LACTIC ACID FERMENTATION OF PASTEURIZED AND POWDERED MILK AND OPTIMIZING THE FACTORS AFFECTING THE FERMENTATION PROCESS

A PROJECT SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE BACHELORS DEGREE IN MICROBIOLOGY

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DECLARATION

I hereby solemnly declare that the research work embodying the results reported in this project entitled "LACTIC ACID FERMENTATION OF PASTEURIZED AND POWDERED MILK AND OPTIMIZING THE FACTORS AFFECTING THE FERMENTATION PROCESS" submitted by the signatory has been carried out under the supervision of Professor Dr. Naiyyum Choudhury, Coordinator of Biotechnology & Microbiology Program, MNS Department, BRAC University. This project work has been implemented in the Microbiology Laboratory of MNS Department. It is further declared that the project work presented here is original and has not been submitted to any other institution for any degree or diploma.

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~ DEDICATED TO MY PARENTS ~

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ABSTRACT

Yogurt is a basic dairy product that has been consumed for centuries as a part of the diet, even when its beneficial effects were neither fully known nor scientifically proven. With time, yogurt has been continuously modified to obtain a product with better appeal and nutritional effects. In this study, both spontaneous and inoculated fermentation of pasteurized and powdered milk was performed in order to study the quality of the yogurts. In addition to that, bacteria were isolated from both types of yogurts and their microscopic characteristics had been observed under the microscope. Several factors are required to produce a good quality yogurt; such as, temperature, pH, NaCl concentration, liquid and powdered milk concentration etc. These fundamental factors were optimized in such a way that the yogurt was produced in a short incubation period and the efficiency of the fermentation process was enhanced due to optimal conditions. Moreover, the quality and texture of the yogurt remained unchanged up to 5 days.

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LIST OF ABBREVIATION & SYMBOL

SERIAL NO.	TITLE	ABBREVIATION
1	HCl	Hydrochloric Acid
2	NaCl	Sodium Chloride
3	NaOH	Sodium Hydroxide
4	LAB	Lactic Acid Bacteria
5	MRS	de Man Rogosa Sharpe
6	Ca, Na	Calcium, Sodium
7	sp.	Species
8	˚C	Degree Celsius
9	Kg, g, ml	Kilogram, gram, Milliliter
10	*et al*	And friends

INTRODUCTION

1.1 IMPORTANCE OF THIS STUDY

Yogurt (also spelled yoghurt) is a semisolid fermented milk product that originated centuries ago and has evolved from many traditional Eastern European products. It is eaten all over the world. The partial digestion of the milk caused by the fermentation of the starter culture makes yogurt easily digested, even by people who cannot tolerate milk. It is a rich source of protein and calcium, and the fermentation process makes these nutrients easier to absorb.

All yogurts must contain at least 8.25% solids not fat. It can be made from ready-made yogurt or starter culture. The main (starter) cultures in yogurt are *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. The function of the starter cultures is to ferment lactose (milk sugar) to produce lactic acid. The increase in lactic acid decreases pH and causes the milk to clot, or form the soft gel that is characteristic of yogurt. The fermentation of lactose also produces the flavor compounds that are characteristic of yogurt. The acidic condition also restricts the growth of food poisoning pathogens and some spoilage bacteria. So, whereas milk is potential source of food poisoning and only has a shelf life of few days, yogurt is safer and can be kept for up to ten days, under proper storage conditions.

The secret to tasty yogurt is in the proper control of the temperature at various stages. If the temperature is too low, the culture grows too slowly to adequately acidify milk and to achieve a good texture. In addition, there is a subtle difference in the taste because the formation and secretion of metabolites which contribute to the overall taste are dependent on the growth rate. The window of proper fermentation is quite small, i.e. from 37 ºC to 44 ºC. In general, as the temperature is raised up to 44 ºC, the rate of culture metabolism is higher, and the yogurt is sweeter. Faster growth also prompts the yogurt to set faster. When the desired acidity is reached, yogurt is quickly cooled to halt further fermentation and metabolic activity. This cooling step must be done quickly to control the acidity of the yogurt, which has a profound effect on the taste.

Except for *L. bulgaricus* and *S. thermophilus*, other bacterial cultures, such as *Lactobacillus acidophilus*, *Lactobacillus* subsp. *casei*, and Bifido-bacteria may be added to yogurt as probiotic cultures. Probiotic cultures benefit human health by improving lactose digestion, gastrointestinal function, and stimulating the immune system. Ongoing studies are revealing further health benefits to this great food, such as reducing yeast infections and colon cancer.

1.2 OBJECTIVE OF THE PRESENT STUDY

The main objective of this project work is to observe the two different methods of fermentation process; e.g. spontaneous and inoculated fermentation method and studying the necessary factors affecting the fermentation process. There are many aspects which are very crucial for carrying out a fermentation process. Among them temperature, pH, NaCl concentration, liquid and powdered milk concentration are the most basic and fundamental factors. These conditions will be optimized to improve the fermentation of milk and produce a fine quality yogurt with a smooth texture and firm consistency. Therefore, the factors affecting the overall method will be thoroughly studied and optimized in the laboratory to accomplish a enhanced fermentation process.

1.2.1 Specific Objectives

The specific objectives of this study are as follows:

- Spontaneous fermentation method
- Inoculated fermentation method (from commercial yogurt & stock culture of LAB)
- The cell morphology of bacteria
- The effect of temperature
- The effect of pH
- The effect of NaCl concentration
- The effect of powdered milk concentration in liquid milk
- The effect of powdered milk concentration in water, etc.

1.3 RESEARCH HYPOTHESIS

In milk fermentation, lactic acid bacteria produce lactic acid, reduce the pH and therefore coagulate the milk. Desirable strain of lactic acid bacteria (LAB) can be added to the fermentation process. Similarly, live cultures from commercial yogurt sample can also be used as a starter culture for the fermentation process. In order to yield a good quality yogurt, the various factors necessary to carry out fermentation can be adjusted to improve the overall process. If the physiochemical properties; e.g. temperature, pH and concentration of both liquid and powdered milk are increased or decreased, the quality and flavor of the yogurt will be enhanced. Moreover, if the incubation period is reduced into 5-6 hours from 18-24 hours; the bacteria will grow more rapidly and ferment the milk sample in a shorter period of time to produce yogurt.

1.4 SCOPE & LIMITATION OF THIS STUDY

In spontaneous fermentation, bacteriophage may contaminate the milk and alter the quality of yogurt. On the other hand, in inoculated fermentation, undesirable smell and off-flavor may come from the yogurt if the starter cultures are of poor quality or become contaminated. For this reason, fresh, recently purchased culture or freshly grown bacterial culture should be used each time to make yogurt. Adding culture to very hot milk (>115°F) can kill bacteria; this is why thermometer should be used to carefully control the temperature of milk. Too hot or too cold of incubation temperature can slow down culture growth; so the temperature of the incubator must be adjusted beforehand.

Yogurt generally has a 10-21 day shelf life when made and stored properly in the refrigerator below 40°F (4.4˚C). Molds, yeasts and slow-growing bacteria can spoil the yogurt during prolonged storage. Ingredients added to yogurt should be clean and of good quality. Introducing microorganisms from yogurt add-ins can reduce shelf life and result in quicker spoilage. Yogurt samples with visible signs of microbial growth or any odors other than the acidity of fresh yogurt should be discarded straightaway. Whey collects on the surface of the yogurt. This process is called syneresis. Some syneresis is natural. Excessive separation of whey, however, can be caused by incubating yogurt for too long or by agitating the yogurt while it is setting. Moreover, if the yogurt has over-set or incubated too long; it must be refrigerated immediately after a firm coagulum has formed.

1.5 EXPECTED RESULTS

In this study, both spontaneous and inoculated fermentation method of milk fermentation will be performed in the laboratory. Live cultures of lactic acid bacteria (LAB) will be taken and used from both commercial yogurt and stock culture. The major physicochemical properties (temperature, pH, NaCl concentration, powdered milk concentration etc.) affecting the fermentation process will be optimized to enhance the fermentation performance. Additionally, a shorter incubation period for the lactic acid fermentation of pasteurized and powdered milk samples will be determined in order to produce yogurt in a small period of time. The findings of this study are expected to contribute to the production of a good quality yogurt.

CHAPTER: 02

LITERATURE REVIEW

2.1 LACTIC ACID FERMENTATION OF MILK

Yoghurt is a fermented milk product obtained from milk by the lactic acid fermentation through the action of *Streptococcus salivarius* subsp. *thermophiles* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (FAO/WHO, 1977). When a sufficient quantity of lactic acid is produced, the milk coagulates and this coagulated milk is called yogurt. The word "yoghurt" is derived from Turkish "jugurt", used to describe any fermented food with an acidic taste (Younus *et al.*, 2002). Historically, yoghurt was made by fermenting milk with indigenous microorganisms. Yoghurt having high nutritional and therapeutic properties is being highly consumed and produced (Karagul *et al.*, 2004). Yoghurt is stored at 2- 4ºC throughout the distribution chain for avoiding risk of spoilage from yeasts (Tamime *et al.*, 2000) and also for preventing further activity by starter culture. The probiotic yogurt, having probiotic effect is a fermented milk product with adjuvant microorganisms. A high population of probiotic organisms in the colon contributes to good intestinal health. Consequently consumption of products such as yogurt containing viable probiotic organisms adds benefit to human gut health. Moreover, yogurt is an excellent source of calcium, phosphorus, potassium and contains significant quantities of general vitamins. Yoghurt could be used for feeding, owing to its higher Ca/Na ratio (Demott, 1985). Yogurts vary in appearance, flavor and ingredients. The quality and composition of yoghurt of applied bacterial cultures affects the quality of the yoghurt obtained as a result of the milk fermentation processes. There is a symbiotic relationship between the two species of bacteria, *Lactobacillus bulgaricus* and *Streptococcus thermophiles* and so there is more rapid acid development than in single strain culture (Rasic *et al.*, 1978; Tamime *et al.*, 1980). Various combinations of starter cultures are selected during manufacturing of yoghurt to achieve desirable characteristics of product and to provide the consumers with a wide choice of therapeutic benefits. Depending on its activity, manufacturer usually adds 2-4 % yogurt starter culture. Now a days, there has been an increasing trend to fortify the dairy product with fruits (natural fruit juice, pulp, dry fruits) (Desai *et al.*, 1994; Ghadge *et al.*, 2008). Aesthetic value of new product can be increased by using fruit juice as a functional pigment in the fermented milk with an array of colors and flavor properties (Coisson *et al.,* 2005). Euterpeoleracea juice can be used as functional pigment for yoghurt, which is dark purple in color having high anthocynin and phenolic content (Coisson *et al.,* 2005). Yogurt is a functional food. The functional food includes probiotics, prebiotics and synbiotics. Probiotics can be defined as "live microbial feed supplements that beneficially affect the host animal by improving its intestinal microbial balance" (Champagne *et al.*, 2005). The "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon". Symbiotic is a combination of probiotics and prebiotics that "beneficially affects the host by improving the survival and the implantation of live microbial dietary supplements in the gastro-intestinal tract by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health promoting bacteria" (DiRienzo *et al.*, 2000).

Yogurt provides two significant barriers against pathogen growth; e.g. heat and acidity (low pH). Both are necessary to ensure a safe product. Acidity alone has been questioned by recent outbreaks of food poisoning by *E. coli* O157:H7 that is acid-tolerant. *E. coli* O157:H7 is easily destroyed by pasteurization (heating). Therefore, milk sample must be boiled before making yogurt at home or commercially pasteurized milk should be used to make yogurt.

2.2 CLASSIFICATION YOGHURT

There are different types of yoghurt produced worldwide. However particular yogurt may be subdivided into different groups based on the following aspects:-

A. Set Yogurt

This type of yoghurt is incubated and cooled in the final package and is characterized by a firm jelly like texture. Set yoghurt is fermented in a retail container, filled after milk inoculation and is incubated in an incubation room at a suitable temperature normally 40- 43°C for approximately 2 hours and 30 minutes to 4 hours (Desai *et al.*, 1994).

B. Stirred Yogurt

This type of yoghurt is incubated in a tank and the final coagulum is "broken" by stirring before cooling and packing. The texture of stirred yoghurt will be less firm than a set yoghurt somewhat like a very thick cream. A little reformation of coagulum will occur after packaging. Stirred yogurt is a non-Newtonian fluid, obtained by promoting the growth of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* at a mild temperature (between 40°C and 43°C) until a desired acidity level is reached. In stirred yoghurt, milk is inoculated and incubated in a fermentation tank, the yoghurt gel being broken up during the stirring, cooling and packaging stages. Due to several factors there may be variations in the rheological properties of stirred yoghurt. These can be of a physical nature such as those related with total solid content, milk composition and type of starter culture or processing conditions; such as, homogenization, thermal pre-treatment of the milk and post-incubation stages (including: stirring, pumping, cooling and packaging) (Tamime *et al.*, 1980).

C. Drinking Yoghurt

It also has the coagulum "broken" prior to cooling. In drinking yoghurt, the agitation used to break the coagulum is severe. Very little reformation of coagulum may occur.

D. Frozen Yoghurt

Frozen yoghurt is inoculated and incubated in the same manner as stirred yoghurt. However, cooling is achieved by pumping through a chiller or freezer in a fashion similar to ice-cream. The texture of the finished product is mainly influenced by the freezer and the size and distribution of the ice crystals are produced.

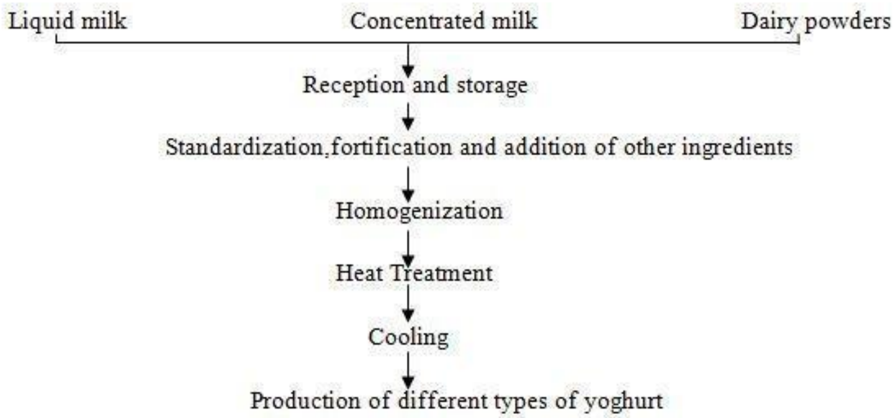
E. Concentrated Yoghurt

This type of yoghurt is inoculated and fermented in the same manner as stirred yoghurt. Following the breaking of the coagulum, the yoghurt is concentrated by boiling off some of the water, which is often done under vacuum to reduce the temperature required. Heating of low pH yoghurt can often lead to protein being totally denatured and producing rough and gritty textures. This is often called strained yoghurt due to the fact that the liquid that is released from the coagulum upon heating used to strain-off in a manner similar to making soft cheese.

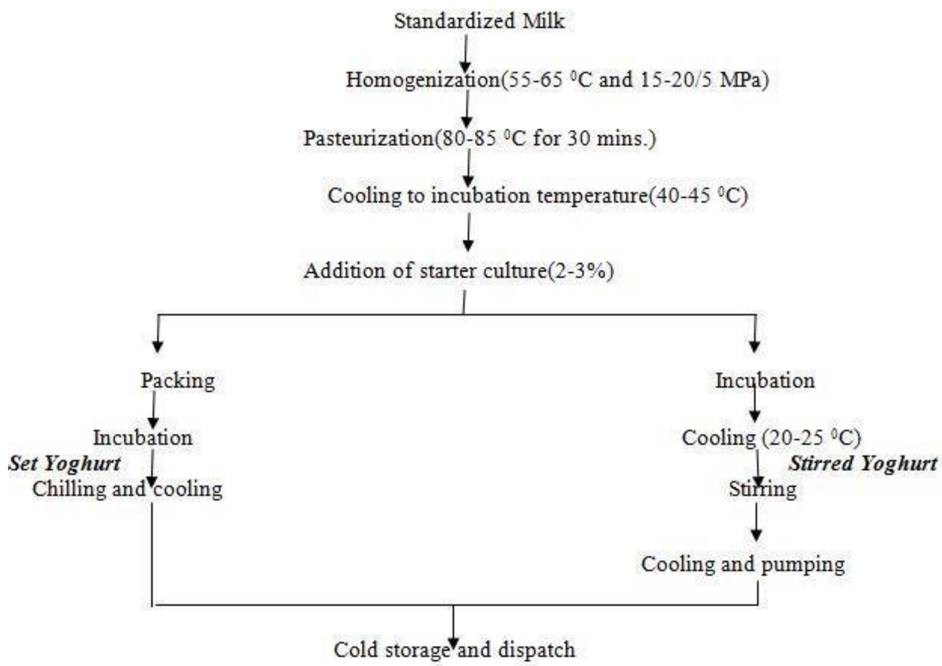
F. Flavored Yoghurt

The flavors are usually added at or just prior to filling into pots. Common additives are fruit or berries, usually as a puree or as whole fruit in syrup. These additives often have as much as 50% sugar in them; however, with the trend towards healthy eating gaining momentum; many manufacturers offer a low sugar and low fat version of their products. Low or no sugar yoghurts are often sweetened with saccharin or more commonly with aspartame.

2.3 PRODUCTION OF DIFFERENT TYPES OF YOGHURT



Flowchart: Showing Processing of Yoghurt



Flowchart: Showing Processing of Set and Stirred Yoghurt (W.J Lee and J.A Lucey, 2010)

2.4 HEALTH BENEFITS OF YOGHURT

- Probiotic yoghurt is aimed at reducing medical conditions; such as constipation and diarrhea by restoring the beneficial microbial population in the colon.
- It is beneficial to our digestive system, especially stomach and colon. Cow's milk is preferred for preparing yoghurt as having low fat.
- It provides immunity; protect us from cold, cough and strengthen body's defense mechanism.
- It strengthens the collagen in the skin and is good for our skin.
- It lowers the blood pressure, bad cholesterol and risk of heart attacks.
- Yoghurt is a source of natural proteins; it is safer for those having problem in tolerance of lactose. Yoghurt is rich in calcium; so it protects the bones against osteoporosis and arthritis.
- It discourages vaginal yeast infections.
- It helps in cutting down calorie and thus helps in burning fat.
- By daily consumption of yoghurt, disease causing bacteria are flushed out from the colon and therefore, help in protecting against colon cancer.
- Consumption of yoghurt can shut down *Helicobater pylori*; the bacterium responsible for most peptic ulcers.

2.5 COMPOSITION AND NUTRITIONAL FACTS OF MILK

Nutrients in milk can differ depending on the age, breed of cattle or goat, the season of the year and the food it is fed or grass the animal grazes. On average, milk has 4% fat, 3.4% protein and 4.8% milk sugar (lactose) and provides approximately 730 kcal in energy per liter. As such, a serving of 250 ml glass would therefore provide 180 kcal, which is 10% of an under five year old child's energy needed for a day and 6% of a symptomatic HIV positive adult's energy needed. Milk also provides valuable vitamins; e.g. vitamin A (for eyes, skin, hair, mucous membranes and immunity), vitamin B's (important for metabolism of carbohydrates in foods to produce energy) and vitamin D (important for strong bones and teeth), as well as minerals, calcium and phosphorus; both of which are important for strong bones.
(Source: http://www.fao.org/docrep/v1650t/v1650T0s)

Table A: Showing Health benefits when milk is fermented

Effect of fermentation	**Changes in milk**	**Effects on health**
Increase in lactic acid bacteria (LAB) population	▪ Reduced lactose content in milk ▪ Reduced number of pathogenic bacteria	▪ No diarrhea and bloating ▪ Improved gut health ▪ Prevention and protection from bacterial vaginitis and fungal infections in women ▪ Ability to digest remaining lactose in the fermented milk and use as an energy source
Breakdown to short chain peptides	Identification of casein peptides and whey peptides with functional properties	▪ Easier digestion ▪ Some with antihypertensive effects ▪ Some with pain relief effects ▪ Some with immune enhancing properties ▪ Some with calcium binding bone building properties
Increased acidity	Sharpness in taste	Acidity prevents pathogenic bacterial growth in milk

*Reference for changes in energy requirements in HIV: - http://whqlibdoc.who.int/publications/2003/9241591196.pdf

*Reference for energy requirements in the lifecycle: ftp://ftp.fao.org/docrep/fao/007/y5686e/y5686e00.pdf

2.6 MICROBIOLOGY OF YOGHURT STARTER CULTURES

There is a symbiotic relationship between the two species of bacteria i.e. *Lactobacillus bulgaricus* and *Streptococcus thermophiles* that's why there is more rapid acid development than in the single strain culture (Rasic *et al.*, 1978; Tamime *et al.*, 1980).

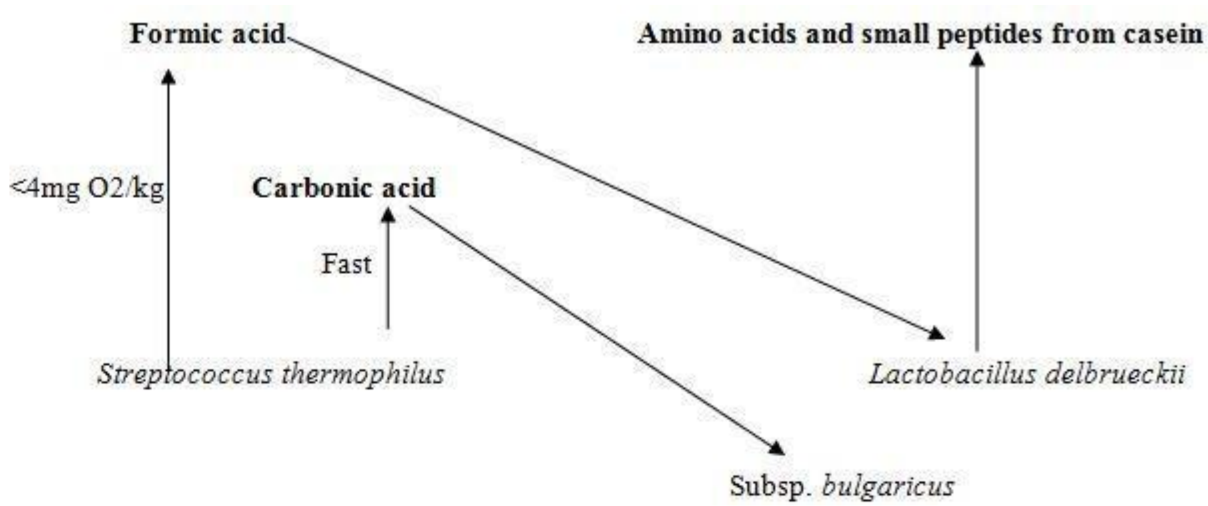


Figure: Showing symbiosis between *Streptococcus thermophilus* and *Lactobacillus delbrueckii* Subsp. *bulgaricus.*

As shown in the above figure, *Streptococcus thermophilus* produces lactic acid and small quantities of formic acid, which promotes outgrowth of *Lactobacillus delbrueckii* Subsp. *bulgaricus.* On the other hand, *Lactobacillus delbrueckii* subsp. *bulgaricus* species produce amino acids to stimulate the growth of *Streptococcus thermophilus* Chemical composition of the milk base (total solids and fat content) have significant effects on the activity of starter cultures. Ozer and his co-worker have studied the behavior of starter cultures in concentrated yoghurt produced by different methods. They discovered that production method and total solid content in milk that influenced the growth and activity of starter cultures (Ozer and Robinson, 1999).

2.7 CHARACTERISTICS & IDENTIFICATION OF LACTIC ACID BACTERIA (LAB)

(A) *Lactobacillus delbrueckii* subsp. *bulgaricus*

It is a gram-positive rod shaped filamentous, non-motile, non-spore forming bacteria. It requires a low pH (5.4 to 4.6) to grow effectively so the bacterium is regarded as acidouric or acidophilic. The bacterium has complex nutritional requirements including the inability to ferment any sugar except lactose, from which it produces lactic acid that helps to preserve yoghurt. It is often helpful to sufferers of lactose intolerance whose digestive systems lack the enzymes to break down lactose to simpler sugars. While fermenting milk, it produces acetaldehyde, which is one of the main yoghurt aroma components.

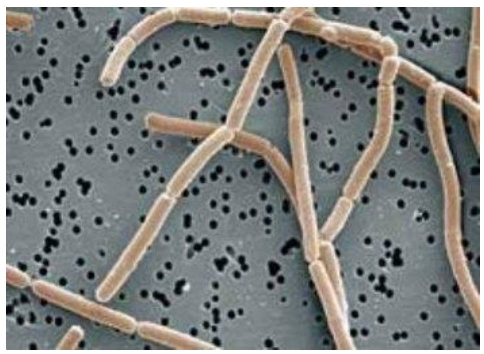


Figure: Microscopic observation of bacterial cells (source: www.bacterianamehere.pbworks.com)

(B) *Streptococcus thermophilus*

The genus *Streptococcus* comprises several harmful pathogenic species such as *Streptococcus pyogenes*or, *Streptococcus pneumoniae*, together with a single, “Generally Recognized As Safe species”- *S. thermophiles* and *S. thermophilus* are widely used for the manufacture of dairy products (Cvetan, 1998; Fox, 1993).

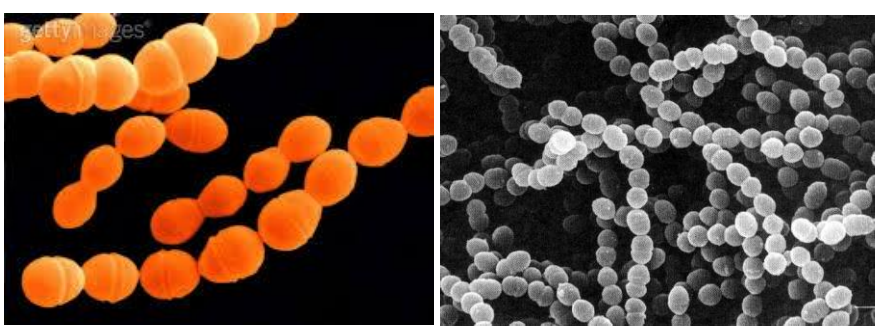


Figure: Microscopic observation of bacterial cells

(Image source: http://www2.unibas.it/parente/Starter/gruppi.html)

(C) *Lactobacillus acidophilus*

Lactobacillus acidophilus shows a range of health benefits which include: providing immune support for infections and cancer, reducing occurrence of diarrhea in human, aiding in lowering cholesterol, improving the symptoms of lactose intolerance. Studies have shown that dietary supplementation with *L. acidophilus* decrease the number of colon cancer cells in a dose dependent manner (Rao, 1999). The better survival of *L. acidophilus* when added along with the starter was presumably due to the transient tolerance of the culture to hydrogen peroxide (Speck, 1976).

Figure: Microscopic observation of bacterial cells

(D) *Bifidobacterium bifidium*

Bifidobacteria are one of the most important group of intestinal organisms with regard to human health.*Bifidobacterium* spp. belongs to the dominant anaerobic flora of the colon. The main species present in human colon are *Bifidobacteriumadolescentis, B. bifidum, B. longum*subsp.*infantis, B. breve*and *B. longum* (Holzapfel, 2001). *B. bifidum* was found to be tolerant to the acidity of a model gastrointestinal tract system, with only a 20 % decrease in numbers as the pH decreased from 5.0 to 1.8 over an 80 min period (Holzapfel, 2001). Important property of probiotic bifidobacteria is acid tolerance, enabling the cells to survive gastric acidity and volatile fatty acids produced during fermentation in the intestine (Charteris, 1998). In the presence of yoghurt starter organisms the growth of bifidobacteria seems to be suppressed. Bifidobacteria have a higher resistance to acid and bile present in the gastrointestinal tract than yoghurt bacteria. The ingestion of bifidus fermented milk led to an increase in total bifidobacteria which was related to the colonic transit of the exogenous bifidobacteria (Bouhnik, 1996). In yoghurt like product, addition of probiotic cultures to the normal starters generally results in slower growth of the probiotic strains than if they were added alone in milk (Roy, 1996;

Samona, 1994). These starters produce environments that inhibit the growth of not only pathogens and spoilage microorganisms but also of probiotic (Vinderola, 2002). The phenomenon could partially be related to the production of bacteriocins or other inhibitors such as lactic and other organic acids and hydrogen peroxide produced by the starter cultures (Maus, 2003; Vinderola, 2000). In addition, starter cultures grow faster, acidification occurs rapidly, and fermentation times are much shorter in their presence which resulted in reduced availability of nutrients (Shah, 2000); thus probiotic cultures do not have time to grow extensively. Many authors also reported the capacity of bifidobacteria to synthesize galacto-oligosaccharides (Hung and Lee, 2002; Lamoureux, 2002; Saxelin, 1999).

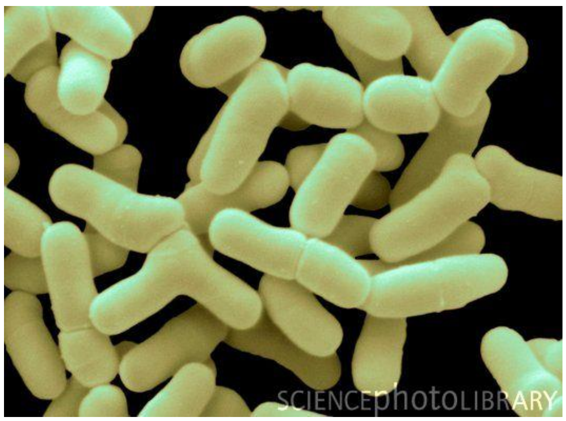


Figure: Microscopic observation of bacterial cells
(Image source: http://barefootprovisions.com)

2.8 FACTORS AFFECTING FERMENTATION OF MILK

There is several factors necessary milk fermentation process. Some of them are described below:

2.8.1 The Effect of pH on Yogurt

The ideal pH of the finished product of yogurt should be 4 to 4.1. However this pH also depends on adding fruits or flavoring agent to the yogurt. After fermentation when the yogurt is ready to eat, the pH should be 4. The pH of yoghurt is decreased if the amount of skimmed milk powder is increased. The yogurt mixture coagulates during fermentation due to the drop in pH. The streptococci are responsible for the initial pH drop of the yogurt into approximately 5.0. The *Lactobacillus* species are responsible for further decrease in pH into 4.5.

Shaker *et al* studied the effect of milk fat content on the acid development during fermentation and rheological properties of plain yoghurt. They indicated that increasing milk fat content increased the initial pH of the samples and the rate of decreasing pH during incubation of high fat samples was lower than others (Shaker *et al*, 2000).

A. Change in pH from 6.6 to 6.0

When the pH of the milk is decreased from 6.6 to 6.0 then the electrostatic repulsion decreases; as the net negative charge on the casein micelles decreases. The size of the casein micelles remains unchanged as the small amount of colloidal calcium phosphate is solubilized at pH > 6.0

B. Change in pH from 6.0 to 5.0

When the pH changes from 6.0 to 5.0 then there is decrease in electrostatic repulsion and steric stabilization occurs which are responsible for the stability of casein micelles in the original milk. Due to the decrease in the pH, the negative charge on casein micelles decreases and the charged tails of casein curls up or may shrink.

C. At pH 6.0

At this pH, the electrostatic repulsion between the exposed phosphoseriene residues increases due to the increase in the rate of solubilization of colloidal calcium phosphate, which weakens the internal structure of casein micelles.

D. At pH 5.0

The decrease in electrostatic repulsion between casein molecules occurs at pH 5; as the net negative charge on the casein decreases. When the pH of milk becomes close to the isoelectric point (pH 4.6) then casein-casein attractions is amplified due to increased hydrophobic and electrostatic charge interactions (Horne, 1998). The three dimensional network is formed consisting of clusters and chains of caseins due to the acidification process (Mulvihill and Grufferty, 1995).

2.8.2 The Effect of Rheological Property of Yogurt

Food rheology is the study of the deformation and flow of food materials (Rao, 1999). It can be classified as a pseudoplastic material (contains a yield stress that has to be exceeded for flow to be initiated) that can be either a viscoelastic fluid (stirred yoghurt) or viscoelastic solid (set yoghurt). Viscoelastic indicates that the material is having some of the elastic properties of an ideal solid and some of the flow properties of an ideal (viscous) liquid. Yogurt exhibits time-dependent shear thinning behavior but is not a true thixotropic material, since structural breakdown due to shear is not completely reversible once the shear stops.

2.8.3 The Effect of Physical Properties of Yogurt

It has been found that addition of protein (i) improves water holding capacity (ii) decreases the syneresis effect and (iii) stabilizes the pH of the samples during storage (Athanasios G. Stefanakis *et al*). The physical and sensory properties of yoghurt gels are highly influenced by the protein content and the total solids content of the yoghurt milk. A study on the effect of milk supplementation (whey, casein hydrolysate and milk protein) on the acidification and microbiological stability of fermented milks showed that acidifying activity was greatly improved with casein hydrolysate, with a reduction of the fermentation time by 55% in comparison with the other supplementation (Oliveria *et al.*, 2001).

Abd-El Salam *et al.,* (1982) showed that total solids content had no adverse effect on starter activity or coagulation time. Increasing milk total solids from 16 to 23% had significant effect on decreasing rate of pH during fermentation (Ozer *et al.*, 1998). The incubation time for the milk at pH 4.6 was shorter than the time of retentive (Tamime *et al.*, 1989). The increase in milk fat content influences the growth and activity of starter cultures in samples with two levels of total solid (12 and 23%) (Mahdian *et al*., 2007). The chemical composition of the milk base especially total solids has the major effect on the acceptability of concentrated yoghurt. Concentrated yoghurt containing less than 20% total solid was assessed as thin and tasteless whereas, greater than 25% total solid became gummy and bitter (Robinson, 1977).

2.9 CURRENT STATUS OF YOGURT PRODUCTION IN BANGLADESH

In Bangladesh, curd (Dadhi) is one of the most popular fermented milk products which are made in mud pots, usually from cow's milk, sometimes from buffalo milk. The bacteria used are *Lactobacillus bulgaricus*, *Lactobacillus plantarum* and *Streptococcus thermophiles* and/or *Lactotococcus lactis*. In making curd, the inoculum is higher than is usual for other fermented milk products (approximately 5%-8%). The traditional fermented dahi is prepared without any care of quality control and hygienic conditions and contain a lot of contaminants which may not be friendly to the body; it still enjoys loyal following in rural communities in Bangladesh and a must dessert after an improved heavy meal. In addition to poor quality control and hygiene during manufacturing, dahi are sold almost in every open market and products are kept on shelf at ambient temperature without any covering while very few retailers keep their product in refrigeration. Therefore, periodical inspection on the quality of the product in necessary, then again is not in practice. Very few researchers are conducting the study of market dahi quality on region basis and considering the investigation upon renowned brand dahi of Bogra, Muslim, Bonoful, Aarong and Grameen Soktee* to check and compare their quality with the dahi prepared from fresh raw milk by following standard procedures (Bhattahacharya, K.P., 1980).

2.10 CURRENT STATUS OF YOGURT PRODUCTION IN THE WORLD

Alternative methods to improve quality of low-fat yoghurt become an area of considerable research interest. Yoghurts that have past their 'best before' date constitute a waste that has to be environmentally treated. It can be used as a source for lactic acid production by *Lactobacillus casei*. Production of yoghurt generates a residue highly supplemented with sugar and fruits syrups that can be metabolized to lactic acid. Rheological properties were not seriously affected by freezing, while the drying step resulted in an overall structural weakening of the reconstituted products, possibly as a consequence of the mechanical energy required for mixing with water. However, its viscoelastic properties were retained and the original strength could be recovered by modulating the amount of water. Mortality levels of bacterial population were reduced when sucrose and blueberries were added as ingredients. Addition of herbs or their active components like oils could be an effective strategy to improve functionality of milk and milk products with respect to the health benefits, food safety and bio preservation. These components should improve therapeutic quality without any adverse effect on sensory and rheological attributes.

CHAPTER: 03

MATERIALS & METHODS

3.1 MATERIALS

- Aarong Full-cream Pasteurized Milk (1 liter)
- Milk Vita Pasteurized Milk (1 liter)
- Dano Milk Powder (1000 g)
- Ultra Doi
- Shakti Doi
- Bacteria Stock Culture
- *Lactobacillus* MRS agar
- *Lactobacillus* MRS Broth
- Distilled water

3.2 REAGENTS & CHEMICALS

Name of the reagents	
✓ 0.85% Normal saline	✓ Crystal Violet
✓ 1 M NaOH	✓ Gram's Iodine
✓ 32% HCl	✓ Safranin
✓ Ethanol	✓ NaCl

3.3 INSTRUMENTS

Name of the instruments	
✓ Laminer Airflow	✓ Vortex mixture
✓ Compound Microscope	✓ Refrigerator
✓ Autoclave	✓ Balance machine
✓ Incubator	✓ Spirit lamp

3.4 SPONTANEOUS FERMENTATION METHOD

200 ml Milk Vita pasteurized milk was taken in a 250 ml beaker and boiled. Then the milk was cooled to room temperature and 100 ml milk was added to two separate plastic containers. No inoculum or bacterial culture was added into the containers. After that, the containers were incubated at 36˚C for 20 hours.

Smears were prepared on glass slides taking loopful of yogurt from each of the spontaneously fermented milk containers and Grams' staining was performed. The microscopic characteristics of the presumptive organisms were observed under a compound microscope at 100X magnification.

3.5 INOCULATED FERMENTATION METHOD

In inoculated fermentation method, live culture of bacteria was used to ferment milk.

- 100 ml "Milk Vita" milk was boiled and cooled to room temperature. 1 teaspoon of yogurt from the spontaneously fermented milk container was added into milk poured in the plastic containers. They were incubated at 37˚C for 18 hours.
- 200 ml milk was boiled and cooled to room temperature. 1 teaspoon of yogurt from "Shakti Doi" and 1 teaspoon of yogurt from "Ultra Doi" was taken and added into 100 ml milk poured into each of the plastic containers. They were incubated at 45˚C for 22 hours.

Inoculum was taken separately from these set of experiments and inoculated into fresh "Milk Vita" pasteurized milk. Likewise, this process was done three times by taking 1 tsp. of yogurt from each batch and inoculated into fresh "Milk Vita" pasteurized milk separately, until pure culture was obtained from the yogurts. In addition, the incubation period was shortened in each fermentation batch to an optimum period of time (from 22 hours to 5 hours). Besides the fermentation process, the microscopic characteristics of the presumptive organisms present in the fermented yogurts were also observed in parallels.

3.5.1 Reviving Bacterial Culture

Five different bacterial strains were taken which were named S1, S2, LP, Aarong, LAB and streak plated on to MRS agar medium and incubated at 37˚C for 48 hours. Isolated colonies were transferred to MRS broth and incubated at 37˚C for 48 hours. Since the bacterial growth was slow, the plates and tubes were kept more than 24 hours in the incubator.

3.5.1.1 Inoculated fermentation using Bacterial Stock Culture

A loopful of bacterial colony from MRS agar plate and 10 ml culture from MRS broth tube were added to 100 ml "Milk Vita" pasteurized milk (after boiling and cooling) in each container and stirred well. The containers were incubated at 37˚C for 24 hours. After 24 hours, the physical appearances of the yogurts were observed and recorded in a table.

3.6 SELECTION OF OPTIMUM CONDITION FOR THE FERMENTATION PROCESS

For selecting the optimum condition for lactic acid fermentation of milk several factors are associated. The effect of temperature, pH, NaCl concentration, powdered milk concentration in water and liquid milk were conducted to determine an optimum condition for the fermentation process in order to produce a good quality yogurt.

3.6.1 Effect of temperature

To optimize the fermentation temperature, fermentation was carried out at 45˚C, 55˚C and 60˚C. All other conditions were kept constant. The initial pH of the milk was measured 6.5 after calibrating the pH meter with pH 7.0. 100 ml Aarong full-cream pasteurized milk was taken in each container and a thermometer was used to carefully control the temperature of milk before adding the inoculum.

3.6.2 Effect of pH

To study the effect of pH on lactic acid fermentation of pasteurized milk, the fermentation process was carried out at pH 4.5, 5.5, 6.5 and 8.5 in a volume of 100 ml Aarong full-cream milk in each container to find out the optimum pH. While there were variations in pH, all other conditions were held constant. The initial pH of the milk was measured 6.5 after calibrating the pH meter with pH 7.0. 32% HCl was added drop by drop to reduce the pH into 4.5 and 5.5 respectively and 1 M NaOH was used to increase the pH to 8.5. 1 tsp. of inoculum was added to each milk containers. The containers were incubated at 37˚C for 5 hours. The quality and texture of the yogurts as well as their final pH were recorded in a table.

3.6.3 Effect of NaCl concentration

To study the effect of NaCl concentration for yogurt production, the fermentation process was carried out with an initial pH 6.5 of Aarong full-cream pasteurized milk. At first, the 300 ml milk was measured by a measuring cylinder and then boiled. After cooling, 100 ml milk was taken into three separate plastic containers. To find out the optimum concentration of NaCl, three different concentrations of NaCl (0.1 %, 0.5 % and 1.0%) were prepared and added to the milk containers. 1 tsp. of inoculum was added to each container and they were incubated at 37˚C for 5 hours. While there were variations in NaCl concentrations, all other conditions (milk volume, inoculum size, pH & temperature) were kept constant. After 5 hours of incubation, the quality and texture of the yogurts at different NaCl concentrations were observed and recorded in a table.

After determining the optimum concentration of NaCl in yogurt production, 1 tsp. was taken from the corresponding yogurt container and transferred to five containers having 100 ml freshly boiled Aarong full-cream pasteurized milk. All of them contained 0.1 % NaCl concentration and they were incubated at 37˚C for 5 hours. The changes in pH of one of the five containers were measured at 1-hour intervals up to 5 hours. The quality and texture of the yogurts at different NaCl concentrations were observed and recorded in a table.

Furthermore, smears were prepared on glass slides taking loopful of the watery portion of yogurt from each of the five containers and Grams' staining was performed. The glass slides were placed under a compound microscope and the microscopic characteristics of the presumptive organisms were observed under 100X magnification.

3.6.4 Effect of Powdered Milk concentration in Liquid Milk

To study the effect of powdered milk concentration in yogurt production, the fermentation process was carried out with an initial pH 6.5 of Aarong full-cream pasteurized milk. At first, the 500 ml milk was measured by a measuring cylinder and then boiled. After cooling, 100 ml milk was taken into five separate plastic containers. To find out the optimum concentration of powdered milk, five different concentrations of Dano powdered milk (2%, 4%, 6%, 8% and 10%) were prepared and added to the containers having liquid milk and dissolved properly. 1 tsp. of inoculum was added to each container and they were incubated at 37˚C for 5 hours and 30 minutes. While there were variations among powdered milk concentrations, all other conditions (milk volume, inoculum size, pH & temperature) were kept constant. After incubation period, the quality and texture of the yogurts at different concentrations of powdered milk were observed and recorded in a table.

3.6.5 Effect of Powdered Milk concentration in Water

To study the effect of powdered milk concentration in yogurt production, the fermentation process was carried out using distilled water. At first, the 500 ml distilled water was measured by a measuring cylinder and then boiled. After cooling, 100 ml water was taken into five separate plastic containers. To find out the optimum concentration of powdered milk, five different concentrations of Dano powdered milk (2%, 4%, 6%, 8% and 10%) were prepared and added to the containers having distilled water and dissolved properly. 1 tsp. of inoculum was added to each container and they were incubated at 37˚C for 5 hours and 30 minutes. While there were variations among powdered milk concentrations, all other conditions (milk volume, inoculum size, pH & temperature) were kept constant. After incubation period, the quality and texture of the yogurts at different concentrations of powdered milk in distilled water were observed & recorded in a table.

CHAPTER: 04

RESULTS

4.1 SPONTANEOUS FERMENTATION

Observation & result:-

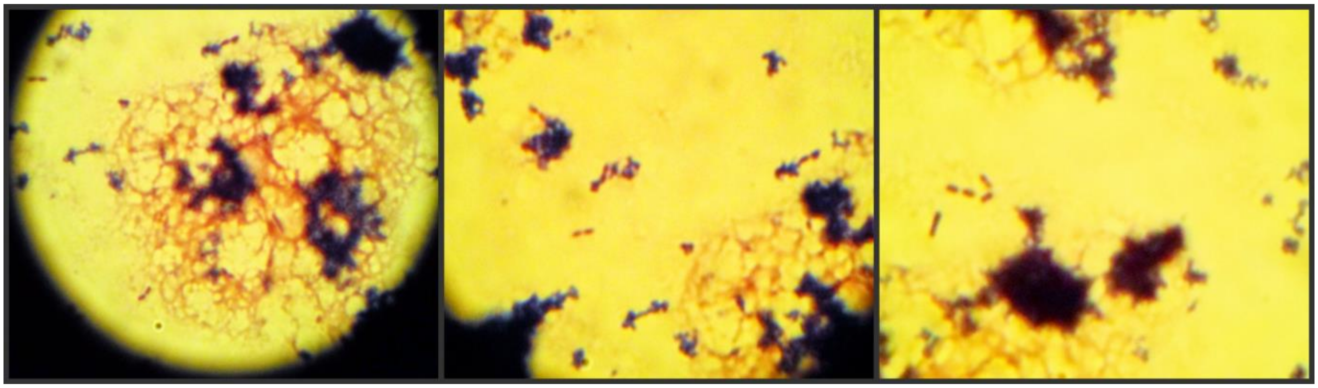
After 20 hours of incubation period, the yogurts were observed and their physical characteristics were recorded in the following table:

Figure 1: Showing yogurts made by spontaneous fermentation

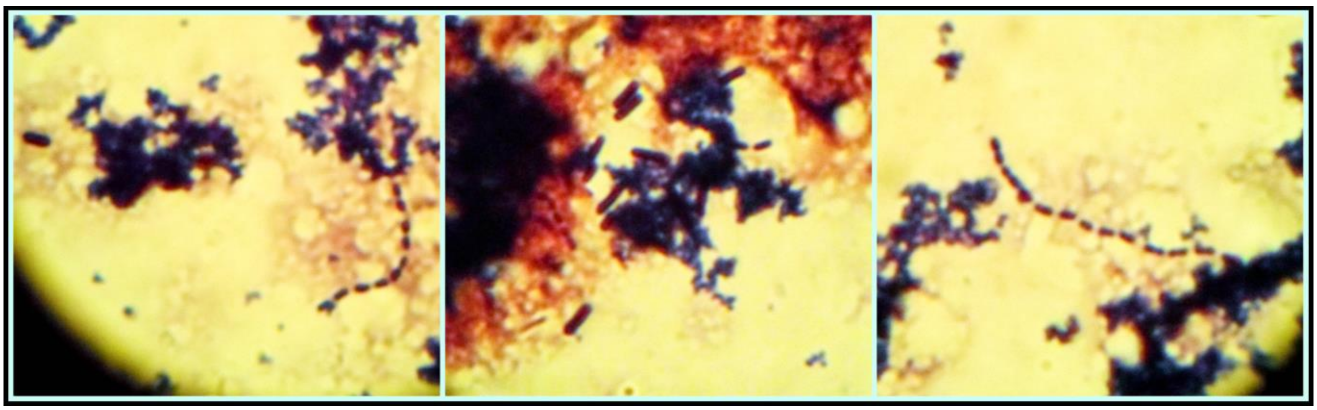
Table 1.1: Characteristics of yogurts made by spontaneous fermentation

Sample name	Color	Aroma	Texture	Consistency	Presence of water
Red container	Off-white	Slightly acidic	Smooth	Firm, thick	+
Yellow container	White	Pleasant smell	Smooth	Firm, thick	Absent

Microscopic Characteristics:-



(a)



(b)

Figure 2 (a & b): The cell morphology observed under a compound microscope

Table 1.2: Tabular presentation of microscopic characteristics of the cells

Isolates	Shape	Arrangement	Grams reaction	Presumptive organism
Isolate no.1 (figure a)	Diplococci	Pairs	Positive	*Streptococcus thermopilus*
Isolate no.2 (figure b)	Diplococci	Long chain	Positive	*Lactococcus lactis*
Isolate no.3 (figure b)	Rod shaped	Single	Positive	*Lactobacillus bulgaricus*

4.2 INOCULATED FERMENTATION

4.2.1 Observation & result:-

After 22 hours of incubation period, the yogurts were observed and their physical appearances were recorded in the following table:

Figure 3: showing yogurts inoculated from Shakti & Ultra Doi

Table 2.1: Characteristics of yogurts made by inoculated fermentation

Inoculated from	Color	Aroma	Texture	Consistency	Presence of water
Shakti doi	Off-white	Pleasant smell	Smooth	Firm	++
Ultra doi	Off-white	Pleasant smell	Smooth	Loose, broken	+++

4.2.1.1 Observation & result:-

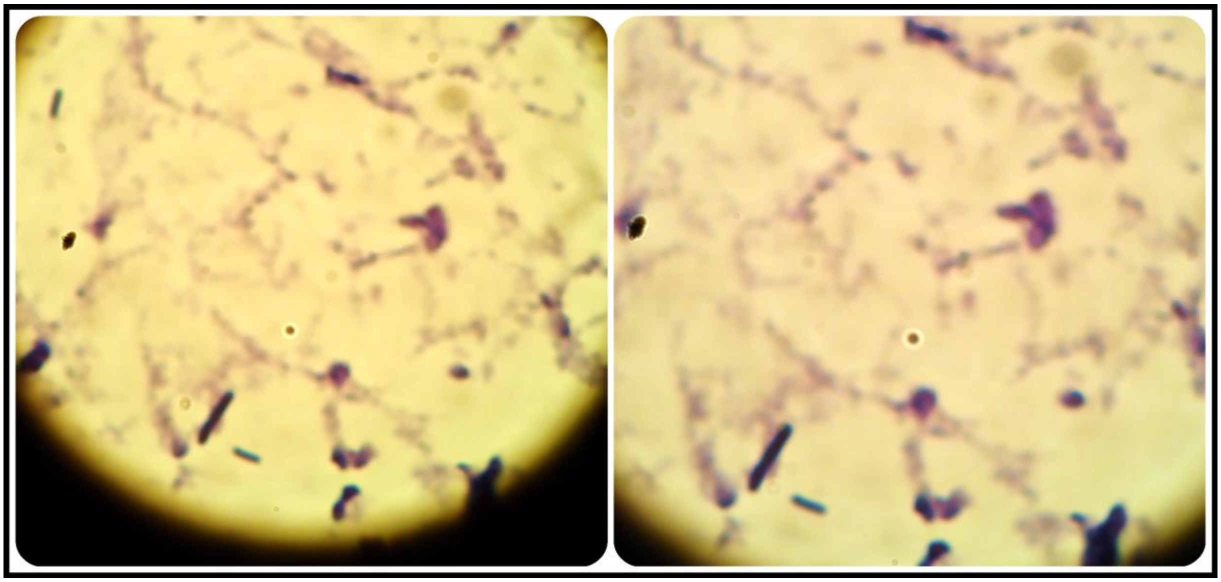
After 18 hours of incubation, the yogurts were observed and their physical appearances were recorded in the following table:

Figure 4: Sub-cultured from spontaneously fermented yogurt (fig.1) & Ultra doi (fig.2)

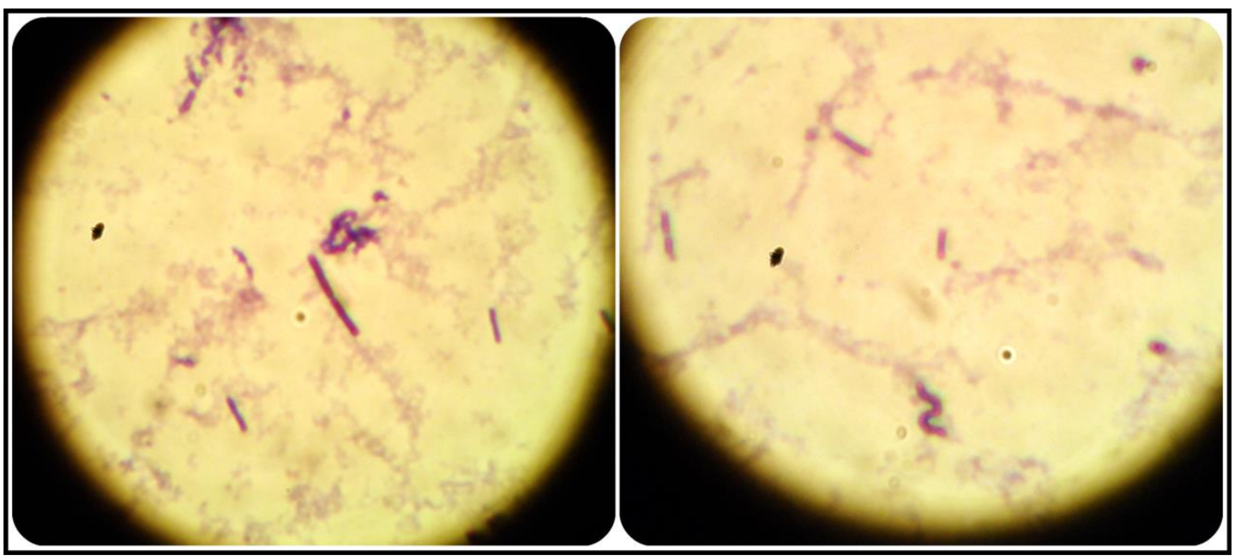
Table 2.2: Characteristics of yogurts made by inoculated fermentation

Inoculated from	Color	Aroma	Texture	Consistency	Presence of water
Spontaneously fermented	Off-white	Slightly acidic	Rough	Thin	++++
Ultra doi container	White	Pleasant smell	Smooth	Firm, thick	+

<u>Microscopic Characteristics:-</u>



(a) organism from spontaneously fermented yogurt



(b) organism from ultra doi container

Figure 5 (a & b): The cell morphology observed under a compound microscope

Table 2.3: Tabular presentation of microscopic characteristics of the cells

Sample name	Shape	Arrangement	Grams reaction	Presumptive organism
Spontaneously fermented	Long and short rods	Single	Positive	*Lactobacillus delbrueckii* subsp. *bulgaricus*
Ultra doi container	Rods	Single, pairs	Positive	*Lactobacillus delbrueckii* subsp. *bulgaricus*
Ultra doi container	Spiral	Single	Positive	*Lactobacillus delbrueckii* subsp. *bulgaricus*

4.2.1.2 Observation & result:-

After 6 hours of incubation, the yogurts were observed and their physical appearances were recorded in the following table:

Figure 6: showing continuous fermentation process

Table 2.4: Characteristics of yogurts made by inoculated fermentation

Inoculated from	Color	Aroma	Texture	Consistency	Presence of water
Green container	Off-white	Pleasant smell	Smooth	Semi-solid	++
Yellow container	White	Slightly acidic	Smooth	Firm, thick	Absent

Microscopic Characteristics:-

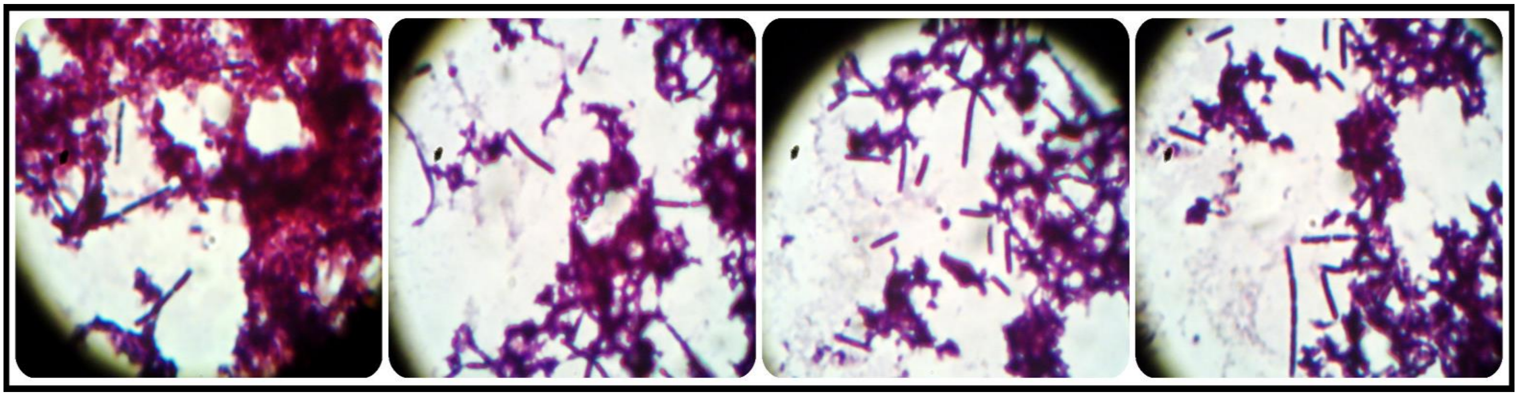


Figure 7: The cell morphology observed under a compound microscope

Table 2.5: Tabular presentation of microscopic characteristics of the cells

Sample name	**Shape**	**Arrangement**	**Grams reaction**	**Presumptive organism**
Green container	Rods	Short chains	Positive	*Lactobacillus delbrueckii* subsp. *bulgaricus*
Yellow container	Rods	Long chains	Positive	*Lactobacillus delbrueckii* subsp. *bulgaricus*

4.2.1.3 Observation & result:-

After 5 and 6 hours of separate incubation periods, the yogurts were observed and their physical appearances were recorded in the following table:

Figure 8: showing yogurts after 5 and 6 hours of incubation period at 37˚C

Table 2.6: Characteristics of yogurts made by inoculated fermentation

Inoculated from	**Color**	**Aroma**	**Texture**	**Consistency**	**Final pH**	**Presence of water**
Set A (5 hrs)	White	Slightly acidic	Smooth	Firm, thick	6.3	+
Set B (6 hrs)	Off-white	Acidic smell	Grainy	Firm	6.2	++

Microscopic Characteristics:-

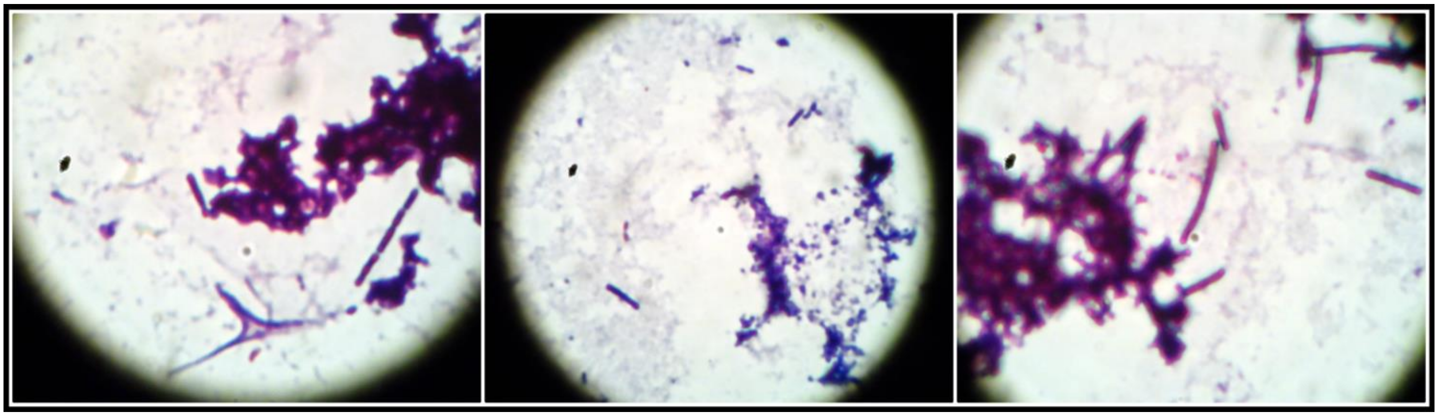


Figure 9: The cell morphology observed under a compound microscope

Table 2.7: Tabular presentation of microscopic characteristics of the cells

Sample name	**Shape**	**Arrangement**	**Grams reaction**	**Presumptive organism**
Set A (5 hrs)	Rods	Pairs, short chains	Positive	*Lactobacillus delbrueckii* subsp. *bulgaricus*
Set B (6 hrs)	Rods	Dispersed	Positive	*Lactobacillus delbrueckii* subsp. *bulgaricus*

4.2.B Inoculated fermentation using MRS Agar plate & MRS Broth Culture

Observation & result:-

After 24 hours of incubation, the yogurts were observed and their physical appearances were recorded in the following table:

Figure 10: Inoculated fermentation of milk using bacterial culture from MRS agar plate

Table 3: Characteristics of yogurts made by using bacterial stock culture

Inoculated from	Color	Aroma	Texture	Consistency	Presence of water
MRS agar	Yellow	Unpleasant smell	Grainy, pitted	Thin	+++
MRS broth	Yellow	Unpleasant smell	Irregular	Very thin	+++

4.3 Effect of temperature

<u>Observation & result:-</u>

After 5 hours of incubation period, the effect of different temperatures on milk fermentation was observed and recorded in the following table:

Figure 11: Effect of temperature at 45˚C (a), 55˚C (b) & 60˚C (c & d) respectively

Table 4: Effect of temperature on lactic acid fermentation of milk

Sample name	**Characteristics of yogurts on different temperatures**					
	Color	**Taste**	**Aroma**	**Texture**	**Consistency**	**Presence of Water**
Set A (45˚C)	White	Sour	Pleasant smell	Smooth	Moderately firm	++
Set B (55˚C)	Off-white	-----	-----	-----	Semisolid with water	+++
Set C (60˚C)	White	Sour	Pleasant smell	Smooth	Firm, thick	Absent

4.4 Effect of pH

Observation & result:-

After 5 hours of incubation period, the quality and characteristics of the yogurts in different pH were observed and recorded in the following table:

(a) (b) (c)

Figure 12: Effect of pH 5.5 (a), pH 6.5 (b) and pH 8.5 (c) respectively

<u>Table 5: Effect of pH on lactic acid fermentation of milk</u>

Sample names	Color	Aroma	Texture	Consistency	Presence of water	Final pH
pH 4.5	----	-----	----	----	----	----
pH 5.5	White	Sweet smell	Coarse, clumps of yogurt	Thin	Water floating around the clumps of yogurt	4.7
pH 6.5	Off-white	Sweet smell	Smooth	Thick, firm	Absent	5.8
pH 8.5	White		Smooth	Thick, firm	Small amount of water	6.3

4.5 Effect of NaCl concentration

<u>Observation & result:-</u>

After 5 hours of incubation period, the quality and characteristics of yogurts in different concentrations of NaCl were observed and recorded in the following table:

(a)

(b)

Figure 13 (a & b): Effect of NaCl concentrations (0.1 %, 0.5 % and 1.0 %)

Table 6.1: Effect of NaCl concentration on lactic acid fermentation of milk

Effect of NaCl concentration					
Concentration of NaCl	**Color**	**Aroma**	**Texture**	**Consistency**	**Presence of water**
0.1 %	Off-white	Sweet smell	Smooth, glossy	Firm, thick	Absent
0.5 %	Off-white	Sweet smell	Smooth, glossy	Firm, thick	Absent
1.0 %	Off-white	Sweet smell	Smooth, glossy	Semi-solid, loose	Water around the yogurt

4.5.1 Observation & result:-

After 5 hours of incubation period, the quality and characteristics of 5-set of yogurts in same concentration of NaCl were observed as well as the changes in pH were recorded in the following tables:

(a) (b)

(c)

Figure 14 (a, b & c): yogurts inoculated from 0.1 % NaCl conc. yogurt previously prepared

Table 6.2: Effect of NaCl concentration on lactic acid fermentation of milk

Effect of NaCl concentration						
Serial no.	**Concentration of NaCl**	**Color**	**Aroma**	**Texture**	**Consistency**	**Presence of water**
1	0.1 %	White	Sweet smell	Smooth	Semi-solid	++
2	0.1 %	White	Sweet smell	Smooth	Firm, thick	+
3	0.1 %	White	Sweet smell	Smooth	Firm, thick	Absent
4	0.1 %	White	Sweet smell	Smooth	Firm	+
5	0.1 %	White	Sweet smell	Smooth	Firm	+

Table 6.3: pH of milk measured at 1-hour intervals

Initial pH of milk	pH after 1 hour	2 hour	3 hour	4 hour	5 hour
6.5	6.3	6.2	5.4	5.0	4.7

4.5.2 Microscopic Observation:-

The cell morphology of *Lactobacillus* sp. was observed under 100X magnification of a compound microscope and rod shaped single cells were found.

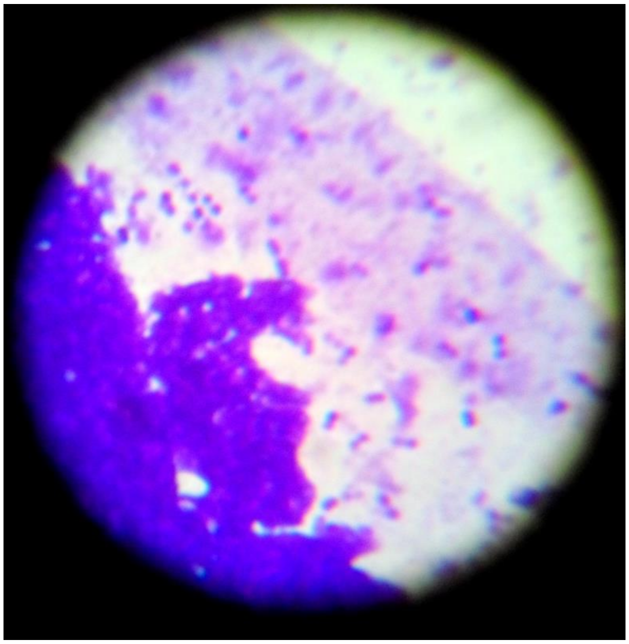


Figure 15: The cell morphology observed under a compound microscope

Table 6.4: Tabular presentation of microscopic characteristics of the cells

Isolates	Shape	Arrangement	Grams reaction	Presumptive organism
Isolate no.1	Coccus	Pairs	Positive	*Streptococcus thermophilus*
Isolate no.2	Diplococci	Pairs	Positive	*Lactococcus lactis*

4.6 Effect of powdered milk concentration in liquid milk

<u>Observation & result:-</u>

After 5 hours & 30 minutes of incubation period, the quality and characteristics of yogurts in different concentrations of powdered milk were observed and recorded in the following table:

Figure 16: Effect of powdered milk conc. (2%, 4%, 6%, 8% and 10%) in liquid milk

Table 7: Effect of powdered milk concentration in liquid milk

Concentrations of PM	Color	Aroma	Texture	Consistency	Presence of water
2 %	Off-white	Pleasant smell	Smooth, glossy	Firm, thick	Absent
4 %	Off-white	Pleasant smell	Smooth, glossy	Moderately firm & thick	+++
6 %	Off-white	Pleasant smell	Smooth, glossy	Moderately firm & thick	++
8 %	Off-white	Pleasant smell	Smooth, glossy	Firm & thick gel like	+
10 %	Off-white	Pleasant smell	Smooth, glossy	Very firm, thick gel like	Absent

*PM= Powdered Milk

4.7 Effect of powdered milk concentration in water

Observation & result:-

After the incubation period, the quality and characteristics of yogurts in different concentrations of powdered milk were observed and recorded in the following table:

(a)

(b)

Figure 17 (a & b): Effect of powdered milk conc. (2%, 4%, 6%, 8% and 10%) in water

Table 8: Effect of powdered milk concentration in water

Concentrations of PM	Color	Aroma	Texture	Consistency	Presence of water
2 %	-------	-------	-------	------	------
4 %	Off-white	Pleasant smell	-------	Semi-solid, loose, broken	++++
6 %	Off-white	Pleasant smell	Smooth	Semi-solid, loose	+++
8 %	Off-white	Pleasant smell	Uneven	Moderately firm	++
10 %	Off-white	Pleasant smell	Smooth, glossy	Firm, thick gel like	+

*PM= Powdered Milk

CHAPTER: 05

DISCUSSION

5.1 SPONTANEOUS FERMENTATION

The yogurts made by spontaneous fermentation contained no inoculum in them still they presented good qualities. They were white in color, had pleasant aroma, smooth texture and firm consistencies. Also, the presence of water was very limited (figure 1, table 1.1). Moreover, the microscopic observation of the glass slides showed that the yogurts seemingly contained *Streptococcus* and *Lactobacillus* species in them (figure 2, table 1.2). Therefore, the natural flora of milk triggered the fermentation of lactic acid and as a result the milk was spontaneously fermented and hence it is called spontaneous fermentation process.

5.2 INOCULATED FERMENTATION

The yogurts inoculated directly from Shakti doi and Ultra doi showed the same characteristics as their starter cultures, which had a sweet smell and they were semi-solid with water (figure 3, table 2.1). Since, the inoculum size was large, it did not mix well with the milk and so, it presented no different qualities than the starter culture (Shakti doi & Ultra doi).

In the consecutive batches of yogurt, the quality and texture indicated massive improvement (table 2.2, 2.4, 2.6). Moreover, the incubation time was shortened in each fermentation batch and therefore, the quality of yogurt had been improved. In addition, the microscopic characteristics of bacterial cells from every batch have been observed to ensure that they presumably contained pure culture of *Lactobacillus* species (figure 5, 7, 9). Further biochemical tests can be performed to confirm their presence.

The stock culture used to inoculate the milk sample was too old and kept at -4˚C for a very long time. At first, they were transferred to *Lactobacillus* MRS Agar and MRS broth in order to revive *Lactobacillus* species. They took almost 48 hours to grow onto the agar plates and in broth cultures and after that they were transferred to fresh pasteurized milk and incubated overnight. The yogurts inoculated from the stock culture presented very poor qualities. The smell was very unpleasant and undesirable, the color was too yellow, the texture was very grainy and the consistency was semi-solid with water (figure 10, table 3).

Therefore, it can be concluded that the corresponding stock culture was contaminated and thus it produced a very poor quality yogurt which was discarded afterwards.

5.3 Effect of temperature

To find out the optimum temperature for yogurt production, fermentation process was carried out using three different temperatures (45˚C, 55˚C, 60˚C) and the most suitable and effective temperature was determined as the optimum temperature for this study.

The yogurt incubated at 45˚C was moderately firm, water was present on top the yogurt and smell was pleasant (figure 11-a). The second one was incubated at 55˚C and it was semi-solid with water (figure 11-b). Furthermore, the taste & smell wasn't pleasant. Thus, it was a very poor quality yogurt. On the other hand, 60˚C yogurt was the finest and most firm between the other two yogurts. It had a pleasant smell, smooth texture and thick consistency with no presence of water in the yogurt [figure 11 (c & d), table 4]. Therefore, the optimum temperatures for yogurt production were determined 45˚C and 60˚C respectively.

5.4 Effect of pH

To find out the optimum pH for yogurt production, fermentation process was carried out using four different pHs (4.5, 5.5, 6.5 & 8.5) and the most suitable and effective pH was determined as the optimum pH for this study.

At pH 6.5, the most satisfactory quality of yogurt among the four was seen, since it had a sweet smell, smoother texture, firm consistency and had no presence of water in it (figure 12, table 5). On the contrary, there were chunks of solid floating in the water in case of pH 5.5 and it could not be classified into a good quality yogurt in any way. Furthermore, yogurt at pH 8.5 had a smooth texture and thick consistency but then it had an unpleasant smell. Additionally, when the pH of the milk was decreasing to 4.5, the milk started to curdle and the pH dropped rapidly to 3.8. Therefore, this part the experiment could not be performed since the particular milk container gave no results. Consequently, the suitable pH for yogurt production was determined at pH 6.5 undeniably.

5.5 Effect of NaCl concentration

The higher the concentration of NaCl, the less firm the yogurt was. Therefore, yogurt containing 1.0% NaCl was loose in consistency and gave a watery texture, since salt caused water to come out from the yogurt. On the other hand, 0.1 % and 0.5 % NaCl concentration in yogurt gave more firm and thicker consistency as well as smoother texture (figure 13, table 6.1). So, lesser concentration of salt is more suitable for a good quality yogurt production. Therefore, the optimum concentration of NaCl for yogurt fermentation was determined at 0.1% and 0.5% NaCl correspondingly.

In subsequent batches of continuous fermentation, the yogurts presented similar physical characteristics (figure 14, table 6.2) and the pH of milk every 1-hour interval were measured (table 6.3). The microscopic characteristics of cells isolated from yogurts were observed under compound microscope where they appeared as cocci shaped arranged in pairs. Presumably, these organisms were *Streptococcus* species (figure 15, table 6.4).

5.6 Effect of powdered milk concentration in liquid milk

The higher the concentration of powdered milk was, the thicker and finer the yogurt was. The yogurt containing 2%, 8% and 10% powdered milk concentration showed thick, firm, smooth gel like consistency and pleasant aroma (figure 16, table 7). On the other contrary, yogurt having 4% and 6% powdered milk were moderately firm with the presence of water (table 7). Therefore, the most favorable concentrations of powdered milk in liquid milk for yogurt fermentation were determined 2%, 8% and 10% respectively.

5.7 Effect of powdered milk concentration in water

The higher the concentration of powdered milk was the thicker and better-quality the yogurt was. The 2% powdered milk concentration did not show any characteristic of yogurt production and thus fermentation did not take place (figure 17). Correspondingly, yogurts containing 4% and 6% powdered milk were semisolid with water. In addition, 8% yogurt was moderately firm and 10% yogurt was thick, firm and showed a gel like consistency (figure 17, table 8). Therefore, the ideal concentration of powdered milk in water for yogurt fermentation was determined 10% respectively.

CHAPTER: 06

CONCLUSION

In this study, the physicochemical properties affecting lactic acid fermentation of milk were studied and an optimum condition for a good quality yogurt production has been achieved. Having stated before, the significant factors that affect the fermentation process are: temperature, pH, NaCl concentration, liquid and powdered milk concentrations. These factors required for the fermentation process were optimized and the performance of the fermentation process was enhanced remarkably. The suitable temperature for lactic acid fermentation was determined 45˚C and 60˚C correspondingly and optimum pH was 6.5; optimal concentrations of NaCl were achieved 0.1% & 0.5% while, powdered milk concentrations in liquid milk were 2%, 8% and 10% respectively. In addition, ideal concentration of powdered milk concentration in water was carefully chosen 10%. The yogurt produced by means of these optimum conditions showed thick gel-like consistency, smooth texture and contained a pleasant smell which ensures an efficacious fermentation and they were incubated for up to 5 hours. The cell morphology of the organisms taken from these yogurts were observed under the compound microscope after performing Gram's stain where they appeared as long rod shaped bacteria in pairs and in long chains. Additionally, some cocco-bacilli were also seen under the microscope which was in pairs and in chains. All these morphological characteristics confirmed the presence of lactic acid bacteria (LAB) in the corresponding yogurts. Further biochemical tests are required to confirm the presumptive organisms.

CHAPTER: 07

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CHAPTER: 08

APPENDIX

Appendix - A

8.1 MICROBIOLOGICAL MEDIA

Media used in this study, were prepared using methods with appropriate compositions. All media were sterilized by autoclaving at 121˚C and 15 lb. pressure for 20 minutes.

8.1.1 Composition of Lactobacillus MRS Agar media

Name of the ingredients	Amount (Gms/litre)
Proteose peptone	10.00
Beef extract	10.00
Yeast extract	5.00
dextrose	20.00
polysorbate	80.00
Ammonium citrate	2.00
Sodium acetate	5.00
Magnesium sulphate	0.10
Manganese sulphate	0.05
Dipotassium phosphate	2.00
Agar	12.00

8.1.2 Composition of Lactobacillus MRS Broth

Name if the ingredients	**Amount (Gms/litre)**
Dextrose	20.00
Proteose peptone	10.00
Beef extract	10.00
Yeast extract	5.00
Sodium acetate	5.00
Ammonium citrate	2.00
Dipotassium phosphate	2.00
Magnesium sulphate	0.10
Manganese sulphate	0.05

8.2 REAGENTS & CHEMICALS

There are various reagents that were used for different purposes during the project work. Some of them were used directly and some were prepared in the lab. The most commonly used reagents and their composition are shown below:

Serial No.	Name of the reagent	Manufacturer
1	Crystal violet	Sigma, Nentech
2	Distilled water	Invitrogen
3	Ethanol (absolute)	Merck, Germany
4	Ethanol (70 %)	
5	Ethanol (95 %)	
6	Gram's Iodine Solution	
7	Hydrochloric Acid (32%)	Merck
8	Sodium Hydroxide (1M)	
9	Immersion Oil	Sigma
10	Lactobacillus MRS Agar	Hi Media Laboratories Ltd, India
11	Lactobacillus MRS Broth	Hi Media Laboratories Ltd, India
12	Safranin-O	Sigma, Loba
13	Sodium Chloride	Merck

Composition of 1M NaOH

- NaOH pellets - 40 g
- Distilled water

Composition of Crystal Violet

- Crystal violet - 2 g
- Ethanol (95%) - 20 ml
- Ammonium oxalate - 0.8 g
- Distilled water - 80 ml

Composition of Gram's Iodine Solution

- Iodine crystals -1 g
- Potassium Iodide -2g
- Distilled water -300 ml

Composition of Decolorizer

- Acetone - 1 volume
- Ethanol (95%) - 1 volume

Composition of Safranin-O

- Safranin O - 0.25 g
- Ethanol (95%) -10 ml
- Distilled water - 90 ml

Appendix - B

Instrument name	**Model no.**	**Origin**
Autoclave	WAC-47	Daihan Scientific, Korea
Balance machine	Core series	Adam, UK
Compound microscope	Model- CX-21	Olympus
Incubator (37˚C)		SAARC Engineering
Incubator (45˚C)	Model - DSI300D	Taiwan
Laminer airflow cabinet		SAARC Engineering
pH meter	pHep, HI-98107	HANNA Instruments
Refrigerator	Model-0636	Samsung
Vortex mixture		VWR International
Water bath		Korea

Appendix – C

Serial no.	Name of the Glassware and Other Equipment	Origin
1	Beaker (250 ml)	Iwaki, Japan
2	Conical flask (250 ml)	Pyrex
3	Measuring cylinder (100 ml & 250 ml)	MC, China & Pyrex, UK
4	Glass rod	China
5	Glass slide	China
6	Petri dishes	Germany
7	Screw cap testube (10 ml)	Pyrex
8	Reagent bottle (250 ml) with dropper	China
9	Inoculating loop	India
10	Spatula	India
11	Spirit lamp	China
12	Bunsen burner	Bangladesh
13	Aluminum Foil	USA
14	Thermometer	China