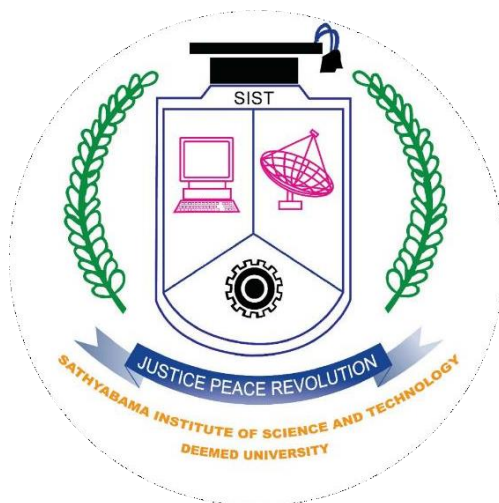


ISOLATION AND CHARACTERIZATION OF BACTERIA FROM HOME MADE VEGAN CURD

Submitted in partial fulfilment of the requirements for the award of
Bachelor of Technology degree in Biotechnology

by

S.KIRTANA (37230038)



**DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO AND CHEMICAL ENGINEERING**

**SATHYABAMA
INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

Accredited with Grade "A" by NAAC

JEPPIAAR NAGAR, RAJIV GANDHI SALAI, CHENNAI – 600 119

MARCH - 2021



SATHYABAMA

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DEPARTMENT OF BIOTECHNOLOGY

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of S.KIRTANA (37230038) who carried out the project entitled "Isolation and characterization of bacteria from home made vegan curd" under our supervision from January to March 2021

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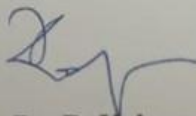
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This is to certify that **S. KIRTANA**, B. Tech Biotechnology, Sathyabama Institute of Science and Technology, Chennai has carried out the Project titled **"ISOLATION AND CHARACTERIZATION OF BACTERIA FROM HOME MADE VEGAN CURD "** under the guidance of Dr. R. Kalpana, Head – R&D Centre, National Agro Foundation, Taramani, Chennai – 600113 during January 2021 to March 2021.



Dr. R. Kalpana
Head- R&D Centre
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DECLARATION

I S.KIRTANA (37230038) hereby declare that the Project Report entitled “**Isolation and characterization of bacteria from home made vegan curd**” done by me under the guidance of **Dr. S UshaNandhini** and **Dr.R.Kalpana** at National Agro Foundation, Anna university Tharamani campus submitted in partial fulfillment of the requirements for the award of Bachelor of Technology degree in Biotechnology.

DATE:

PLACE: Chennai

SIGNATURE OF THE CANDIDATE

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ABSTRACT

Curd is a traditional food which is high in nutrition and helps in boosting immunity. Curd has been consumed worldwide and has health gut bacteria that fights against harmful pathogenic bacteria. Curd is coagulated using milk. Since some people experience lactose intolerance, plant based curd is advised for their intake. In this study we will explore the good gut bacteria and other microorganisms present in the homemade vegan curd. This plant based curd is made of peanut milk and rice milk and green chilli pediceles are added for fermentation process that helps in curdling of milk. Peanuts are rich in antioxidants and they have health gut bacteria like *Bradyrhizobium*, proanthocyanins, polyphenols and fibres. Rice contains bacteria like *Bacilli*. Green chillies consist of *Lactobacilli* and is also a starter for lacto fermentation. The homemade vegan curd are rich in protein and carbohydrates. The bacterias which are present in homemade vegan curd are analysed through plating methods..

Keywords: Lactofermentation, Curdling, bacilli, *Bradyrhizobium*, Plating.

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LIST OF SYMBOLS AND ABBREVIATIONS

NA – Nutrient Agar

NB – Nutrient Broth

EMB – Eosin Methylene blue agar.

TPC – Total plate count

VP - Vogues Proskeur

CONC-Concentration

MM – millimeter

CFU- Colony Forming Units

E.COLI- Escherichia Coli

CHAPTER 1 - INTRODUCTION

1.1 AN OVERVIEW

Curd is obtained by coagulating milk that is normally called curdling. It is often caused by adding edible acidic substance like lemon juice or vinegar, then permitting it to coagulate. The accrued acidity causes the milk proteins to breakdown at its iso-electric point and the solids separate from the whey liquid.

Curd could be a nice probiotic product that contains sensible helpful bacterium necessary to regulate the gut activity, soothe the inflamed digestive gut, control water absorption, heal wounds and prevent indigestion. The live bacterium found in curd, fight disease-causing germs, and keeps the gut and internal organ healthy and protected.

Common psychotropic bacterium in milk are species of Micrococci, Bacilli, Staphylococci, Lactobacilli, Pseudomonas, and Coliforms. The generic Pseudomonas species are a unit, the foremost common, and usually have the maximum impact on quality.

Curd could be a source of supply of essential Vitamins. A cup of fat free curd contains Vitamins in same amount as that in milk and these include Vitamin A, Vitamin B₁₂ and Vitamin D. In addition, it maintains the osmotic balance in the digestive gut by the presence of balanced sodium and potassium ions. If pH factor is disturbed, the colloid will lose its structure.

Milk is composed of several compounds, primarily fat, protein and sugar. The protein in milk is normally suspended in a colloid solution, which means that the small protein molecules float around freely and independently. Along with the suspended fats, these protein molecules refract light, so the milk appears to be white. Normally these protein molecules repel each other, but when the pH of their solution changes the clumps are formed. (Sorit Gupta, 2019).

The regular consumption of curd produces a higher level of immunity boosting interferon as these bacteria cultures stimulate infection fighting white cells in the blood stream with anti-tumor effects. (Maltock, 2007). It is widely consumed as a functional food due to its good taste and is also rich in potassium, calcium, protein and in vitamins and also acts as an excellent vehicle to deliver probiotics to the customer. (Reid et al, 2003).

Regular consumption of curd is thought to be beneficial in strengthening of the immune system, improves in lactose digestion, blood glucose management (Yadav, Jain, & Sinha, 2007). And the reduction of constipation, diarrhoea, colon

cancer, inflammatory bowel diseases and allergies (Adolfson, Meydani & Russel ,2004).

1.2 BACTERIA PRESENT IN CURD

Curd contains the bacteria *Lactobacillus acidophilus* which is the most abundant and beneficial. Others that also play an important role are *Lactobacillus pedicoccus*, *Lactococcus lactis* and *Lactococcus cremoris* etc, whereas other dairy products contain eubacteria *Lactobacillus thermophilus* and true bacteria *Lactobacillus bulgaricus*.

The presence of *Lactobacillus* in the digestive gut and in the milk products enhances the bio-availability of vitamins especially Vitamin A and B₁₂ and minerals such as calcium, phosphorus, sodium and potassium, thereby helping in the development of bones and teeth and maintaining the osmotic balance of sodium and potassium in the cells. These probiotics or the helpful bacteria within the curd facilitate gut health and optimal functioning of the alimentary canal.

The curdling of milk is a chemical change. The type of change which leads to the formation of new product with different properties and the obtained product cannot be reversed again to the reactant is called as chemical change.

When milk is curdled, the liquid form of milk gets converted into solid due to the secretion of acids by the bacteria, and the protein in milk (casein) precipitates and leads to the formation of curd. In the curdling of milk, whether domestic or industrial, milk is first brought to boil.

On cooling to around 35 degrees centigrade, it is curdled by addition of a small quantity of seed curd. The *Lactobacillus* bacteria present in the seed curd, get activated, and multiply. The bacteria utilize the galactose sugars in milk and form carboxylic acids which reduce the pH of the milk and hence curdling occurs due to the precipitation of the milk protein casein.

Bacteria in curd are probiotics and they facilitate to keep our gut/digestive system healthy and strengthen the immune system. Probiotics contain live micro-organisms and when ingested along with food or nutritional supplements provide good health and protection from various diseases.

For being considered as a probiotic, just like the probiotic drink or probiotic milk or probiotic food the product should contain a particular strain of live bacteria that are immune to the hydrochloric acid in the stomach; biliary juices and must reach the intestine live to offer the benefits. Therefore, any product that is probiotic could be considered as a standardized product.

Usually in curd there are both good gut bacteria and bad gut bacteria. But some people are allergic to milk and hence lactose intolerance occurs. Majority of the bacteria should be intestine friendly and should produce health gut bacteria, only then the curd is fit for consumption and they do not cause any infections or harm to the human body,

1.3 VEGAN CURD

Vegan curd made from plant-based sources, like peas, soy, coconut, cashews, and almonds, are often a healthy addition to the diet of individuals. The most effective ones tend to contain no added sugars

Individuals who are lactose intolerant and cannot consume milk and milk products, can opt for vegan curd since it is lactose free. A variety of non-dairy curds starting from peanut-rice curd, cashew curd, cashew-rice curd, soy curd, and peanut-soy curd, are a few to illustrate

Coconut curd is mildly sweet and slightly bitter with a definite coconut flavour. It may be utilized in place of dairy farm curd in many recipes. The thickness of the curd depends on the type of milk that is utilized. Full-cream canned coconut milk can yield a creamy curd whereas the lighter homespun milk can end in curd that has a thick consistency.

Most vegan curds have probiotics and is available commercially and also can be prepared at home in an ambient temperature. The shelf life of the vegan curd normally ranges up to three to seven days while refrigerating. They are healthy compared to the milk curds. Peanut milk, coconut milk or soy milk gives consistent results and makes the curd rich. These are usually fermented in pots to get the curd thick and consistent.

1.4 FERMENTATION

Fermentation is a process used in food industry from the past. For all food borne microorganisms, factors either promote or inhibit their growth. The factors include temperature, oxygen, water activity, nutrients and pH. These are the key factors that have an effect on microorganism growth.

Fermentative microbes preserve food and create them safe for consumption by increasing acid content or alcohol content of foods or reducing water conjointly aiding the fermentation method. Concentration or level of salt or sugar facilitate restart of microorganism growth.

Fermentation is the metabolic process that converts sugar into acids, gases, or alcohols by microorganisms in available or environmentally controlled conditions. Yeasts for example, produce alcohol under anaerobic conditions, while most bacteria produce toxins and acids under aerobic conditions.

Yeast and plenty of alternative microbes normally use fermentation to produce different compounds to hold on for their survival, in their surrounding environment. Alcoholic fermentation one mole of glucose into two moles of ethanol and two moles of carbon dioxide, producing two moles of ATP in the process. (Aren van Waarde; Maria Verhagen, 1993)

.Consumption of fermented milk products causes a slight reduction in stomach which reduces the risk of pathogen transmit and the effects of low gastric juice secretion problem. Since fermented milk products are highly consumed foods around the world, nutritional components are delivered into the human diet.

The production of curd in a fermented milk product has been started in Middle East and spread all over the world. Compared to milk, curd is more nutritious and an excellent source of protein, calcium, phosphorous, riboflavin, thiamine, vitamin B12, folate, niacin, magnesium, and zinc. Since lactose in milk is converted to lactic acid during fermentation and due to the presence of lactose fermenting bacteria in curd it can be consumed without any adverse effect.

1.5 PROBIOTICS

Probiotics are the health gut bacteria which provides health benefits to the body. Live probiotic cultures are part of fermented dairy products, other fermented foods and probiotic fortified foods.

One of the concerns throughout the scientific literature resides in the viability and reproducibility on a large scale of observed results for specific studies, as well as the viability and stability during use and storage and finally the ability to survive in stomach acids and then in the intestinal ecosystem. (Corthier G and Marteau P, 2011).

Probiotics regulates immune responses which enhance healthy reactions to dangerous infectious organisms, and they suppress excessive inflammation. These health gut bacteria are normally found in healthy human gastrointestinal tract. There are also several different strains of probiotic strains available and are in use, which also have different but overlapping benefits.

These probiotic organisms work through several interrelated mechanisms to promote health at a molecular level. They conquer the dangerous organisms in the intestines

and boost the immunity and also reduces the risk of toxin mediated diseases and infections.

These probiotics causes an intestinal inner lining which prohibits from causing any harmful infections to the human body. The most common probiotics in curd are Lactic Acid Bacteria which is also known as LAB and Bifidobacteria.

Some of the other probiotics are *Bifidobacterium longum*, *Bifidobacterium breve*, *Brifdobacterium infantis*, *Brifdobacterium bifdum*, *Brifdobacterium adolescentis*, *Streptococcus cremoris*, *Steptococcus diaceylactis*, *lactococcus cremoris*, *Lactococcus lactis*, *Lactobacillus acidophilus*, *Lactobacillus fermeum* and *Latobacillus casei*. These kind of probiotic strains can be seen in curd.

CHAPTER 2 – LITERATURE SURVEY

Many genera of bacteria produce lactic acid as a primary or secondary end product of fermentation, the term lactic acid bacteria is conventionally reserved for genera in the order *Lactobacillales*, which includes *Lactobacillus*, *Leuconostac*, *Pediococcus*, *Lactococcus* and *Streptococcus* *Cornobacterium*, *Enterococcus*, *Oenococcus*, and *Tetragenococcus*, *Vagococcus*, and *Weisella* (Todar's online textbook of bacteriology, Keneeth todar, PhD).

Probiotics are the products designed to deliver potentially beneficial bacterial cells to the microbiotic ecosystem of humans and other animals. Lactic acid strains are the common strains that has beneficial bacteria and curd is a very rich source of probiotics. They also have other bacterial species like *Streptococci*, *Micrococci* etc.. (M. Ratna Sudha, 2010).

Many contaminating organisms only spoil the product, thereby reducing its shelf life. Some such as lactic acid bacteria are used in milk processing and they also cause the milk to sour. Other bacteria are pathogenic to man and causes diseases if the milk is consumed untreated. (Sharp et al 1985 & Heeschen 1994).

Different species of lactic acid bacteria have adapted to grow under widely different environmental conditions. They are found in the gaserointestinal tract of animals, dairy products, seafood products, soil and on some plant surfaces (Ring & Gatesoupe, 1998).

The most important role of bacteria is to protect against harmful microorganisms. The ability of the lactic acid bacteria is to prevent and cure a variety of diseases leading to the coining of the term probiotics or prolife. Probiotics are monoculture or mixed

cultures of live microorganisms that might beneficially affect the host by improving the characteristics of indigenous microflora (Hozapfel et al, 1998).

Bacterias are inhibitory strains that are produced by both gram positive and gram negative strains. Gram positive bacteria are produced by lactic acid bacteria which are used in fermented foods and dairy products. The antimicrobial effect of lactic acid are for thousand years and they have some sort of acetaldehyde flavour in the curd. They have been used in the food fermentation for more than 4000 years. They also have a flavour inducing metabolite and it is called as diacetyl (Yadav et al 2007).

The taxonomic position and physiology of strains are investigated by phenotypic and genotypic methods. These are based upon their biochemical test characteristic and enzyme characteristics and growth temperatures and cell wall analysis of the proteins. Later they are used to find the lactic acid bacteria using the strains (G Klein et al 1998).

The reliable identification and correct naming of bacterial strains are not only primary strains but also for tracking of the probiotic strains and a non fraudulent labelling of fermented milks. Genera *Lactobacillus* and *bifidobacterium* include a large number of species and strains exhibiting important properties in an applied context. (Giovaana E Felis et al. 2007)

Lactic acid bacteria are among the most important probiotic microorganisms which are usually associated with the human gastrointestinal tract. Traditionally lactic acid bacteria have been classified based on the phenotypic properties example: morphology, mode of glucose fermentation, growth of different temperatures, lactic acid configuration, and fermentation of various carbohydrates. Thus some species are not distinguishable by phenotypic characteristics. This is applicable for the species like *Lactobacillus acidophilus* and *Lactobacillus casei* and *Lactobacillus paracasei* (Petra Haberer, 2001).

The concept of curd has led to widespread consumption of food preparations containing food microbes and probiotic microbes. The human gut bacteria also plays an important role in nutritional process in the body. (R. Balamurugan, 2014).

The more friable curd of the acid product presumably makes it easier to digest. Fermented milk products are safer than fluid milk in many countries where transport, pasteurization and refrigeration facilities are inadequate. The introduction of fermented products such as cheese and yoghurt to the diet of the civilization. Consumption of fermented milk products are linked with several types of human health benefits partly because of their human benefits. (Nurul Abduvan Choudary, 2018).

In Vitro digestibility of fermented milk products were available and the products were studied. Size of curds with various stages of digestion was determined by screening method and amount of nitrogen in each curd size was estimated by analysing lyophilized curd samples. In spite of much higher protein content resembled human milk fairly is digestion. Fermented milk products could be easily digestible foods for infants, children and adults even when gastrointestinal disturbances prevail. (Mjolkcentralen Arla, 2010).

The presence of calcium ions form white creamy lumps. As it is rich in its nutrient content it is easily subjected to contamination by many kinds of microorganisms present are highly interrelated. The presence of pathogens indicator of higher deficiencies of colonies and counted them. (Sivakumar N, 2010).

Many different types of fermented milks are manufactured around the world. Approximately 400 generic names are applied to traditional and industrial products, including special fermented milk with health aspects attributed to probiotic microorganisms. Among the fermented foods those originating from eastern Europe and central Asia. (Barbarous Ozer, 2014).

Fermentation is a naturally widely used method of natural food fermentation and preservation that has consequences on nutritional value of the transformed food. Fermented dairy products are increasingly investigated in view of their ability to exert health benefits beyond their nutritional qualities. (Gregory Pimental, 2018).

Starter cultures are those microorganisms that initiate and carry out the desired fermentation essential in manufacturing cheese and fermented dairy products such as dahi, lassi, yoghurt and the starter culture is defined as an active microbial preparation deliberately added to initiate desirable changes during preparation of fermented products. (Subrota Hati, 2013).

CHAPTER 3 – AIM AND OBJECTIVE

3.1 AIM

To isolate and characterize the bacteria present in the Homemade Vegan Curd.

3.2 OBJECTIVES

3.2.1 To prepare a homemade vegan curd using peanut milk and rice milk with green chilli pediceles.

3.2.2 To compare and perform nutritional analysis between milk curd and vegan curd.

3.3.3 To isolate and identify the bacteria present in the vegan curd using microbiological techniques

3.3.4 To explore the probiotics present in the homemade vegan curd.

CHAPTER 4 – MATERIALS AND METHODS

4.1 MATERIALS

4.1.1 VEGAN CURD

1. 125 grams of raw peanuts.
2. 125 grams of rice
3. 200 ml of water
4. 12 green chilli pediceles.
5. Nut strain bag.
6. Blender.

4.1.2 MILK CURD

1. 50 ml of raw milk
2. 1 green chilli
3. Lemon.

4.1.3 CHEMICALS FOR NUTRITIONAL ANALYSIS

1. Petroleum benzene
2. Ammonia solution
3. Sodium sulphate
4. Sulphuric acid
5. Copper sulphate
6. Methyl red indicator
7. Sodium hydroxide

4.1.4. ISOLATION OF BACTERIA

- 1.Total plate count agar
2. Nutrient agar
- 3.Iodine solution
- 4.Kovac's indole reagent
- 5.Nutrient broth
- 6.Potassium hydroxide
- 7.Tryptone broth
- 8.Methyl Red
- 9.Triple sugar iron agar
- 10.Urease agar
- 11.Bile salts
- 12.Sodium citrate
- 13.Crystal violet solution
14. Gram's iodine
15. Decoloriser
- 16.Saffranin.

4.1.5 APPARATUS FOR NUTRITIONAL ANALYSIS

1. Soxhlet extractor
2. Round bottom flask
3. Condensor
4. Mantle

5. Distillation flask
6. Siphon
7. Kjeldahl flask
8. Kjeldahl trap
9. Conical flask
10. Kjeldahl apparatus.

4.1.6 APPARATUS FOR MICROBIOLOGICAL TECHNIQUES

1. Petri Dish
2. Inoculation loop
3. Bunsen burner
4. Filter paper
5. Scissors.
6. Parafilm
7. L rod
- 8 Cotton rolls
9. 250 ml Conical flasks
10. 30ml Test tubes
11. Spatula
12. Microscopic glass slide
13. Eppendorf tubes
14. Micropipette

4.1.7 INSTRUMENTS

1. Autoclave
2. Laminar flow
3. Hot air oven
4. Magnetic stirrer
5. pH meter
6. Test tube racks

4.2 METHODOLOGY

4.2.1 Preparation of vegan curd

125 grams of raw peanuts and raw rice are soaked in 250 ml of water overnight. The soaked peanuts are washed thoroughly and blended along with 250 ml of water. This process is repeated for two times.

After blending the mixture is filtered using a nut strain bag. Later the peanut milk is separated and heated at a medium flame. Meanwhile 125 grams of raw rice is washed thoroughly and blended and later the extract is filtered. The rice milk is added to the peanut milk and stirred continuously in a medium flame. After pasteurization the milk is allowed to come back to its lukewarm temperature. The milk should be in lukewarm temperature so that fermentation process takes place easily. When the milk is extremely hot fermentation of milk does not take place. When the milk is in luke warm temperature green chilli pediceles are added on the top and the vessel is closed for 24 hours and is kept in an ambient temperature.



Fig 4.1 Green chilli pediceles



Fig 4.2 Fermentation



Fig 4.3 Homemade vegan curd

4.2.2 PREPARATION OF MILK CURD

200ml of fresh raw milk is heated at a medium flame. Four tablespoons of milk is taken in a separate vessel and one full green chilli and a tea spoon of lemon juice is added and is kept aside for 24 hours.

The heated milk is later poured in a container and kept in an ambient temperature overnight. Later, the milk which contains green chilli and lemon juice is added to the fresh heated milk and left undisturbed for 12 hours.

If the temperature is cold the curd can be kept near a microwave oven or stored in a heat place. If the temperature is normal the curd takes 24 hours to set. The setting of the curd depends upon the surrounding temperature. The maximum time for setting of the curd is around 12 to 24 hours or sometimes 48 hours.



Fig 4.4 Homemade milk curd

4.2.3 NUTRITION ANALYSIS BETWEEN MILK CURD AND VEGAN CURD

4.2.3.1 Soxhlet Method (Fat test)

The apparatus is rinsed with petroleum ether and is kept in hot air oven for 120 degree celcius.5gram of curd sample was weighed and placed it under thimble.The thimble was attached to the soxhelet extractor.

In a apparatus curd was added along with ammonia solution and petroleum benzene and was mixed thoroughly until the pressure was released. Later, 150ml round bottom flask was taken and washed with petroleum ether and dried thoroughly.

Later the round bottom flask was filled with 90ml of petroleum ether and is placed on the mantle and was heated for six hours and the same process was repeated again for several hours and the extraction unit was removed from the condensation unit allowing the sample to cool down. The conical flask was later kept in hot air oven to evaporate the solvents and weight of the fat is noted.



Fig 4.5 Soxhlet extraction



Fig 4.6 Milk curd fat



Fig 4. 7 Vegan curd fat

CALCULATION :

$$\text{Fat\% (w/w)} = \frac{\text{Weight of the extracted fat}}{\text{Sample weight} * 100}$$

Vegan curd

Weight of the empty dish = 128.225

Weight of the dish with fat = 128.454

Milk Curd

Weight of the empty dish = 134.370

Weight of the dish with fat = 134.445

4.2.3.2 Kjeldahl method (Protein test)

This method is helped in oxidising the organic compounds the basic principle was discovered by Johan Kjeldahl hundred years ago.

This method has three process digestion, distillation and titration. Digestion is a process where in a kjeldahl flask 5grams of curd sample and 0.5grams of copper sulphate and 25ml of sulphuric acid is added and kept for protein digestion. After 24 hours the flask appears to be colourless.

The liquid is cooled down by adding distilled water and 40ml of sodium hydroxide and 5 gel crystals are added and transferred to a kjeldahl round bottom flask. After protein extraction. Receiver solution is prepared by adding 40ml of sulphuric acid and 12 drops of methyl red indicator. After the kjeldahl method is complete. The receiver solution is titrated against 50%NaOH after it reaches to 150 ml.



Fig 4.8 Kjeldahl extraction



Fig 4.9 Receiver solution



Fig 4.10 Magnetic stirrer

4.2.3.3 pH value for vegan curd

In the digestive system stomach helps to digest the food without causing any harm to the human body. Hydrochloric acid produced in the body helps in the digestion of the food.

But when the amount of acid goes beyond a certain limit due to indigestion, pain and irritation causes in the stomach. When there is normal pH in the body there is no harm. Different types of body has different values. The food that is consumed regularly has a certain pH that helps the body to undergo proper digestion.



Fig 4. 11 pH value for vegan curd

4.2.3.4 Titratable acidity

Natural acidity of milk is due to its constituents such as casein, albumin, citrates, phosphates and carbon dioxide. The acidity can be measured by titrating milk against standard alkali using an indicator like phenolphthalein and is expressed as terms of lactic acid. 10ml of curd is taken in a beaker and 2 to 3 drops of phenolphthalein indicator was added and shaken well and later titrated with standard

NaOH solution. The titration was completed in 20 seconds. The titratable acidity of milk curd and vegan curd was performed.



Fig. 4.12 Acidity value



Fig 4.13 Vegan curd with indicator solution

Calculation :

$$\% \text{ acid} = \frac{N \cdot V \cdot M}{S \cdot 10}$$

N = Normality of the standard NaOH solution used for titration.

V = Volume of Standard NaOH solution used for the titration.

M = Molecular weight of the predominant acid in the sample divided by the number of hydrogen ions in the acid molecule that are titrated.

S = Sample size in millimetres.

Titre value 6

Acidity value 0.6 %

4.2.4 ISOLATION OF BACTERIA FROM VEGAN CURD

4.2.4.1 Preparation of Nutrient agar

- Suspend 1.6 grams of nutrient agar powder in a 250ml conical flask which contains 60ml of distilled water.
- Mix and dissolve them completely
- Cover the conical flask using cotton balls and wrap a parafilm on the neck of the flask
- Sterilize by autoclaving at 121 degree celcius for 15 minutes at 15Lbs.

After sterilizing pour the liquid into the petridish and wait for the medium to solidify.



Fig 4.14 Nutrient agar

4.2.4.2 Serial dilution method

Dilution process is usually done to make a solution weaker and to reduce its concentration. In microbiology serial dilution technique is performed to decrease the

bacterial concentration so that it is easier to count the colonies on the agar plate. Ten test tubes,

200ml of distilled water and six petridishes were autoclaved for sterilization at 121 degree celcius for 15 minutes at 15 Lbs. Each test tube is filled with 9ml of distilled water and named as 10^{-1} to 10^{-9} respectively. 1ml of small amount of well mixed curd is added to the 10^{-1} test tube and using a 1000ml micropipette the curd is diluted upto nine test tubes. 1ml of the solution is diluted in all the nine test tubes and the last 1ml dilution is discarded using a micropipette.

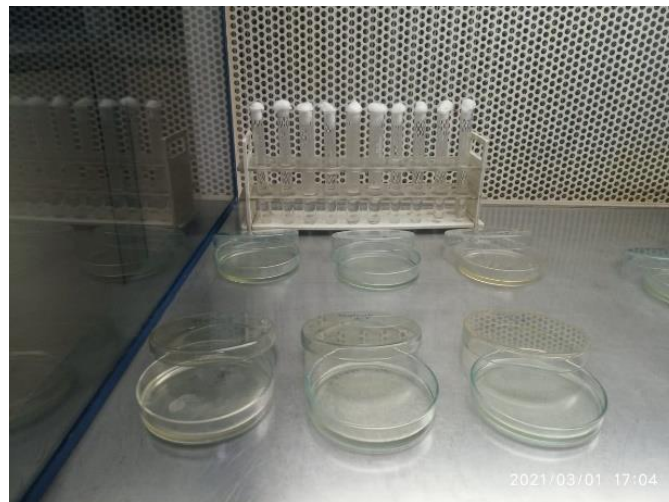


Fig 4.15 Serial dilution and solidified agar plates

4.2.4.3 Spread plate technique

This technique is used to plate a liquid sample for isolating and counting the bacteria present in that sample. Spread plate technique provides visible and isolated colonies that are evenly distributed in the plate.

To avoid contamination, the L rod which is also known as spreader should be sterilized or wiped with ethanol to kill the unwanted bacteria. From serial dilution three plates are of duplicate to compare the colony growth from the original.

1ml of liquid sample is poured on the agar plate and by using L rod the bacteria is evenly spread through the surface of the plates. After spreading the bacteria the plates are incubated at 37 degree celcius for 24 hours and later the colony growth is noted.

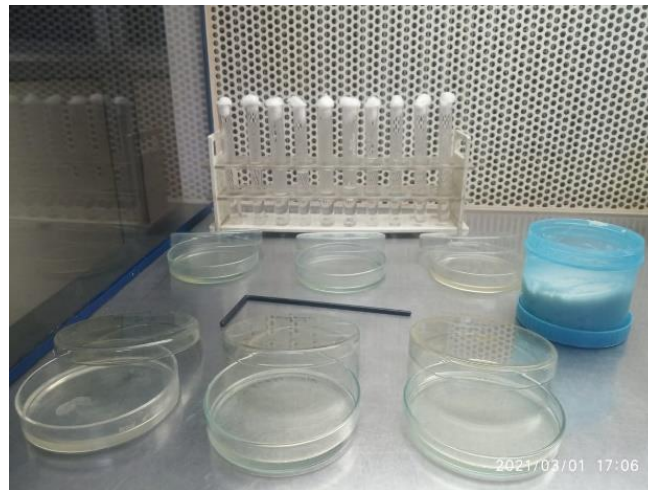


Fig 4.16 Spread plate technique

4.2.4.4 Colony growth

A visible mass of microorganisms is called as colony growth. Bacteria needs a special nutrient medium to grow and be visible and they grow on solid media as colonies. The colonies that are grown on the agar plates may be different.

They differ in size, shape and colour and growth. After incubating the plate for 24 hours visible colonies are identified and are characterized.

The temperature should be ranged around 40 when the plates are kept in the incubator. If the temperature increase the bacteria will not survive and it stops its growth. The plates should be wrapped tightly with parafilm to avoid melting of agar and contamination.

4.2.4.5 Streak plate technique

After performing spread plate technique several colonies were grown in the agar plates. Streak plate method is one of the easiest method to isolate a pure bacteria strain from several species of microorganisms.

The dilution and streaking method was first identified by Leffeler and Gaffky in Koch laboratory. This is also a simple and a rapid process. These are done by aseptic techniques and sterile tools are used such as cotton swabs ethanol Bunsen burner and an inoculation loop.

By using this method the bacteria or any microorganisms can be identified. When numerous colonies are grown in a plate it is difficult to identify them as a group. Streaking helps the bacterial colonies to be identified. After the colony growth three plates were selected and different colour colonies were identified and selected. Further streak plate technique were performed on the agar plates and incubated it for 37 degree celcius for 24 hours and later the bacteria is identified easily as the colonies are separately streaked.

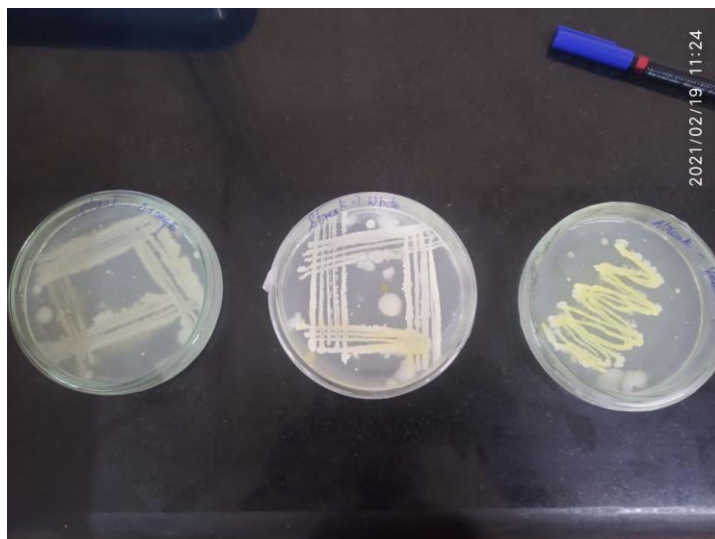


Fig 4.17 Streak plate technique

4.2.4.6 Total plate count

This method is done by pour plate method to count the number of colonies present in a specimen. In this method a fixed amount of inoculum which is 1 ml is poured on a sterilized petridish and 15ml of plate count agar is autoclaved for 15 minutes at 15 Lbs and poured on the half closed petridish. The mixture is now gently rotated and the medium should be covered evenly.

The agar should not spilt in the end of the petridish. Parafilm is wrapped around the petridish and is incubated for 37 degree celcius and observed for the growth.

The agar should be lukewarm so that that bacteria inoculum survives and grows during incubation. When the medium is at high temperature bacteria growth or any colony growth cannot be seen. While pouring the agar the neck of the conical flask should be slightly heated while pouring it on the petridish. Later colonies were counted.



Fig 4.18 Colonies in pour plate technique

4.2.5 CHARACTERIZATION OF THE BACTERIA

4.2.5.1 Gram staining method

This method is performed to differentiate between gram positive and gram negative bacteria. This method was first discovered by the Danish bacteriologist Hans Christian Gram. The cell walls have different physical and chemical properties, by using gram staining technique the thick layer and the thin layer of peptidoglycan in the cell reacts to the stains that are added to the bacteria. For example: thick layer absorbs the violet stain and the thinner peptidoglycan absorbs the red stain which is called as safranin.

Take a clean glass slide and pick a colony from the agar plate by using a sterilized inoculation loop. The inoculation loop is heated thoroughly before scraping a colony.

The bacteria is heat fixed slightly. The slide should not be overheated. Temperature is checked by placing the back of the slide on the wrist.

Sterilized distilled water droplet is added on the slide and after it is dried crystal violet stain is added and kept for one minute on the slide and washed away.

After placing the crystal violet stain for a minute the slide should be washed in a running tap water. Later, Gram's iodine is added and is left for one minute and washed away.

Decolorizer is added after washing off the gram's iodine solution. Adding too much decolorizer can give a wrong result. Ethanol should be on the slide only for 15 seconds. Placing decolorizer for a long time can kill the purple stain.

After washing off the decolorizer safranin solution is added and is kept for one minute on the slide and later washed away with water.

After adding all the reagents allow the slide to dry and view it under microscope.

Table 4.1 Gram staining

APPLICATIONS OF THE EACH DYE.	GRAM STAINING REAGENTS	GRAM POSITIVE CELLS	GRAM NEGATIVE CELLS

PRIMARY DYE	CRYSTAL VIOLET	PURPLE	PURPLE
TRAPPING AGENT	GRAM'S IODINE	PURPLE	PURPLE
COUNTER STAIN	SAFRANIN	PURPLE	PINK OR RED

After adding the stains the slide is viewed under a microscope.



Fig 4.19 Microscope

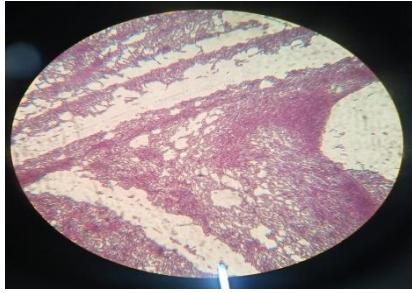


Fig 4.20 Organism A microscopic view

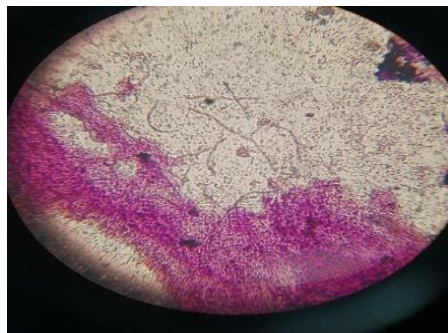


Fig 4.21 Organism B microscopic view

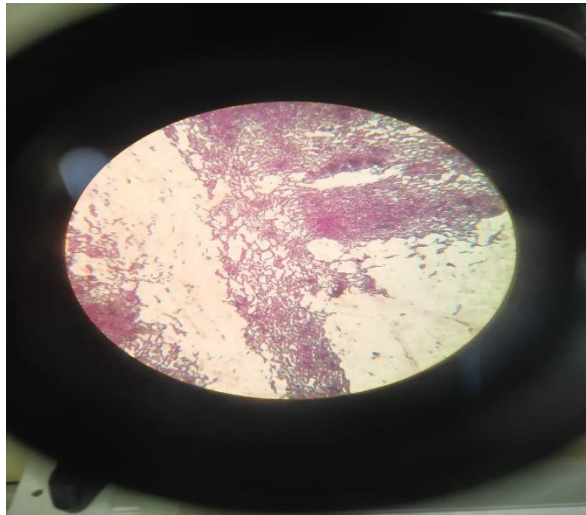


Fig 4.22 Organism C & D microscopic view

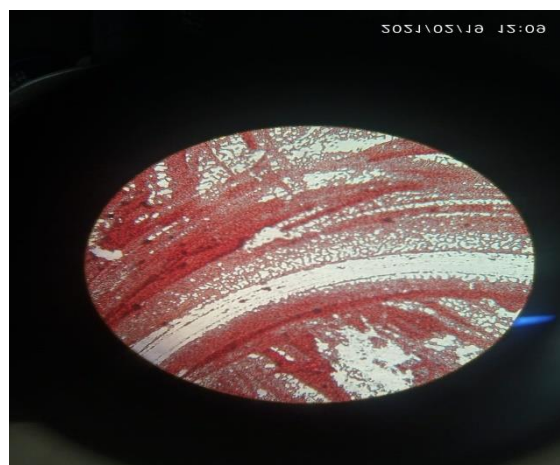


Fig 4.23 Organism E microscopic view

4.2.6 BIOCHEMICAL TESTS FOR BACTERIA

4.2.6.1 Catalase test

Catalase is an enzyme which converts Hydrogen peroxide into oxygen and water. This is a primary test used for identifying gram negative and gram positive organisms and also differentiates aerobic and anaerobic bacteria. Louis Jaccques Thunard was the first scientist to discover the catalase and hydrogen peroxide. Take a clean glass slide the suspected colonies are placed on each slide and a loopful of culture was taken with a wire loop and 3 to 5% of Hydrogen Peroxide was added and the reaction was observed.

4.2.6.2 Urease test

Urease was discovered by Jack bean in 1975. Urease test is performed to identify those organisms that produce ammonia and carbon dioxide. Slant was prepared with Christenson Urea agar and the culture was inoculated in the slant and incubated for 24 hours at 37 degree celcius and later observed for reaction.

4.2.6.3 Voges Prokauer test

VP test is used to detect acetoin in bacterial culture. Voges and Proskauer in 1898 first observed the red colour after adding potassium hydroxide to cultures that were grown on a specific media. Later it was discovered that the production of red colour was due to acetyl-methyl carbinol production. Tryptone broth was prepared for 5ml in a test tube and the culture was added. The test tube was incubated for 24 hours and 1ml of alpha naphthol solution and potassium hydroxide solution was added and shaken well. By adding few drops of solutions the test tube was observed for any colour changes.

4.2.6.4 Methyl Red Reaction

5ml tryptone broth was taken in a test tube and the culture was added. It was incubated for 24 hours and after incubation two three drops of Methyl red solution was added to observe the reaction.

4.2.6.5 Citrate utilization test

This test helps to identify the ability of a microorganism to use citrate as a carbon source. The citrate depends upon enzyme citrase that breaks down citrate to oxalo acetate and carbonat. Sterlized citrate agar was made into slant and bacterial cultures were streaked in the slant and kept for incubation for 24 hours. After incubation tubes were checked for colour change.

4.2.6.6 Indole Production

5ml of tryptone broth was taken in a test tube and culture was added. It was inoculated for 24 hours and add two drops of Kovac's reagent and the test tube is observed for any reaction

4.2.6.7 Starch hydrolysis test

In a clean sterilized petridish nutrient agar was prepared and poured. The culture was streaked on the petridish and was kept in an incubator for 24 hours. After incubation the streaked plate has some growth. Two to three drops of iodine solution was flooded and excess of iodine solution was removed to observe any changes in the Petridish.

4.2.6.8 Bile tolerance test

The culture were grown in a nutrient broth at 37 degree celcius which contains 2%, 4%, 6% of bile salt mixture. The test tubes were checked at an interval of 1 hour, 3 hour and 4 hour. The growth was checked using pour plate technique where 1ml of culture was placed in a nutrient agar. The plates were incubated and checked for plate counts as colony forming units

4.2.6.9 Indole reagent test

A filter paper is taken and culture was placed with a plastic loop and two three drops of indole reagent was added to observe any changes

4.2.6.10 Triple Sugar Ion test

This test is to identify the microorganisms that can ferment sugars. This test was originally discovered by Sulkin and Willet in 1976. A slant was prepared using TSI agar and inoculated with culture by stabbing and streaking the slope. The test tube was inoculated for 24 to 48 hours and later was observed for any black appearance due to H₂S production and gas fermentation.

4.2.7 TOTAL NUMBER OF E. COLI PRESENT IN VEGAN CURD

In a 250 ml of conical flask 1.44 grams of EMB Agar was prepared and diluted in 40 ml of distilled water. Two petridishes were taken and marked as control and the other plate for observing growth of E. Coli. The dishes were autoclaved for fifteen minutes for 15 Lbs and the agar was poured on the petridish to solidify. Meanwhile the curd sample was diluted with distilled water and spread plate technique was performed and incubated at 37 degree celcius for 24 hours.



Fig 4.24 EMB Agar and Petridish

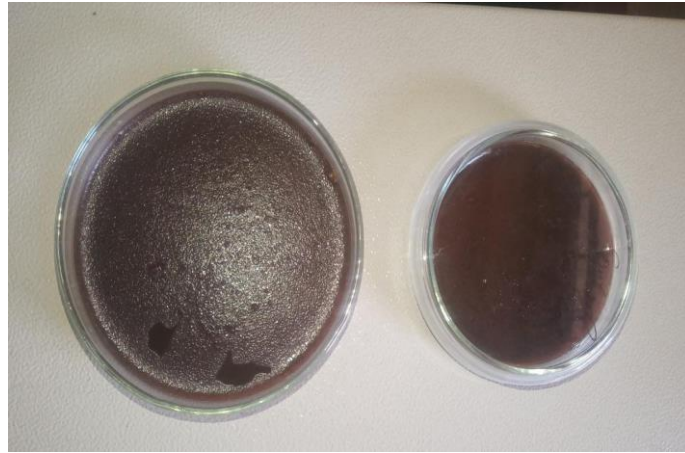


Fig 4.25 E.Coli plate and control.

CHAPTER 5 – RESULTS AND DISCUSSIONS

5.1 NUTRITIONAL ANALYSIS

5.1.1 Soxhelet method (Fat content)

Table 5.1 Fat content value for milk curd

FAT CONTENT	MILK CURD	VEGAN CURD
A	2.58%	4.2%

5.1.2 Kjeldahl method (protein content)

Table 5.2 Fat content value for vegan curd

PROTEIN CONTENT	MILK CURD	VEGAN CURD
A	0.4%	0.7%

5.2 ISOLATION OF BACTERIA

5.2.1 Total plate count

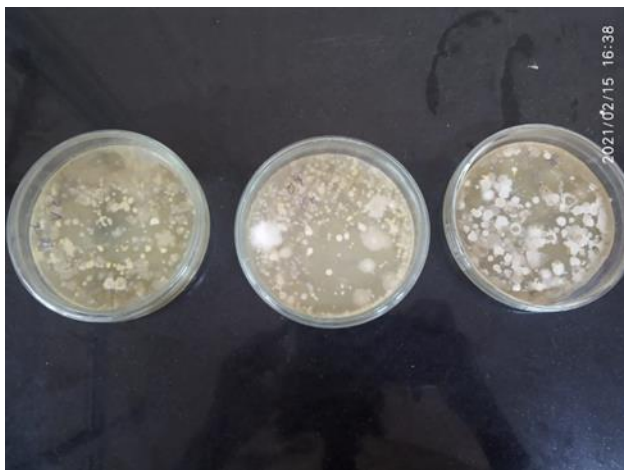


Fig 5. 1 Colony growth on 10^{-4} , 10^{-6} , 10^{-8} plates

Table 5.4 Colony forming unit

DILUTIONS	VEGAN CURD	CFU
4	50	5.0×10^{-5}
6	40	4.0×10^{-6}
8	37	3.7×10^{-8}

5.2.2 Gram Staining

This method is performed to differentiate between gram positive and gram negative bacteria. This method was first discovered by the Danish bacteriologist Hans Christian Gram. The cell walls have different physical and chemical properties, by using gram staining technique the thick layer and the thin layer of peptidoglycan in the cell reacts to the stains that are added to the bacteria. For example: thick layer absorbs the violet stain and the thinner peptidoglycan absorbs the red stain which is called as safranin.

Table 5.5 Gram staining

APPLICATIONS OF THE EACH DYE.	GRAM STAINING REAGENTS	GRAM POSITIVE CELLS	GRAM NEGATIVE CELLS
PRIMARY DYE	CRYSTAL VIOLET	PURPLE	PURPLE
TRAPPING AGENT	GRAM'S IODINE	PURPLE	PURPLE
COUNTER STAIN	SAFRANIN	PURPLE	PINK OR RED

After adding the stains the slide is viewed under a microscope.



Fig 5.2 Microscope

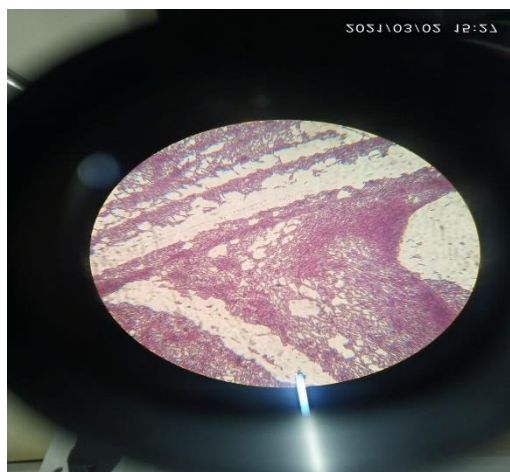


Fig 5.3 Organism A microscopic view

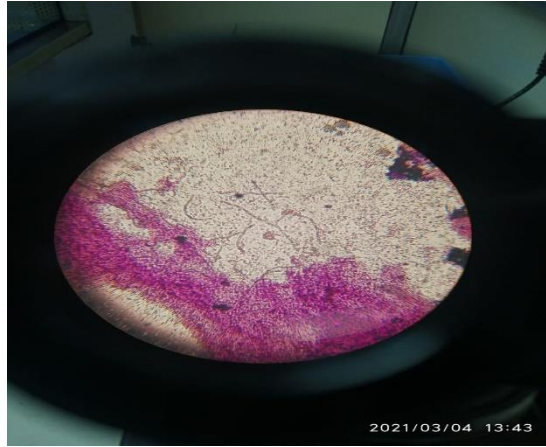


Fig 5.4 Organism B microscopic view

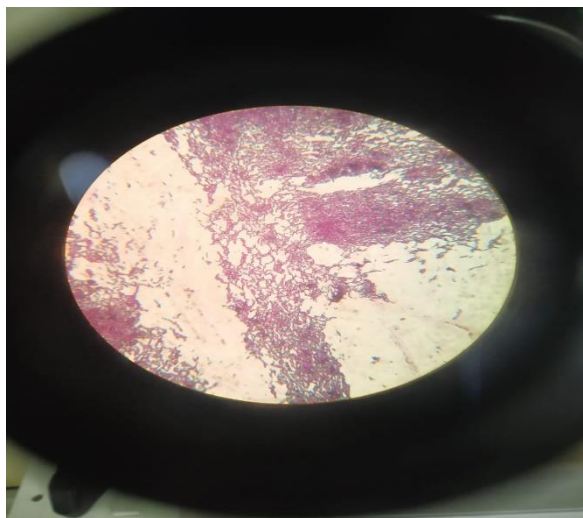


Fig 5.5 Organism C & D microscopic view

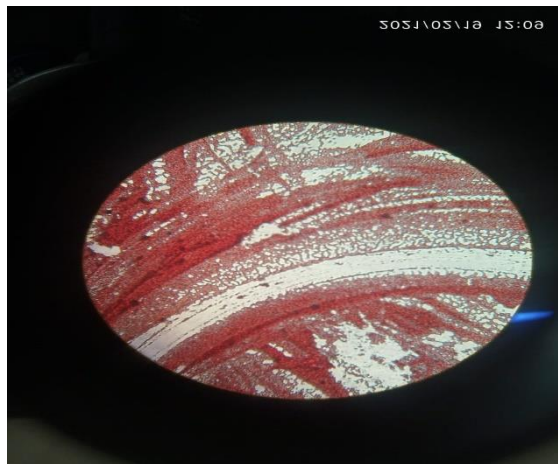


Fig 5.6 Organism E microscopic view

Table 5.6 Identification of bacteria

ORGANISMS	GRAM STAINING RESULTS
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A	Positive, rod shaped
B	Positive, rod shaped
C	Positive, rod shaped
D	Positive, cocci shaped
E	Negative, rod shaped

5.3.3 Biochemical tests

Biochemical tests are usually performed to characterize the bacteria colonies that were grown on the Agar plates. These tests differentiate several organisms.

CHARACTERISTICS	A	B	C	D	E
COLONY MORPHOLOGY	White,opaque buldged	Transparent Bright chained	Single colony, White, opaque	White, Large Opaque smooth	Yellow colony
GRAM STAINING	+	+	+	+	-
SPORE FORMATION	-	-	-	-	-
INDOLE TEST	+	-	-	-	-
METHYL RED	-	-	-	-	+
VP	-	-	-	-	-
CITRATE UTILIZATION	+	+	+	+	-
UREASE	+	+	+	-	-

CATALASE	-	-	-	-	-
TRIPLE SUGAR ION	+	-	+	+	-
STARCH HYDROLYSIS TEST	-	-	-	+	-

Table 5.7.Biochemical tests for bacteria



Fig 5.7 Biochemical tests for A

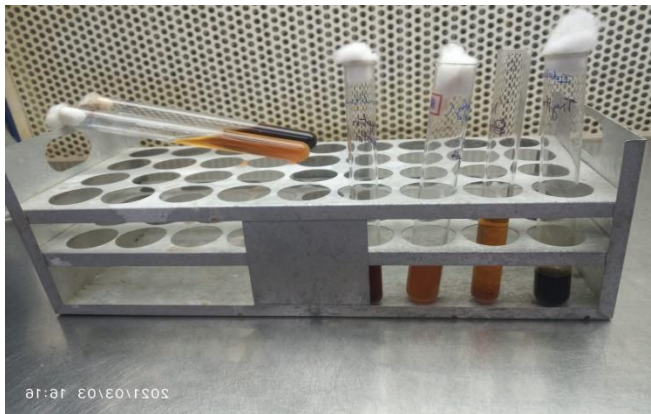


Fig 5.8 Biochemical tests for B

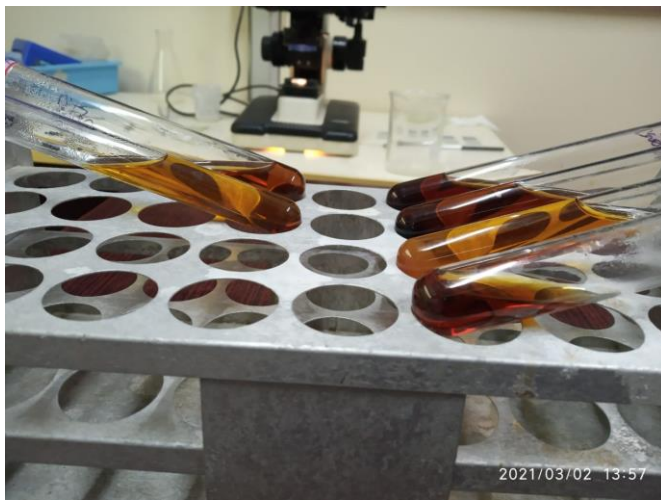


Fig 5.9 Biochemical test for C & D



Fig 5.10 Starch hydrolysis streakplate



Fig 5.11 Starch hydrolysis test



Fig 5.12 Biochemical test for organism E.

4.2.7 TOTAL NUMBER OF E.COLI PRESENT IN VEGAN CURD



Fig 5.13 Total number of E.coli

Table 5.8 E.Coli count

Total plates	Growth observation	Colony count
Control	No growth	None of the colonies
EMB agar plate	colonies were observed after incubation	Ten E.Coli colonies

5.3 CHARACTERIZATION OF BACTERIA

Table 5.8 Characterization of bacteria

ISOLATED BACTERIA	CULTURE CHARACTERISTICS	COLONY MORPHOLOGY
Lactobacillus acidophilus	Small to medium grey colonies	Convex,small, rough edges and white
Lactobacillus lactis	Bright orange	They occur in pairs and short chains
Lactobacillus cremoris	Transparent white chains	non spore forming.
Streptococcus thermophilus	White colony and circular	They occur in chains and pairs and are non motile.
Bacillus anthracis	Flat, slightly convex	Colonies have a sticky consistency with irregular edges and ground glass
Micrococcus	Yellow colony	They grow in clusters

CHAPTER 6 – CONCLUSION

Milk curd has many rich sources and helps in digestion and has good gut bacteria which fights the pathogens in our body. They produce lactic acid bacteria through fermentation. They have the highest nutritive value and contains more probiotics. The bacteria present in the curd is *Lactobacillus bulgaricus*. But due to lactose intolerance in few people milk curd cannot be consumed. As a substitute vegan curd can be taken for consumption and they are good for digestion. The bacteria present in vegan curd are *Lactobacillus acidophilus*, *Streptococcus thermophilus*, *Bacillus anthracis* which are probiotics and are equally healthy and nutritive than the milk curd. This curd can also be prepared by adding toppings and other flavours for good appearance and taste. Vegan curd doesn't cause lactose intolerance. This determines that the organisms present in the Vegan curd fights against harmful infections and has high protein content which is good for the gut and also boosts immunity.

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