

Characterization of Mutational Studies to Enhance the Curdling Activities in *Lactobacillus* sp

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Abstract: Lactic acid-producing bacteria are associated with various plant and animal niches and play a key role in the production of fermented foods and beverages. Utility of related proteomic or gene expression patterns which may improve their use as starter cultures and probiotics. Starter lactic acid bacteria in Cheddar cheese face physico-chemical stresses during manufacture and ageing that alter their abilities to survive and to interact with other bacterial populations. Nonstarter bacteria are derived from milk handling, cheese equipment and human contact during manufacture. Probiotic bacteria are added to foods for human health benefits that also encounter physiological stresses and microbial competition that may mitigate their survival during ageing. Probiotic *Lactobacillus acidophilus*, their survival period over 270 days of ageing and the role of the cheese matrix influences in their survival and production. Present study focused on strain improvement by Mutational studies from UV radiation to enhance the Curdling activities in *Lactobacillus* sp, Isolation of micro-organism from curd sample, Identification of micro organism followed by evaluation of temperature, Enhancement of strain by mutation and Enzyme purification carried out by Ammonium sulphate Precipitation, Ion- exchange chromatography with 2% DEAE and characterization by SDS –PAGE and RAPD analysis. The mutated strain with the bacteria isolated and identified was *Lactobacillus acidophilus* from the curd sample. The maximum reduction in time was obtained in 10 minutes with 15 KDa Size, the beneficial yield in curd production from this strain can improve the process optimization strategy in the dairy technology

Keywords: *Lactobacillus acidophilus*, Strain improvement, Enzyme purification, Ion-exchange chromatography, SDS – PAGE and RAPD analysis

1. Introduction

The art of lactic acid fermentation is one of the oldest technologies that preserve the milk in a wide diversity of flavours, aromas and texture [1]. At present, more than 400 traditional and industrialized fermented dairy products are available in the global market. Among them curd is very popular in part of globe including Indian subcontinent. About 9% of the total milk is converted into fermented dairy products in India [2].

Antioxidant research has become a major scientific pursuit because of the evidence linking oxidative stress with many diseases and because of the potential food preservative applications. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are activated forms of oxygen and nitrogen [3] and other non-radicals, arise from various endogenous and exogenous pathways. ROS and RNS have been implicated in the aetiology of numerous pathological conditions [4]. Oxidative stress results when the oxidant/antioxidant ratio tilts in favour of oxidant factors; it is involved in the aging process and also causes inflammation. Free radicals attack cellular components leading to the oxidation of lipids, proteins and DNA, thus causing structural and functional changes of these molecules [5]. Oxidation of food constituents is also a key event in food spoilage. The higher nutrition value of the curd and its consumption are health beneficial due to decrease in lactose intolerance, immune-modulation, diarrhoea prevention, serum cholesterol reduction, and adverse effects of drugs, etc [6, 7].

Lactobacillus is a genus of gram-positive facultative anaerobic or micro-aerophilic rod-shaped bacteria [8]. They are a major part of the lactic acid bacteria group, named such as because most of its members convert lactose and other sugars to lactic acid. In humans they are present in the vagina [9] and the gastrointestinal tract, where they make up a small portion of the gut flora.

The production of lactic acid makes its environment acidic, which inhibits the growth of some harmful bacteria. Some *Lactobacillus* species are used for the production of yogurt, cheese, sauerkraut, pickles, beer, wine, cider, kimchi, cocoa, and other fermented foods, as well as animal feeds such as silage. Some strains of *Lactobacillus* spp. and other lactic acid bacteria may possess potential therapeutic properties including anti-inflammatory and anti-cancer activities, as well as other features of interest. *Lactobacilli* can also be used to restore particular physiological balance such as in the vaginal ecosystem [10, 11, and 12].

Their role is- (a) to physically protect the vaginal epithelium by building a thick layer separating the epithelium from pathogens, (b) to physiologically keep the balance of the vaginal ecosystem in maintaining the pH at ~ 4.5, (c) generating hydrogen peroxide against pathogens.

It has several nutritional benefits such as vitamin B production, enhances gastrointestinal health acting as antiallergenic, antimicrobial, antihypertensive, anti-tumorigenic and antioxidant. It has therapeutic benefits such as restoration of the ecological balance of intestinal micro-flora, alleviation of lactose intolerance, enhancement of

immunity (Nutritional and therapeutic response). *Lactobacilli* are highly tolerant to low pH and can easily maintain low pH and protect the vaginal eco-system from gram-negative and gram-positive bacteria. It also helps to maintain the endogenous redox balance to prevent several pathophysiological conditions [13].

The previous methods used for introducing mutation in *Lactobacillus* to increase the curd frequency are by (a) recombinant DNA techniques which include isolation of plasmid DNA [14, 15, 16], isolation of total DNA [17] use of restriction endonucleases, DNA polymerases, T4 DNA ligases, construction of mutant strains, construction of mutant vector; (b) by using chemical mutagen like N-methyl-N'-nitro-N-nitrosoguanidine (NTG); (c) by environmental conditions like temperature, high salt concentration, starvation, etc [18].

The determination of curd frequency can be determined by many ways like by observing the structural changes in the curd sample during the fermentation process using scanning electron microscope (SEM), by analyzing the degradation of proteins into peptides and amino acids with the help of SDS-PAGE and amino acid analysis [19]. The anticipated genetic organization of transformants produced during DNA techniques can be analyzed by using PCR [20].

However, the preparation of quality curd depends on the milk, fermentation temperature, and potentiality of the starter culture, fermentation vessels and storage conditions. In the present study the bacterial strain was isolated and identified on the basis of biochemical tests.

The bacterial strain was used as fresh inoculum and mutated for different time intervals and checked for the probiotics, intensity of curd formation and antioxidant activity.

2. Materials and Methods

2.1 Isolation of Bacteria by Pour Plate Method

The curd sample was collected. 1 ml of curd was poured in 10 ml of saline solution. Saline solution was prepared by taking 0.1g of NaCl in 10 ml of distilled water and autoclaved. Saline solution with curd sample was poured on autoclaved petri-plates. Autoclaved nutrient media was poured on it and allowed to solidify. The plate was incubated in inverted position at 37°C for 24 hours.

2.2 Pure Culturing of the Organism

The nutrient agar slants were prepared, sterilized and used for pure culturing of the organism. A loopful of culture from each colony from the culture plates was taken and streaked into the slants. Then the slants were incubated at 37°C for 24 hr.

2.3 Maintenance of Pure Cultures

Pure cultures were maintained by sub-culturing the pure cultures at intervals of every one week followed by incubation at 37°C for 24 hours, after which the cultures were preserved at 4°C.

2.4 Identification of Bacteria

Bacteria were identified on the basis of Gram's staining, endospore staining and biochemical tests.

2.5 Biochemical Tests

- (a) Indole test
- (b) Methyl Red (MR) and voges proskauer (VP) test
- (c) Citrate utilization test
- (d) Catalase test
- (e) Oxidase test
- (f) Gelatin hydrolysis test
- (g) Casein hydrolysis test
- (h) Urease test
- (i) Hydrogen sulphide production test
- (j) Starch hydrolysis test
- (k) Carbohydrate fermentation test

2.6 Preparation of Curd and Collection of whey Fraction

50ml of cow milk was pasteurized at 75°C for half an hour. Then cooled to 37°C and loopful of bacterial culture was inoculated and allowed for fermentation at 37°C. The non-hydrolysed casein was removed by centrifugation at 10,000 rpm for 10 minutes. The collected whey was used for all the assays.

2.7 Induction of Mutation

Physical mutation using UV radiation is used to induce mutation in *Lactobacillus*. Cotton swab method was used for it. LB agar media was prepared and autoclaved. The prepared media was poured in the 4 different petri-plates labelled as 5, 10, 15 and 20 minutes. The media was allowed to solidify. Suspension of pure culture was prepared by adding loopful of pure culture in 1 ml of autoclaved distilled water. The drops of suspension were spread on the solidified media using cotton swabs for all the 4 petri-plates. UV light of laminar air flow was switched on for 5, 10, 15, 20 minutes for the respective petri-plates. The plates were incubated at 37°C for overnight in incubator.

2.8 Analysis of Probiotics

2.8.1 Protease Assay

The activity of protease was estimated by plotting a standard graph of L-tyrosine. Different aliquots from 0.2-1.0 ml of L-tyrosine (1mg/ml) were taken. Volume was made up to 1 ml using distilled water. 5 ml of 500mM sodium carbonate was added to it. To this 1ml of 0.5 M Follin- Ciocalteu reagent was added, mixed with swirling and incubated at 37°C for 30 minutes, allowed to cool at room temperature and filtered. Absorbance was read at 660nm.

The obtained absorbance values were plotted in the standard graph to obtain the respective concentrations of L-tyrosine. The activity was calculated by putting the concentration of L-tyrosine in the formula-

$$\text{Activity} = \frac{\text{micromole of L-tyrosine} \times 11}{1 \times 10 \times 2}$$

Where 11 is the total volume of assay, 10 is the incubation

time, 2 is the value of calorimetric determination and 1 is the volume of enzyme.

2.8.2 Lipase Assay

Seven test tubes were taken and marked as blank, BM1, BM2 (before mutation), M5, M10, M15, M20 (mutated for 5, 10, 15, 20 minutes respectively). 2.5 ml of distilled water was taken in all the test tubes. 1ml of 200mM Tris HCl buffer (pH 7.2) was added to all the test tubes. To this 3.0 ml of olive oil was added to all test tubes, mixed by swirling, equilibrated to 37°C for 30 minutes. After incubation the 1ml content of test tubes were transferred to conical flasks and allowed to cool. 3 ml of 95% ethanol was added to all the conical flasks, mixed by swirling. Four drops of phenolphthalein indicator was added and titrated against 50mM NaOH solution till light pink colour was obtained. Three concordant reading was taken for each sample. Activity of lipase was calculated by putting the volume of NaOH rundown in the formula-

$$\text{Activity} = \text{Volume of NaOH} \times 0.05 \times 1000 \times 2 / 1$$

Where, 0.05 is the molarity of NaOH, 1000 is the conversion equivalent from mili-equivalent to micro-equivalent, 2 is the time conversion factor from 30 minutes to 1 hour and 1 is the volume of the enzyme.

2.8.3 Reductase Assay

Seven test tubes were taken and marked as blank, BM1, BM2 (before mutation), M5, M10, M15, M20 (mutated for 5, 10, 15, 20 minutes respectively). To all test tubes 1.5ml of 0.1M potassium phosphate buffer (pH 6) was added. To this 0.5ml of 0.2mM NADH added to each tube. 0.5 ml of respective enzyme samples are added to the respective test tubes. 0.5ml of distilled water was added to the blank. All the test tubes were incubated for 15 minutes at room temperature. Absorbance was read at 340nm. activity was calculated using the formula

$$\text{Activity} = A_{340} \times 100 \times 3 / 63 \times 0.5 \times 15$$

Where A_{340} is absorbance read at 340 nm, 3 is total volume of assay, 63 is molar extinction coefficient of NADH at 340nm, 0.5 is the volume of enzyme, 15 is the incubation time.

2.8.4 Lactic Acid Assay

Curd for before and after mutation was prepared using the respective inoculums. Then 10ml of the product to be tested is measured and taken in conical flask. 30 ml of distilled water was added to this and mixed well. 5 drops of phenolphthalein indicator was added and titrated against 0.1N NaOH in burette. Titrate till the pink color appeared. Titration was repeated for three concordant readings. Same procedure was followed for all the samples. Percentage of lactic acid in sample was calculated using the formula

$$\% \text{ lactic acid} = \text{Volume of NaOH} \times 0.1 \times 0.09 \times 100 / 10$$

Where 0.1 is the normality of NaOH, 0.09 is the mili equivalent of lactic acid, 10 is the volume of sample.

2.8.5 Antioxidant Activities

2.8.5a DPPH Radical Scavenging Assay

Antioxidant capacity of the whey was determined 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method with some modifications (22). 100 micro litre of whey was added to 1.9ml of 0.1 mM DPPH in methanol up to completing 2ml. After 10 min incubation free radicals scavenging capacity was evaluated by measuring the decrease of absorbance at 517nm. Antioxidant capacity was expressed as mM of TROLOX equivalent (TE)/L of whey, using the calibration curve varying the concentration 20-100 micro gram/ml.

$$\text{Scavenging activity (\%)} = (1 - A_{\text{sample}} / A_{\text{control}}) \times 100.$$

Where A_{sample} was the absorbance in the presence of the sample or reference material and A_{control} was the absorbance of the control containing all the reaction reagents expects the sample or reference material.

2.8.6. Purification and characterization of Enzyme

The mutated bacterium and its original bacterial culture were centrifuged at 10,000rpm for 10 minutes and the supernatant was collected for the isolation of enzyme.

2.8.6a. Enzyme Purification

In purification, many steps such salt precipitation, dialysis, ion-exchange chromatography and SDS-PAGE were used.

2.8.6b Ammonium Sulphate Precipitation

Proteins are usually soluble in water because they have hydrophilic amino acids on their surfaces that attract water molecules and interact with them. Thus solubility is a function of the ionic strength and pH of the solution. Proteins have iso-electric point at which the charges of their amino acids side group balance each other. If the strength of a solution is either very high or very low, proteins will tend to precipitate at their iso-electric point.

The solubility is also a function of ionic strength and as we increase the ionic strength by adding salt, proteins will precipitate. Ammonium sulphate is the most commonly used chemical for precipitation since it gets completely soluble in many solvents and also does not alter pH.

2.8.6c Dialysis

Dialysis is the process of separating molecules in solution by the difference in their rates of diffusion through a semi permeable membrane, determined by their sizes. It is done to remove the small molecules from the crude enzyme.

2.8.6d. Ion-exchange Chromatography

Ion-exchange separations are carried out mainly in columns packed with an ion-exchanger. There are two types of ion-exchanger, namely cation and anion exchangers. Cation exchangers are also called acidic ion-exchange materials because their negative charges result from the ionization of acidic groups. Anion exchangers have positively charged groups that will attract negatively charged anions.

Materials Required

Chromatography column (2% DEAE), protein sample after dialysis, elution buffers. Stationary phase: DEAE cellulose. Mobile phase: Elution buffers

2.8.6e. Pre-processing of column:

- The chromatography column was first rinsed with the methanol and then washed with the distilled water.
- The column packed with the DEAE cellulose (anionic exchanger) was washed with distilled water two times and kept for degassing for 10-15 minutes.

Procedure:

- The matrix was activated using activation buffer I.
- The dialyzed enzyme sample was poured into the column followed by the elution buffer to elute the enzyme. Five ml of elutant was collected per tube. The process of elution was carried out using solutions II, III, IV, V & VI which contains different concentration of NaCl.

2.9 SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis)-

SDS-PAGE is used to separate the proteins according to their size. SDS is a detergent that can dissolve hydrophobic molecules and gives negative charge to it. The SDS results two important features 1) all proteins retain only primary structure 2) all proteins have a large negative charges which means they will migrate towards the anode.

If the proteins are denatured and put into an electric field, they will all move towards the anode at the same rate, with no separation by size. So we need to put the proteins into an environment that will allow different sized proteins to move at different rates. The environment of choice is polyacrylamide, which is a polymer of acrylamide monomers.

Materials required: Separating gel, stacking gel buffer, electrode buffer, separating gel, stacking gel, staining solution and destaining solution.

2.9.1. Protein estimation by Lowry's Method**2.9.2. RAPD (Rapid amplified polymorphic DNA)-****2.9.3. Preparation of broth culture-**

50ml of nutrient broth was prepared and autoclaved. A loopful of inoculum was inoculated and incubated for 24 hrs.

2.9.4. Isolation of genomic DNA-

1ml of overnight culture was taken in Eppendorf tube. It was centrifuged at 6000 rpm for 5 min at 4°C. Supernatant was discarded and 500 micro liters of lysis buffer (25mM Tris HCl, 10mM EDTA, 10% SDS) was added to the pellet.

Incubated at 65°C for ½-1 hour and allowed to come at room temperature. 300 micro liters 3M anhydrous sodium acetate was added to it. Incubated in ice-cold condition for 10 min. Centrifuged at 10,000 rpm for 10 minutes at 4°C. Double volume of ethanol was added to the supernatant, Incubate in ice-cold condition for ½ hour at 4°C.

Centrifuged at 12,000 rpm for 10 minutes, Supernatant was discarded and pellet was dissolved in 50 micro liters of TE buffer (10mM Tris HCl, 1mM EDTA).

2.9.5. Agarose gel electrophoresis-

0.50g of agarose was dissolved in 50ml of TBE buffer (Tris borate buffer). It was heated till it dissolved completely. 2 micro liters of ethidium bromide was added to it. The gel was poured in the casting tray and the comb was inserted and allowed for solidification. After solidification the comb was removed and the tape was also removed which was attached to the gel casting tray.

The gel was with gel plate was placed in electrophoretic tank containing TBE buffer (wells should be dipped in the buffer). Then on a plastic 5 micro liters of loading dye and 5 micro liters of genomic DNA sample was mixed and 10 micro liters of sample was loaded to the well.

The same procedure was followed for the entire sample. The gel was allowed to run till 3/4th and then stopped. The gel was seen under UV-transilluminator to see the bands.

3. Result & Discussion**3.1 Collection of Sample**

Curd was used for the collection of Lactobacillus species. Isolation of microorganism : 1 ml of curd sample was poured in 10 ml saline solution (0.1 gm NaCl in 10 ml of Distilled water).this was poured on petri plate than media was poured .it was allowed for solidification and kept for incubation in inverted position over night.

3.2 Identification of microbes (Gram staining):

In order to identify, whether the bacteria is positive or negative for Gram's stain, Gram staining was done for all the pure cultures. The observation indicated that all the organisms tested were found to be gram positive since all of them have shown purple colour under microscope.

3.3 Analysis of probiotics

Protease assay – the protease assay was done to find the activity of enzyme and estimated using the formula:-

Activity= micromole of tyrosine equivalent released x total volume of assay

Vol. of enzyme X incubation time X vol. of calorimetric determination

Where, Total volume of assay = 11. Volume of enzyme = 1. Incubation time =10. Volume of calorimetric determination = 2.

The activity of protease has increased after mutation (Table 1). This leads to the reduction in curd formation time decreased in the mutated sample which leads to fast formation of curd.

Table 1: Activity of Protease in pure sample and mutated sample

| Sample | Activity |
|-------------------|-----------------|
| 1)Pure culture | 0.165 units/ml |
| 2) After mutation | |
| 5 min mutation | 0.1983 units/ml |
| 10 min mutation | 0.3075 units/ml |
| 15 min mutation | 0.2347 units/ml |
| 20 min mutation | 0.2509 units/ml |

Table 2: The activity of lipase in pure and mutated sample

| Sample | Activity |
|-----------------|--------------|
| 1)Pure culture | 110 units/ml |
| After mutation | |
| 5 min mutation | 120 units/ml |
| 10 min mutation | 180 units/ml |
| 15 min mutation | 150 units/ml |
| 20 min mutation | 130 units/ml |

Table 3: Activity of reductase in pure and mutated sample

| Sample | Activity |
|-----------------|-----------------|
| 1)Pure culture | 0.0336units/ml |
| After mutation | |
| 5 min mutation | 0.1245 units/ml |
| 10 min mutation | 0.2209 units/ml |
| 15 min mutation | 0.1073 units/ml |
| 20 min mutation | 0.2012 units/ml |

Cow milk also contains whey protein such lactalbumin and lacto globin the denaturing of these whey proteins, using proteases, result in creamier yogurt product denaturation is an advantage in yogurt production improves the texture of yogurt.

This principle is also used to create specialized why ingredients with unique functional properties for use in foods. In this experiment the activity protease has increased after mutation which clearly shows that denaturation time has decreased in the mutated sample which leads to fast formation of curd.

Table 4: Percentage of lactic acid in pure and mutated sample

| Sample | Percentage |
|-----------------|------------|
| 1)Pure culture | 0.117% |
| After mutation | |
| 5 min mutation | 0.198 % |
| 10 min mutation | 0.180 % |
| 15 min mutation | 0.171 % |
| 20 min mutation | 0.189 % |

Lipase assay – the lipase assay was done to find the activity of enzyme and estimated by the formula:

$$\text{Activity} = \frac{\text{vol. of NaOH} \times \text{molarity of NaOH} \times 1000 \times 2}{\text{Vol. of enzyme}}$$

Where, Molarity of NaOH = 0.05M. 1000= conversion factor for mili equivalent to micro equivalent 2 = time conversion factor from 30 mint to 1 hour. Volume of enzyme = 1 ml. The activity of lipase is more in mutated sample than

that of in pure culture (Table 2) which leads to reduction in time for the curd formation.

Lipases are used to break down milk fats and give characteristic flavour to cheese. It catalyzes the hydrolysis of fats (lipids). Here, in this experiment there is increase in the activity of two mutated samples (10 minutes and 15 minutes), hence, reduction in time of curd formation.

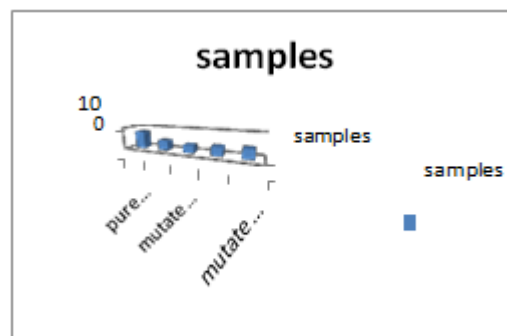
Reductase assay- The reductase test was done to estimate the specific activity of enzyme and estimated using the following formula:

$$\text{Activity} = \frac{\text{Absorbance at 254nm} \times \text{Vol of reaction mixture}}{\text{Vol of enzyme} \times \text{incubation time}}$$

Molar extinction co-efficient of NADH x Vol of enzyme x incubation time

Where, Molar extinction co-efficient of NADH = 63. Volume of reaction mixture = 3 ml. Volume of enzyme = 0.5ml. Incubation time= 15 min

The activity of reductase has increased for mutated sample than for pure sample (Table 3) which leads to reduction in time for curd formation.



Lactic acid – The lactic acid assay was performed and its percentage is estimated on the basis of the formula-

$$\text{Percentage} = \frac{\text{Vol of NaOH} \times \text{Concentration of NaOH} \times 0.09 \times 100}{\text{Vol of sample}}$$

Where, Concentration of NaOH= 0.1. 0.09= mili equivalent weight of lactic acid. Volume of sample= 10ml

The percentage of lactic acid is more in mutated sample than in pure sample (Table 4), which leads to reduction in time for curd formation.

It found primarily in sour milk products the casein is fermented milk is coagulated by lactic acid.

Here, in this experiment increase in the activity of lactic acid after mutation decreases the time of curd formation.

3.4 Mutation of Bacteria using UV-Radiation

There are many methods available for improvement of strains. Among them, mutation is one of the methods used extensively for strain improvement in industrial

microbiology. In order to reduce the time for curd formation the isolated micro-organism was mutated. The nutrient agar plates swabbed with bacteria were exposed to UV-radiation in order to induce the mutation. The plates were exposed to UV-irradiation for 5, 10, 15 and 20 min. The plates with less time exposure to UV-irradiation showed more colony count than the plates with longer time exposure

3.5 Comparative Study of Time of Curd Formation for Pure and Mutated Sample:

Single colony from each of the time exposure points were selected for curd formation study along with original strain. The strain which was exposed to UV-radiation for 10 min had shown maximum reduction in time for curd formation (i.e-77.41%) in comparison to original strain (i.e. 42.85%) (Fig. 3).

This result indicates that, exposure of bacteria for 10 min to UV radiation could have induced mutations in genes encoding enzymes responsible for curd formation since reduction in curd formation time was observed. By mutagenesis, we could able to successfully induce mutations in original strain to reduce the curd formation time.

3.6 Isolation and identification

The mutated bacterium was subjected to various biochemical tests for its identification up to species level. The biochemical results indicated that the bacteria could be a *Lactobacillus* species

3.6.1 Indole test: Bacteria showed positive result. After addition of Kovacs reagent, a cherry red colour appears in the top. The microbe is able to oxidized tryptophan to indole, pyruvic acid and ammonia present on medium because it contain tryptophanase enzyme.

3.6.2 Methyl-red test: Bacteria showed positive results. After addition of MR reagent the colour changed to red. This organism is capable of producing organic acid and is detected by addition of methyl red, a pH indicator.

3.6.3 Voges-Proskauer test: Bacteria showed negative result. After the addition of Barrets reagent the solution didn't change to pink. This is because this organism produce neutral product, acetoin, and colour remain unchanged.

3.6.4 Citrate utilization test: The bacteria showed the positive test where the colour changed from green to blue in bromothymol blue. This indicates the utilization of citrate and conversion of citric acid to sodium citrate (alkaline pH).

3.6.5 Catalase test: Bacteria showed the negative result. Effervescence was not observed after the addition of the H₂O₂. This indicates that the bacteria do not possess the catalase enzyme that degrades H₂O₂ to water and oxygen.

3.6.6 Oxidase test: The bacterium is oxidase positive. When a loopful of test bacteria was added on the oxidase disc it turned to blue colour. This is due to the oxidase enzyme present in bacteria. The bacteria oxidized the N, N-tetramethyl-p-phenylenediamine present on the disc and gives the blue colour.

3.6.7 Starch hydrolysis: Bacteria showed positive result. The clear zone was observed after the addition of iodine. This indicates that the bacteria produce the enzyme alpha-amylase and use starch as a source of carbon and energy.



Figure 1: Bacteria showed the Positive Result.

3.6.8 Casein hydrolysis: Casein, major protein found in milk, it is hydrolyzed by proteinase (caseinase) enzyme. On the agar plate the clear zone indicate positive result

3.6.9 Gelatin test: Bacteria showed positive result. Due to the presence of gelatinase enzyme in the bacteria the tubes content remain liquid after cooling.

3.6.10 H₂S production: Bacteria showed negative test because organism are unable to produce H₂S gas utilizing the ingredient of the medium. Production of H₂S is detected by H₂S indicators (heavy metal salt) which combine with H₂S produced by reduction of sodium thiosulphate and colour changes to black.

3.6.11 Urease test: Bacteria showed negative result because it lack enzyme urease and colour remain unchanged. This indicates no production of the ammonia and remains yellow colour.

3.6.12 Carbohydrate (sucrose) fermentation test: Sucrose showed positive test for acid production indicated by the change in color of phenol red to yellow with gas was observed in durham tube. This indicates that the Sucrose is fermented to produce the acid end product so the medium changes to yellow from red.

3.6.13 Lactose fermentation test: Lactose showed positive result for acid production and no gas production has taken place because bacteria ferment the lactose and color of the media changed.

3.6.14 Fructose fermentation test: Fructose showed positive test for acid production indicated by the change in colour of phenol red to yellow and gas formation was observed in durham tube. This indicates that bacteria fermented the fructose and colour of the media changed to yellow from red.

3.6.15 Maltose fermentation test: Maltose showed positive test for acid production indicated by the change in color of phenol red to yellow but no gas was observed in durham tube. This indicates that the bacteria ferment the maltose to produce acid end product.

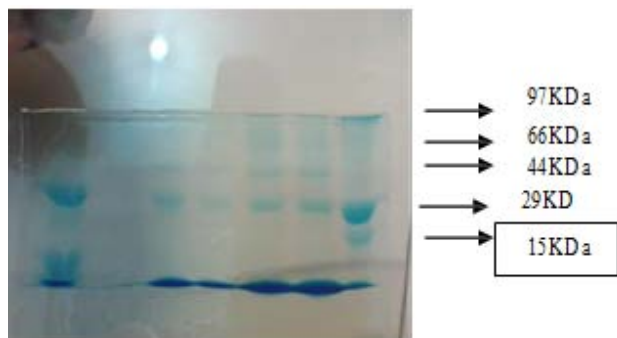


Figure 2: SDS-PAGE analysis of proteins

Marker all blue (Genei Bangalore)

97KDa – Phosphorylase B

66KDa – BSA

44KDa – Ovalbumin

29 KDa- Glutathione S-Transferase

15KDa - Lysozyme

3.7 RAPD

The same sample was used for PCR (RAPD). The smears reflects the amplification of protein bands, denser smear was seen for the mutated culture.

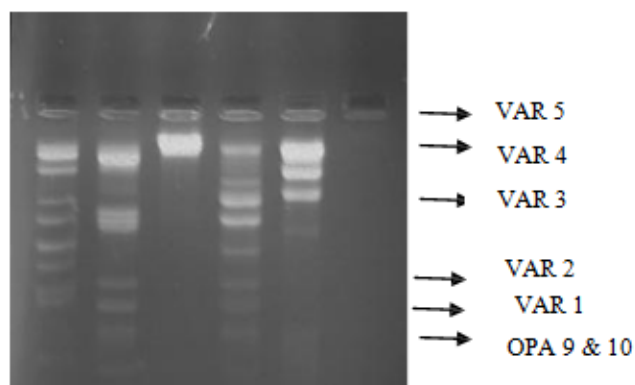


Figure 3: RAPD

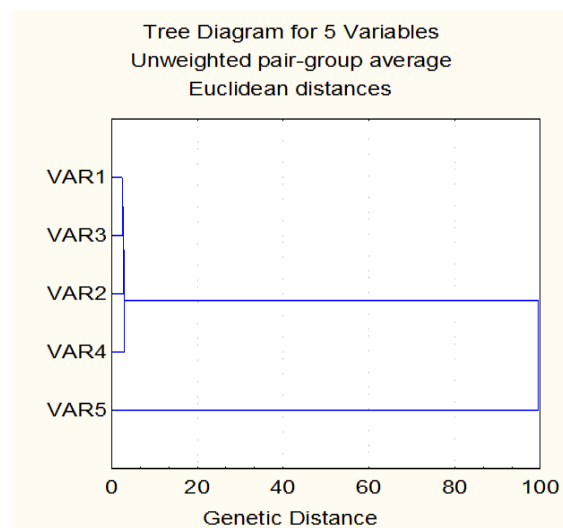
VAR1- Pure culture

VAR2- 5 min mutated culture

VAR3- 10 min mutated culture

VAR4- 15 min mutated culture

VAR5- 20 min mutated culture



3.8 Antioxidant Activity

The DPPH and ABTS radical was scavenged by antioxidants by single electron and hydrogen atom transfer mechanism and transformed in to a yellow or colourless product. The scavenging activity of whey against these radicals was expressed as TE/L of whey. DPPH scavenging activity of the control milk lactobacillus acidophilus was found to be 0.28 mM of TE/L. The DPPH scavenging activity of mutated milk Lactobacillus acidophilus was found to be 0.71 mM of TE/L.

3.9 Enzyme Characterization

3.9.1 Enzyme Purification

The enzyme from the mutated strain was isolated and was subjected to various purification steps such as salt precipitation, dialysis, and ion-exchange chromatography.

The amount of protein in different elution buffer was determined by Lowry's method.

The protein content in the elution buffer of supernatant was estimated by Lowry's method

The sample with highest protein concentration was used for SDS Electrophoresis.

3.9.2 Protein Analysis by SDS-PAGE:

In order to test the presence of protein, SDS –PAGE was performed since the enzymes which are involved in curd formation (Fig. 2). The SDS-PAGE analysis indicated the presence of proteins which may also include the enzymes responsible for curd formation.

4. Future Prospective

Lactobacillus acidophilus has been reported to be beneficial probiotic organisms that provide excellent therapeutic benefits. The biological activity of probiotic bacteria is due in part to their ability to attach to enterocytes. This inhibits the binding of enteric pathogens by a process of competitive exclusion. Attachment of probiotic bacteria to cell surface receptors of enterocytes also initiates signalling events that result in the synthesis of cytokines. Probiotic bacteria also exert an influence on commensal micro-organisms by the production of lactic acid and bacteriocins. Lactobacillus strains can be good candidates as probiotic therapeutic agents for anti-inflammation.

Probiotic bacteria can modulate immune responses in the host gastrointestinal tract to promote health. The genomics era has provided novel opportunities for the discovery and characterization of bacterial probiotic effector molecules that elicit specific responses in the intestinal system. Nutrigenomic analyses of the response to probiotics have unravelled the signalling and immune response pathways which are modulated by probiotic bacteria. These approaches may lead to improved stratification of consumers and to subpopulation-level probiotic supplementation to maintain or improve health, or to reduce the risk of disease [21].

The crystal structure of tannase produced by *Lactobacillus plantarum*, as tannase catalyzes the hydrolysis of the galloyl ester bond of tannins to release gallic acid, although the enzyme is useful for various industries [22].

Some genetic features of *lactobacillar fructan* hydrolases were elucidated, information about their enzymology or mutational analyses were scarce. *Lactobacillus casei* IAM1045 exhibits extracellular activity degrading inulin. After partial purification of the inulin-degrading protein from the spent culture medium, several fragments were obtained by protease digestion. Based on their partial amino-acid sequences, oligonucleotide primers were designed, and its structural gene (*levH1*) was determined using the gene library constructed in the *E. coli* system.

The *levH1* gene encoded a protein (designated as LevH1), of which calculated molecular mass and pI were 138.8-kDa and 4.66, respectively, that the variable domain and [beta]-sandwich module, besides the [beta]-propeller module, are important for inulin-degrading activity of LevH1 [23].

Major factors shaping codon and amino acid usage variation *Lactobacillus sakei* 23K were investigated. It included 13 other *Lactobacillus* species for a comparative analysis. Furthermore, 24 codons that were found to be optimally used by *L. sakei* and its comparative study with 13 *Lactobacillus* species might provide some useful information in their further study of molecular evolution and genetic engineering [24].

5. Conclusion

The bacteria form the curd more efficiently after improving the strain with the mutation. The bacteria isolated and identified was *Lactobacillus acidophilus* from the curd sample. The maximum reduction in time was obtained in 10 minute mutated sample of *Lactobacillus acidophilus*. Here, in this experiment increase in the activity of lactic acid after mutation decreases the time of curd formation. So, we can suggest that the mutated strain of *Lactobacillus acidophilus* can reduce the curd formation time more efficiently and the strain can be used commercially in the dairy industry.

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