MICROENCAPSULATION OF PROBIOTICS WITH MILK BASED EXTRUSION METHOD

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ABSTRACT :
Probiotics are live in microorganisms that contains more health benefits. Probiotics are not utilized completely in colony because of low gastric pH. Microencapsulation of probiotics will improve the viability of probiotics and utilization of probiotics in colony. Probiotics are isolated from goat milk with the help of MRS broth. Isolation of Probiotics such as Lactobacillus from the culture is done by using cell suspension method with the help of Sodium chloride. Isolated probiotics is microencapsulated by extrusion method using Sodium alginate. Microencapsulation of specific probiotic Lactobacillus is microencapsulated probiotic enhances the gut health.
Microencapsulated probiotics has higher rate of survival. Hence, the probiotics will be protected and utilized in the better way.
Keywords: Goat Milk, Lactobacillus, Probiotics, Microencapsulation.

INTRODUCTION

Probiotics:

Probiotics are live microorganisms that are good for human health. Probiotics are human beneficial microorganisms. They are generally considered as safe to consume. Probiotics are good for gut. It improves the gut microbiota. Live probiotics are important for fermentation in the production of dairy products. Probiotics is used in production of fortified products. Probiotics has health benefits such as reducing gastrointestinal discomfort, improve immune health, relieving constipation. Probiotics are often known as good bacteria and friendly bacteria. [1]

Some examples of probiotic are *Saccharomyces bulgaricus*, *, Lactobacillus spp. Bifidobacterium* is a good the bacteria that normally present in human gut. *Bifidobacterium* is present in food products like Kimchi (spicy fermented cabbage), Kombucha (fermented tea), Miso (fermented brown rice). *Saccharomyces boulardii*is a good the fungi which is usually present in dairy products such as yogurt, cheese and present in mangosteen fruit, Kombucha.[2]

Probiotics in milk:

The first discovered probiotic is *Lactobacillus bulgaricus* in Bulgarian yogurt. It was discovered in 1905 by Stamen Grigorov. Milk usually contains probiotic in genus of *Lactobacillus*. Lactic acid bacteria are used as food fermenting bacteria. They ability to prevent food spoilage and can improve the nutritive value of the foods.

Lactobacillus has different strains which can help improve the gut health. This is a type of bacteria that makes lactic acid by breaking down carbohydrates. *Lactobacillus* is good for digestion, treating the chronic constipation and irritable bowel syndrome. The association between *Lactobacillus* and humans gives mutual benefits. The mutual benefits are *Lactobacillus* helps in digesting certain dietary substrates and returns the accommodation, nutrients from human. *Lactobacillus* commercially used as health supplements and also used in products of food technology sector.[3]

Goat's milk and cow's milk:

There are about 170 species present under the *Lactobacillus* genus. The cow's milk usually contains 10 to 12 species of *Lactobacillus.* Whereas the goat's milk contains 19 to 25 species of *Lactobacillus*. Goat's milk has protein content when compared to cow's milk.

Comparatively Goat's milk can be easily digestible .[4] Each milk is good for bone health. Both the milk contains probiotic which helps in digestion and improve intestine health.

MICROENCAPSULATION:

Microencapsulation is a process in which the small particles are coated with another substance to small capsules. Microencapsulation is the process by which tiny solid particles or droplets of liquid are surrounded or coated with a continuous film of polymeric material to produce capsules in the micrometre to millimetre range. The product obtained is called as Microcapsules. Microencapsulation is technique used for making capsules. It is technique used for formulation of products.

Microencapsulation is done for protecting sensitive compounds, ensuring the safe delivery. The compound or material that is encapsulated is called core and the coating substance is called encapsulant.[5]

MICROENCAPSULATION OF PROBIOTICS:

Probiotics are microencapsulated for protection. Microencapsulation is used to maintain viability of probiotic bacteria during processing and storage of diary and food products. Encapsulations protect the core compound from adverse environmental conditions. Probiotics are destroyed in acid present in gastrointestinal tract and not completely utilized and absorbed by the body .[6]

Microcapsules of probiotics are used as nutrient supplements like protein supplements. Proteins supplements is taken when lack of protein is identified. Like that the in order to improve the gut microbiota, the probiotic microcapsules are used. And also used to treat gastrointestinal problems.

EXTRUSION METHOD:

Extrusion is the type of method used in microencapsulation. Extrusion is a process to create a specific shaped object by pushing a material through pressure. Extrusion is process in which the hydrocolloid solution is simply made, to the microorganisms are added, and the cell suspension is extruded through a syringe needle in the form of free-falling droplets into a hardening solution.[8]

MATERIALS AND METHODOLOGY

Materials:

- Probiotic strains: Lactobacillus.
- Milk and Dairy products: Whole milk and Skimmed Milk powder, MRS agar.
- Encapsulating Agents: Hydrocolloids-Alginate and Gelatin, Polysaccharides Starch and Chitosan, Prebiotics-Fructo-oligosaccharides.
- Solvents and Buffers: Distilled water, Calcium chloride, Phosphate Buffer
- Equipment: Incubators, Homogenizers and mixers, Centrifuge, Magnetic Stirrer, Test tube, Petri-plates, Pipets, L shaped rod, Inoculation loop.

COLLECTION OF GOAT MILK:

Fresh goat milk was collected from nearby goat farm. The collected milk is filtered to remove large impurities. The milk is the pasteurized by heating and suddenly cooling to inhibit the growth of pathogens and remove any contaminants. A small portion of the milk is isolated for serial dilution. [9]

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Fig 1: Image Courtesy: https://primalprey.com.au/product/raw-goat-milk/

SERIAL DILUTION OF GOAT MILK:

Serial Dilution of goat milk can help in adjusting the concentration of certain components which includes fats, proteins, or bioactive compounds before microencapsulation. Before initiating serial dilution, dilution factor is decided such as 1:10, 1:5, etc. Here the common tenfold dilution (1:10) is used. Add a measured volume of goat milk to a sterile test tube containing a diluent. As mentioned above, for 1:10 dilution 1ml of goat milk with 9ml of diluent (water) is mixed. Mix the dilution in the vortex mixture. [10]

For subsequent dilutions, take 1ml from first dilution and add it to a new test tube containing the same volume of diluent (1:10 dilution). This gives 2nd diluted sample. Continuing this process, take same volume from the previous dilution and mixed with the fresh diluent, until desired number of dilutions is achieved. Each tube is labelled with dilution factor or the final concentration of the sample. Serial dilution controls the concentration of components in goat milk, which is crucial for ensuring consistency and quality in the final microencapsulated product. [11]

PREPARATION OF LACTOBACILLUS MOTHER CULTURE:

The process of preparing *Lactobacillus* mother culture for microencapsulation involves cultivating a concentrated, pure culture of lactobacillus bacteria. Determine the Lactobacillus strain of choice by evaluating its desired probiotic traits, compatibility with the encapsulation process, and stability in the final product. (Kishoree Krishna Kumaree et al.2015) Ensure the Culture's identity have been established through microbial testing which includes Gram staining and biochemical assays. Adequate growth medium is choosed, ideally MRS (de man, Rogosa, and Sharpe), which is frequently used for cultivation of lactic acid bacteria. Sterilize the medium to eliminate the contaminants. [12]

Adding the lactobacillus started culture to the sterile growth medium in aseptic conditions to initiate the inoculation process. Incubating the culture at 37°C for a period of 24-48 hours, adjustment is done based on the strain's growth characteristics, to facilitate bacterial proliferation. Growth progress is tracked through optical density (OD) measures or viable cell count to verify the culture attains the targeted concentration. To increase the bacteria concentration, centrifugation is used to concentrate the cells [13]

This process entails spinning the culture at a high speed to form a bacterial pellet, which is then resuspended in a reduced volume of sterile saline or growth medium. The concentrated Lactobacillus culture is stored at 4°C if it will be used shortly or it can be frozen at -80°C for long term storage. To protect the cells during freezing, cryoprotectant is added such as glycerol. The lactobacillus culture that has been prepared is subsequently combined with encapsulating materials like sodium alginate, skimmed milk powder for the extrusion method. [14]

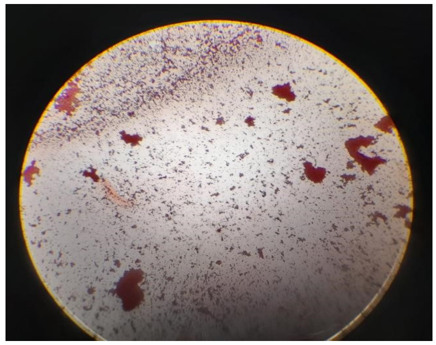

Fig 2: Microscopic view of *Lactobacillus*

Prior to microencapsulation, it is crucial to conduct tests on the cell culture to assess cell viability, purity, and the presence of any contamination. A high-quality culture is necessary to guarantee the production of a reliable and efficient microencapsulated product. [15]

MASSIVE GROWTH OF LACTOBACILLUS:

Growth medium is utilized for rapid and high-density bacterial growth such as MRS (de Man, Rogosa, and Sharpe) broth. The medium was enhanced by adjusting nutrient concentrations and extrusion method for microencapsulation method is proceeded using the prepared Lactobacillus culture. A high-quality starter culture that has been used and confirmed its purity and viability. The bioreactor is inoculated with the prepared Lactobacillus inoculum under a aseptic conditions. [16] Temperature is maintained at 30-37°C, depending on the strain, pH is maintained around 5.5-6 5, as Lactobacillus prefers slightly acidic conditions. Some Lactobacillus requires controlled aeration while others are microaerophilic or anaerobic. Oxygen level is adjusted according to the culture.

PREPARATION OF *LACTOBACILLUS* PELLET:

Once the culture has reached the desired density, the cells should be harvested by centrifuging the culture broth. The centrifugation is done at 5000rpm for 10min for pelleting the cells. Carefully decant the supernatant to avoid disturbing the pellet. The cell pellet is resuspended in a Phosphate buffered solution (PBS) to wash away residual media components. The centrifugation and washing process is repeated 2-3 times and ensure removal of impurities. After final wash, the excess supernatant is removed, the leftover pellet is dense and moist. A small sample is plated on an agar medium or a

viability assay to ensure the active of cells. The Lactobacillus pellet is then mixed with encapsulating agents like sodium alginate and skimmed milk powder. Ensuring the uniform distribution of bacteria in the encapsulation matrix. The microencapsulated Lactobacillus is stored under -4°C to maintain its viability and stability, typically in a cool and dry place. Quality control test was conducted to assess the viability of the encapsulated Lactobacillus.

PURIFICATION OF *LACTOBACILLUS* PELLET:

It is essential to purify a *Lactobacillus* pellet for microencapsulation to eliminate unwanted substances and enhance the concentration of viable bacteria. This purification process enhances the stability and effectiveness of the microencapsulated product. Centrifuging the resuspended cells at the same speed as previously done and discard the procedure 2-3 times, using fresh sterile saline or PBS each time. This critical step helps in reducing contaminants, metabolic byproducts, and any remaining growth medium that interferes with the microencapsulation process. [17]Following the purification steps, centrifuge the cell suspension one final time to concentrate the Lactobacillus cells in a pellet. Discard the supernatent and carefully gather the purified pellet. The Cell viability assays

to ensure that the purification process has not compromised the viability of the Lactobacillus cells. Microbiological tests confirm the purity of the culture, guaranteeing the absence of contamination from other organisms.
Isolation and Purification of Lactic Acid Bacteria from Milk for Probiotic Applications. 2023. This study aims to isolate and purify lactic acid bacteria (LAB) from milk, evaluating their probiotic potential. LAB strains are isolated using selective media and identified through molecular techniques. The research focuses on the strains' ability to survive gastrointestinal conditions and their antimicrobial properties, contributing to the development of effective probiotic dairy products.
Identification of Probiotic Bacteria from Milk Using Centrifugation and Filtration Techniques. This research explores the use of centrifugation and filtration techniques for the purification of probiotic bacteria from milk, aiming to optimize these methods to enhance recovery and purity. The study evaluates different parameters for centrifugation and filtration, analyzing their impact on bacterial viability and purity. [18]
Microencapsulation of Probiotics Using Extrusion Techniques: A Review. 2024. [19] This review paper explores various extrusion techniques for the microencapsulation of probiotics, discussing their effectiveness in protecting probiotics during processing and storage. It covers methods, challenges, and advancements in extrusion technology for probiotic applications. [20] This study investigates the use of extrusion-based microencapsulation techniques for enhancing probiotic delivery systems. The research evaluates the impact of extrusion parameters on the stability and release of probiotics in various formulations. [21] This paper reviews advancements in encapsulation techniques for environmental applications, including coacervation and spray drying. [22] This study explores microencapsulation methods for enhancing the stability and delivery of bioactive compounds in food.

MICROENCAPSULATION OF *LACTOBACILLUS* PELLET:
The incorporation of probiotic bacteria as nutrient rich food in an area that has widely opened in recent years. However, it is important to note that the viability and abundance of these bacteria in the intestinal tract can be influenced by various factors such as diet, stress and medication. Therefore, while these bacteria may have potential health benefits, their effectiveness may vary from person to person in their intestinal tract. The oral intake of the majority of bacteria leads to significant Viability loss due to their transit through the stomach, primarily caused by the elevated levels of acids and bile salts. This reduction in viability diminishes the effectiveness of the supplement being administered. Encapsulating these probiotics in microcapsules is a novel approach aimed at minimizing cell mortality during gastrointestinal transit, while also providing means to regulate the release of cells throughout the intestinal system .[23] The inoculated culture was incubated in the shaker for 24 hours at 37℃. A volume of 1.5ml of overnight cultured probiotic bacteria in MRS broth was distributed into four autoclaved Eppendorf tubes and subjected to centrifugation at 10,000 rpm for 10 minutes at a temperature of 4℃. The resulting cell pellet was washed twice with saline solution. Subsequently, the cells were resuspended in normal saline in preparation for the encapsulation process.
A total of 1.75g each of sodium alginate and skimmed milk powder was combined with 5ml of sterilized water to achieve 1:1 ratio. This resulting mixture was then extruded into a 0.2M of CaCl2 solution, which served as the hardening agent. The Sodium alginate-skimmed milk cell matrix was dispensed into CaCl2 using a 21G needle from a height of 10cm, resulting in the creation of calcium alginate beads that encapsulated probiotic cultures. The hardening process for the calcium alginate microcapsules was conducted for a duration of 30minutes. Following this, the microcapsules were collected , rinsed with distilled water, and then suspended in a saline solution.[24] To evaluate the impact of skimmed milk addition, separate alginate microcapsules were also prepared and assessed for their efficiency.
Efficiency of Encapsulation: The probiotic bacteria derived from microencapsulated cells were released in a phosphate buffer at pH7. The resulting solubilized suspension underwent serial dilution and was subsequently plated onto MRS agar plates.

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These plates were incubated at 37°C for a duration of 24 to 48 hours, after which the colonies were counted to assess the microencapsulation efficiency. [25]Encapsulation efficiency = (Number of colonies from the beads / Number of colonies in cell suspension) * 100
Production yield (%) = (Mass of resulting microcapsules) *100/ (Total mass of the materials used)[26]

RESULT AND DISCUSSION:

Collection of goat milk:

The obtained goat milk has good fat content ranges from 3.5 – 4% fat. The protein content of goat is 3.5% with higher concentration of whey protein than cow milk. The pH level of goat milk is around 6.6. The collected and stored goat milk has 100,000 CFU (Colony forming unit). The goat milk is stored in the refrigerator at 4°C and lasts for 8 days.

Fig 3: Goat milk

SERIAL DILUTION OF GOAT MILK:

The concentration of goat milk in the test tube are decreased from 100% to 0.01%. The titration curve determines the optimal dilution ratio of the milk which is represented in graphical method. Serial Dilution of goat milk by inoculating with bacteria helped in the observation of microorganism's growth at different milk concentrations. Goat milk dilution helped to assess the enzyme activity lactoperoxidase

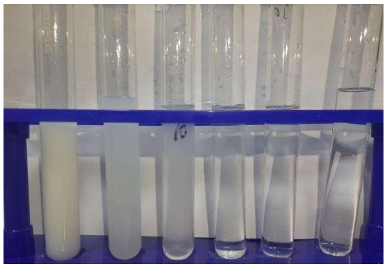

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Fig 4: Serial Dilution

PREPARATION OF *LACTOBACILLU*S MOTHER CULTURE:

Lactobacillus colonies are grown in the culture medium by increasing the cell density and biomass. The pH of the grown Lactobacillus in the medium is decreased to 5.5. The culture produces lactic acid contributing to the pH reduction. The culture became thicker and more viscous due to production of exopolysaccharides. The viability of lactobacillus cells increased from 10^{-3} to 10^{-4}. The mother culture is scaled up larger production and it is used as inoculum for other fermentations.

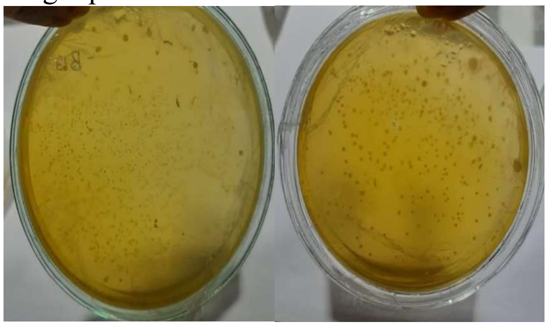

Fig 5: Lactobacillus growth

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MASSIVE GROWTH OF LACTOBACILLUS:

The cell count is increased typically reached 10^12 CFU/ml. The sugars are converted into lactic acid leads to decrease in pH and increase in acidity. Dense *Lactobacillus* growth enhanced beneficial compound production like bacteriocins, antimicrobial peptides and exopolysaccharides. The strain of *lactobacillus* is maintained that leads to the genetic stability.

The contaminants are controlled effectively for maintaining pure and healthy culture.

Fig 6: Massive growth of Lactobacillus

PREPARATION OF LACTOBACILLUS PELLET:

The *lactobacillus* pellets concentrate and stabilizes the bacteria into compact and maintaining stable shelf life. A dense and pelletized structure maintains good handling and storage of *lactobacillus* conveniently. The pellet is restored by simple rehydration process maintaining its active state. The obtained pellet has uniform size, shape and colour. The pellets are compact, and it took only less storage place compared to liquid culture. The obtained pellets can also be stored for months for further analysis.

PURIFICATION OF *LACTOBACILLUS* PELLET:

The purified *lactobacillus* pellets removed impurities and contaminants and achieved high purity product. 90% of impurities and contaminants are removed by purification of *lactobacillus.* Purification of *lactobacillus* helped to decrease the endotoxin content. In order to achieve these factors, Centrifugation, washing techniques, Palletization technique(extrusion), Cryoprotectants are used.

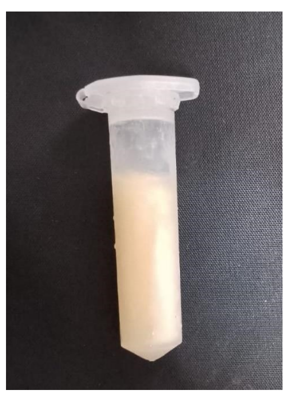

Fig 7: *Lactobacillus* pellet

MICROENCAPSULATION OF PROBIOTICS:

The microencapsulation technique was performed using 0.2M $CaCl_2$ and Sodium alginate of weight 1.75g with *lactobacillus* culture in it with reference to the paper ,Millk based extrusion method for microencapsulation of probiotics. The extrusion method resulted in bead formation containing lactobacillus species. The resulted bead was jelly in texture and was stable at room temperature.

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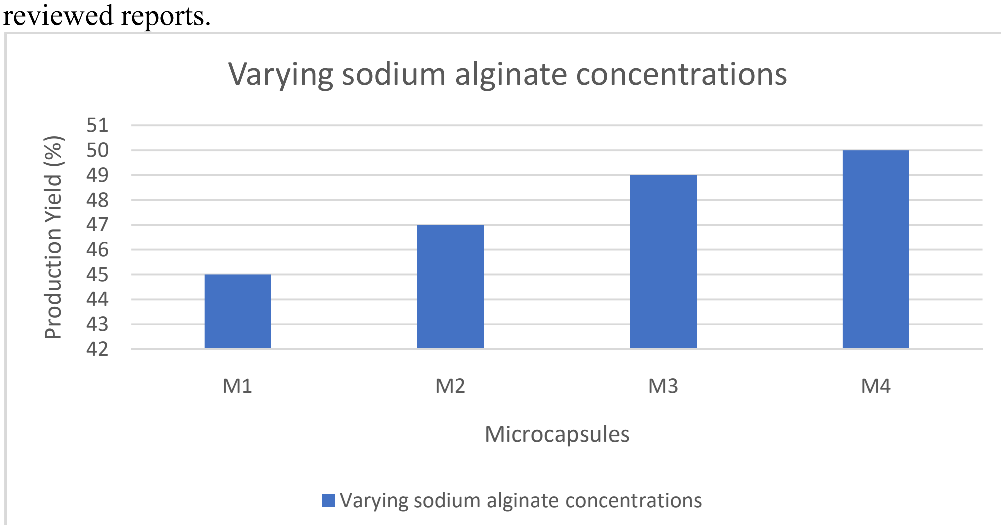
Fig 8: Microspheres with *Lactobacillus*

DETERMINATION OF PRODUCTION YIELD:

The figure below represents the graph of production yield (%) with comparison with 4 different samples obtained by varying the concentrations of the sodium alginate. The four samples are M1, M2, M3, M4 that have concentrations 1.51g ,1.62g, 1.70g, and 1,75g respectively of the sodium alginate concentration among the microcapsule formation. The maximum production was obtained from the M4 synthesis formulation comparatively and obtained results as expected from the reviewed reports.



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