4, the highest dry yogurt weight was recorded at 200 µL, with 72.590 g. The lowest dry yogurt weight was recorded at 300 µL, with 38.814 g. Like the other batches, the result showed variation across concentrations.

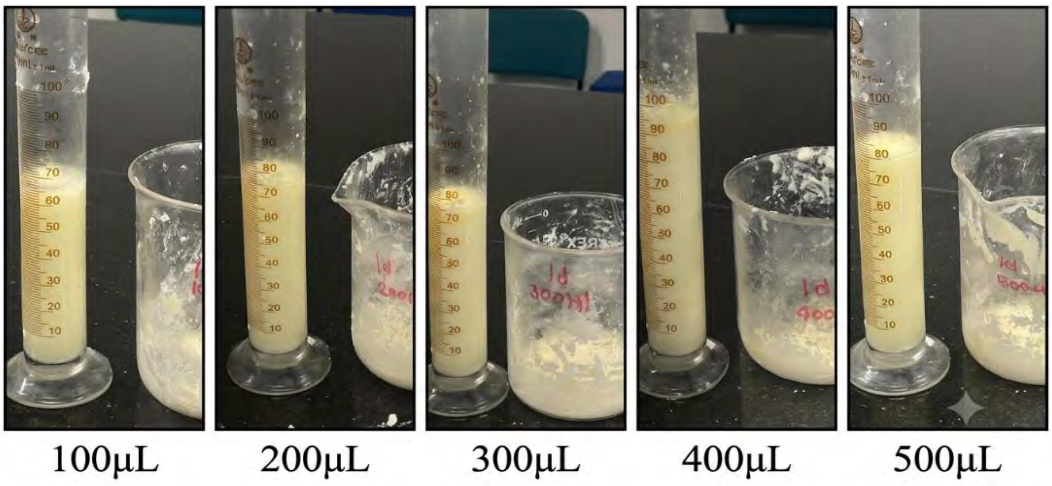


Figure 11: Volume of whey extracted from yogurt from Batch 4

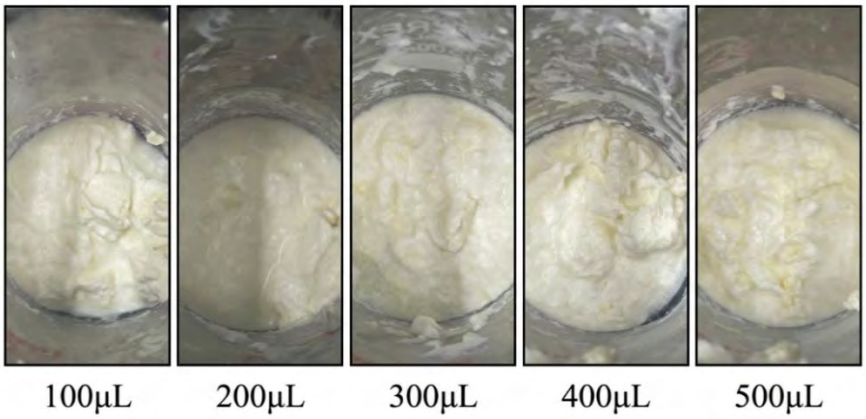


Figure 12: Solid yogurt product prepared from Batch 4

3.4.5 Mean Values of the data

Concentration	Mean pH	Mean liquid volume	Mean dry yogurt weight
100 µL	4.20	58.25 mL	71.112 g
200 µL	4.05	54.75 mL	70.628 g
300 µL	4.07	50.25 mL	69.901 g
400 µL	4.04	68.25 mL	60.692 g
500 µL	4.14	59.00 mL	54.683 g

Table 9: Mean values of pH, Liquid volume & Dry yogurt weight

The mean pH values show that all concentrations were able to produce acidic yogurt-like products. The 400 µL group showed the lowest mean pH, but it did not produce the highest mean dry weight. The highest mean dry yogurt weight was found at 100 µL, followed very closely by 200 µL and 300 µL. The mean dry yogurt weight decreased at 400 µL and 500 µL. It suggests that a higher inoculum amount did not always improve solid yogurt yield.

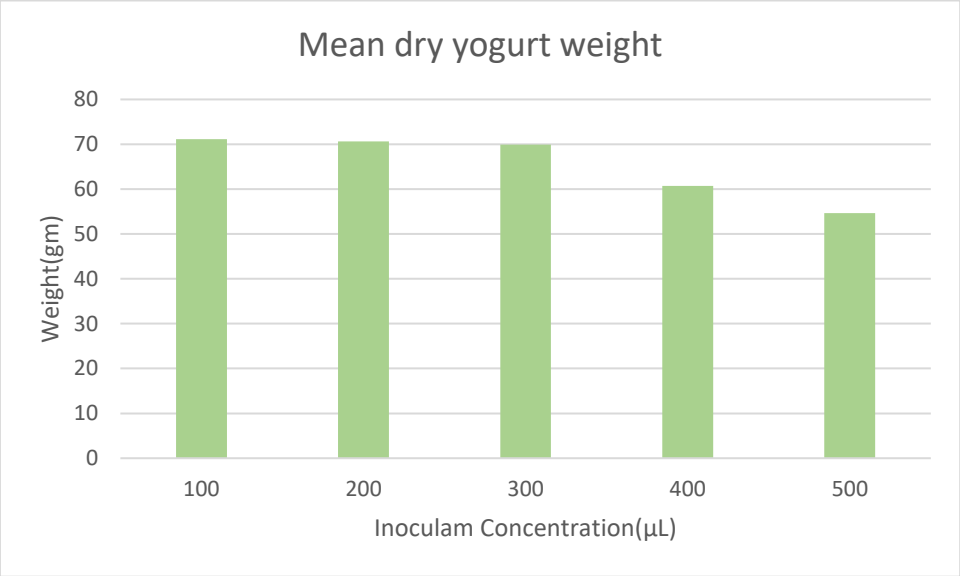


Figure 13: Inoculum concentration versus Mean dry yogurt weight

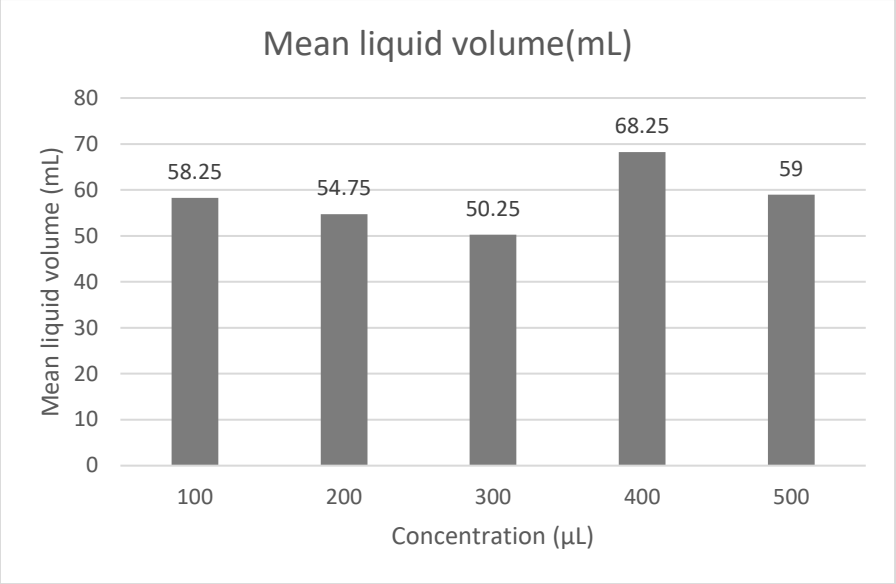


Figure 14: Inoculum concentration versus Mean liquid volume

The best overall fermentation result may be considered around 200–300 µL because these concentrations produced low pH values and relatively high mean dry yogurt weights. However, the variation between batches indicates that further repeated trials are needed before selecting an exact optimum inoculum concentration.

3.6 Gram Staining Observation

Gram staining was planned as a confirmatory morphological test. Lactic acid bacteria, especially Lactobacillus-like isolates, are expected to appear as Gram-positive purple rods, often arranged singly, in chains, or in short groups. In the present dataset, a table for Gram stain pictures was prepared, but the actual microscopic images were not included in the provided data table. Therefore, the Gram staining result should be completed by inserting the microscopic photographs and writing the final observation.

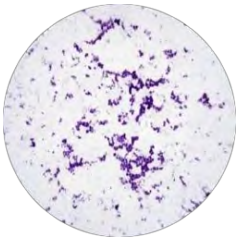
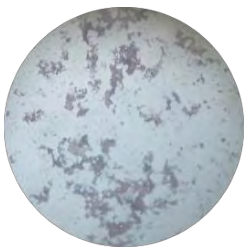
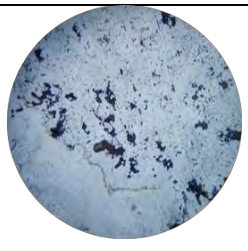
Sample number	Volume	Gram staining observation
1	100 µL	
2	200 µL	
3	300 µL	
4	400 µL	
5	500 µL	

Table 10: Gram Staining

Chapter 4

Discussion

4.1 General Interpretation of Findings

The study demonstrated that lactic acid bacteria (LAB) were able to be recovered from yogurts and used again to ferment milk. The sample that grew most successfully was Sample D, which grew on MRS agar, developed MRS slant culture, formed yogurt, and had measurable changes in pH, liquid volume and weight of dry yogurt. The pH of the milk was an important indicator of the fermentation reaction to be taken into consideration, since LAB generates lactic acid which decreases the pH of milk and triggers casein coagulation that creates the gel structure of yogurt. Acidification was considered a success as most of the batch pH values were in the range 3.98 - 4.19 with one reading of 4.56. The pH values of yogurts indicate effective fermentation, because the range of pH of the yogurts was around 4.6, which is the pH at which yogurt is formed. The pH did not change in a completely linear fashion. Increasing the inoculum size can lead to greater acid production, although the size of 500 µL did not produce the lowest pH. The mean pH was the lowest in the 400µL group and the mean pH was higher in 500µL than in the 200–400µL group. The differences could be attributable to the differential survival of bacteria, mixing, sedimentation, differences in incubation, the size of the beaker, or handling error. In the preliminary dilution test, the pH decreased with dilution, with the 10^{-1} sample with pH of 4.47, and the 10^{-3} and 10^{-4} samples pH>5.0. There were also differences in the weight of the dry yogurt and liquid volume among batches. The highest dry weight was 121.394 g in Batch 2 at 100 µL, while the lowest was 26.801 g in Batch 3 at 500 µL. The mean dry weight of the solid production dropped from 71.112 g at 100 µL to 54.683 g at 500µL, indicating that the solid yield did not improve with higher inoculum. The liquid volume was between 31 mL and 95 mL indicating variations in whey separation and curd strength. Hence, fermentation efficiency should be determined in conjunction with the pH, dry weight, liquid volume, texture and Gram staining.

4.2 Limitations and Challenges Encountered

Several challenges affected the experimental work and reduced the amount of complete data available for analysis. Microbial and fungal contamination occurred in some samples, leading to the discontinuation of affected cultures and loss of experimental data. Sample spoilage during storage also prevented further evaluation of certain isolates. Laboratory interruptions, including power outages and institutional closure, affected incubation continuity and sample preservation. In addition, the candle-jar method used to create reduced-oxygen conditions provided only limited control over the growth environment compared with standard anaerobic systems, which may have influenced LAB cultivation and fermentation performance. Variation among the images of gram stain can be due to differences in smear thickness, uneven staining, over-decolorization, culture age, low cell concentration, or clumping of bacterial cells during slide preparation. Lactobacillus species are generally Gram-positive rods, but they may sometimes appear pleomorphic or Gram-variable, especially in old or stressed cultures.

4.3 Improvement strategies

The experimental work was burdened with several problems and a smaller data set was available for analysis. Some of the samples were contaminated by microbial and/or fungal growth resulting in the termination of the contaminated cultures and loss of experimental data. Some isolates were also not subjected to further evaluation due to sample spoilage during storage. Incubation continuity and sample preservation were interrupted with laboratory disruptions such as power outages and closure of institutions. Furthermore, the candle-jar method created only partially reduced oxygen conditions as compared to the standard anaerobic conditions, which could have affected LAB growth and fermentation characteristics. Microbiological work can become contaminated in a number of ways. The sources that could cause the problem are air exposure, sealing of plates not properly, unsterile handling, and contaminated pipette tips, lack of enough flame sterilization, contaminated glassware and poor sample quality. Some microorganisms such as yeasts or mould or non-LAB bacteria, may already be present in the yogurt samples from local sources. Fungal spores in the air might fall onto the plate surface and grow during incubation and the sample is exposed to the environment during dilution or plating. Sample C could have become fungal contaminated as a result of airborne spores, contamination of the sample material or prolonged processing time. When fungi invade the plate, they will grow on rich media. MRS Agar is more of a general medium that allows the growth of LAB and other organisms may develop if it becomes contaminated. Pathogenic growth may have been present in Sample B, which could account for spoilage. Spoilage in Sample B may have been caused by mixed growth of microbes, late observation, long incubation or unsuitable storage. After the onset of unwanted microorganisms in milk, these microorganisms can cause the milk to emit an unpleasant odor, generate gas, cause proteolysis, or cause spoilage.

4.4 Reasons for Variation Between Batches

The causes of batch variation may be attributed to different bacterial viability and growth phases, differences in the capacity to produce acid, pipetting precision, mixing, milk quantity, incubation temperature, beaker size or oxygen exposure. Small and large beakers can also have different heat transfer, surface area, evaporation, coagulation, whey separation. Inconsistent mixing of the culture may result in an unequal distribution of bacteria between the samples. The fermentation performance was variable, due to the non-molecular confirmation of the isolate, which might have been caused by different strains of LAB.

4.5 Recommendations

To limit the contamination during LAB isolation and growth, improved aseptic handling is necessary. Throughout the experiment, proper media, glassware and work surface sterilizations should be carried out and culture plates and sterile materials handled carefully. Negative controls would be beneficial to monitor for possible environmental or media contamination and to enhance data reliability. It is suggested that a standard anaerobic jar with gas-generating systems be used instead of candle-jar systems for more controlled growth conditions of LAB. Multiple replicates of the experiments would enable statistical analysis that would increase accuracy and repeatability. Molecular and biochemical typing techniques like 16S rRNA gene sequencing and carbohydrate fermentation profile would be recommended as additional means of identification of isolates. Further, performance of fermentation could be better assessed by considering the following additional parameters; titratable acidity, microbial count, water-holding capacity, sensory attributes and shelf-life assessment. The improvements would increase the reliability of the findings and would give a comprehensive assessment of the technological and potential probiotic attributes of the LAB isolates.

4.6 Future Scope

Future studies could be conducted to isolate the LAB strain from Sample D by molecular methods. Strain may also be evaluated for its probiotic attributes, including acid resistance, bile salt resistance, antimicrobial activity, antibiotic sensitivity and safety. The fermentation experiment could be repeated with standardized milk, control fermentation and bacterial cell count. This would facilitate the determination of optimum concentration of inoculum to make yogurt. The nutritional composition, storage stability and possible postbiotic effects of the dry yogurt product can also be further investigated. LAB isolates could be used in a pharmaceutical context for screening and production of antimicrobial substances, enzymes, organic acids or other metabolites. The strain with safe and beneficial properties could be investigated for developing functional foods or probiotics. For use of a probiotic, however, there would be a need for detailed safety testing, strain identification and biological evaluation which would be controlled.

Chapter 5

Conclusion

Improvement of the present study showed that lactic acid bacteria can be isolated from yogurt samples and can be used for the milk fermentation again. Of these samples, sample D gave the most complete results with colonies, growth on slants, yogurt formation, acidification and production of dry yogurt. The pH of most of the batches was in the range of 3.98 to 4.19 which shows good acid production in the yogurt range. There were differences in dry weight of the yogurt and liquid volume between the different concentrations of the inoculum and batches. The biggest average dry yogurt weight was observed at 100 µL, and 200–300 µL exhibited a balanced average value according to the pH and dry weight. The lowest mean pH was found at 400 µL. Some samples were contaminated and/or spoiled during storage and/or in the laboratory, emphasizing the importance of maintaining the care of aseptic handling, proper incubation and the need for replication. Other biochemical and molecular analyses are needed prior to industrial or pharmaceutical use.

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