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Standardisation and fermentation of milk cream with *Lactobacillus plantarum* and *Streptococcus thermophilus* for minimal moisture content and free fatty acids

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Abstract
Milk is a perishable food and can be converted into various fermented products such as cheese, butter, yogurt, butter milk, butter, ghee, curd of which, curd fermentation is considered as the most valuable process as the products none of them go in vein. For instance milk when fermented forms curd of which the upper fatty layer is butter which can be churned out and remaining water is buttermilk which is considered as a natural cooling agent for the body. Another way of producing butter is by the fermentation of cream that is the fatty layer collected from milk. Milk fermentation process has been relied on the activity of lactic acid bacteria, where trans- formation of milk to good quality of fermented milk products is made possible. Milk fermentation is done with the help of certain bacteria like *Lactobacillus* and *Streptococcus* sp. Both of them are promising cultures to be explored in fermented milk manufacture.

Keywords: milk, fermentation, cream, butter, free fatty acid (FFA), moisture

1. Introduction

Milk is the pale creamish-white liquid produced by mammals to nurture their young ones. It is considered as complete diet, and consumed by most of the people. As far as Indian scenario is considered India is the largest producer of milk and milk products. The major components which are present in milk are water, lactose, fat, protein, minerals and vitamins. Milk contains approximately 87% of water and contains 4.7% of carbohydrates in the form of lactose. The most essential constituent of milk is its fat content, probably determining the thickness of the milk. Milk contains approximately 3.4% fat. The percentage of fat varies with the cattle. It contains 3.3% of protein and all essential amino acids. Cream content might vary from cattle to cattle. Cream can be further classified as fresh cream and sour cream depending on the fermentation and the acidity formed [1]. Sour cream is a dairy product obtained by fermenting regular cream with certain kinds of lactic acid bacteria. There are different types of cream, each of which has different fat concentrations [2] single, cream contains 18% of fat whereas double cream normally contains 30% of fat. The bacterial culture, which is introduced either deliberately or naturally, sours and thickens the cream. The production of lactic acid by bacterial fermentation is called souring. Traditionally sour cream was produced by fermenting the cream that was skimmed off from milk and allowed to ferment at a moderate temperature [3].

The bacteria that developed during fermentation thicken the cream and make sit more acidic, a natural way of preserving it [4]. Traditional sour cream contains 18% to 20 % butterfat. Light sour cream has less butterfat than the regular sour cream. Sour cream is not fully fermented similar to many dairy products. Sour cream and cultured cream is manufactured through the processing of cream that includes pasteurization and the addition of lactic acid bacteria [5]. Cream is prepared from milk by centrifugal separation. Fresh cream, (raw cream) is wholesome, thick and glossy with a silken texture that suits dishes both sweet and savoury. The fresh cream comes from fresh milk that is processed and sterilized at high temperatures and then packaged. It contains 25% of milk fat that is unsweetened; it cannot be used as whipping cream. Commercially available fresh creams are best used to flavour, soups, curries etc. Without opening the packet, fresh cream can be store up to 120 days from the packaging. Cream fermentation modified nutritional and textural properties of butter [6].

Butter is a fermented dairy product [7]. It contains 80% of fat, which is solid when chilled and at room temperature in some region and liquid when warmed. It is made by churning the fresh cream or fermented milk to separate the butterfat from butter milk. It is used as spread on bread and a condiment on cooked vegetables, as well as in cooking, such as baking, sauce making, and pan frying. Butter consist of butterfat, milk protein and water, and in some types added salts [8]. Most frequently made from cow's milk, butter can be taken from milk of other mammals also. Butter is water in oil emulsion and is prepared by agitating cream. Butter contains fat in three separate forms; free butterfat, butterfat crystals, and undamaged fat globules. Churning produces small butter grains floating in the water based portion of the cream [9]. This watery liquid is called buttermilk. Commercially available butter has about 80% butterfat and 15% water content, traditionally made butter may have as little as 65% fat and 30% water. Butter made from fermented cream is known as cultured butter. During fermentation, the cream soured, by bacteria convert milk sugars into lactic acid. The cultured cream is prepared by *Lactobacillus*, *Leuconostoc* bacteria. Industrial production requires the use of starter cultures, which are very carefully created, cultivated and maintained.

Milk fermentation is done with the help of certain bacteria like *Lactobacillus sp* [10]. Lactobacillus is a genus of gram positive, facultative anaerobic bacteria. They are a major part of the lactic acid bacteria group they convert sugar to lactic acid [11]. Milk sugars (mainly lactose) are fermented with the major final product being lactic acid [12]. Lactic acid not only inhibits the out-growth of other organisms but also lowers the pH of the food product imparting a special taste and texture to the food in your mouth [13]. Some species of *Lactobacillus* are used as the starter culture for yogurt, cheese, sauerkraut, pickles etc. *Lactobacillus plantarum* is a widespread member of the genus *Lactobacillus*, commonly found in fermented food products as well as anaerobic plant matters. It has the ability to liquefy gelatine [14]. These bacteria are cultured in Man Rogosa and Sharpe agar (MRS) medium and have significant antioxidant activities and also help to maintain the intestinal permeability [15].

Streptococcus thermophilus is a bacterium of wide industrial application. It is a gram positive, cocci bacteria and is thermophilic in nature and grows at a temperature range above 37°. It is non-motile and doesn't form any spores. The use of this organism is in wide application because of their ability to produce lactic acid [16]. *Streptococcus thermophillus* is a gram positive bacterium and a fermentative bacterium, and fermentative facultative anaerobes. It is also classified as lactic acid bacteria. It is the most widely used bacteria in fermentation industry [17]. Live cultures of these bacteria make it easier for people who are lactose intolerant to digest dairy product. The two organisms used in the experiment were *Lactobacillus plantarum* and *Streptococcus thermophilus*, a mesophile and thermophile respectively.

2. Materials and Method

2.1 Sample Collection

The lyophilized cultures of *Lactobacillus plantarum* (MTCC 9483) and *Streptococcus thermophilus* (MTCC9540) were collected from Microbial type culture collection (MTCC) Chandigarh.

2.2 Revival of Culture

The cultures were revived into MRS (Man, Rogosa and Sharpe) agar by spread plate technique. Both the cultures were maintained at 37° for 48 hrs, and then re-inoculated the same in MRS agar.

2.3 Morphological examination of culture

Morphological and cultural examination of cream and pure culture in MRS was carried out by using Gram staining method described by Hans Christian Gram.

Identification of pure culture isolated on MRS agar medium was done with the help of biochemical test like [18].

2.3.1 Catalase test

This test was used to check the production of enzyme catalase. For this test a clean microscopic slide was taken. A loopful of bacterial culture was taken and mixed with 3% of H_2O_2 solution on the slide and the presence of the bubble production was observed.

2.3.2 Oxidase test

Oxidase discs were used for detection of oxidase production by microorganisms. Oxidase reaction was carried out by touching and spreading a well isolated colony on the oxidase disc. The reaction was observed within 5 – 10 seconds at 25 – 30°C. A change later than 10 seconds or no change at all was considered as negative reaction.

2.4 Inoculum Preparation

About 500ml of full cream milk was boiled and cooled to 37°C. 100µL of *L. plantarum* and *S.thermophilus* cultures were inoculated separately into two different beakers and another beaker of mixed culture i.e. 100µLof both *L. plantarum* and *S. thermophilus* [19, 20]. The milk was then allowed to ferment for 48 hours. The titrable acidity was checked with 0.1 N NaOH and phenolphthalein was used as the indicator.

2.5 Fermentation of Cream

The initial acidity of the cream was estimated using titration method and the fat was estimated by Gerbers method. About 100 ml of 40% fat cream was then transferred to 200ml paper cups. All the tests were conducted in triplicates and total number of cups for each organism at one particular temperature amounted to 55 cups, including the control. The total number of cups amounted to 275 cups. Each of the inoculum were added at different concentrations (0.5%, 1.0%, 1.5%, 2.0%, 2.5%, and 3.0%) and incubated at various temperatures 4°C, 18°C, 25°C, 37°C and 45°C respectively. After 48hrs fermentation was stopped by transferring the sample into refrigerator [21].

The cream was churned out to obtain butter. Churning was done with the help of mixer grinder and continued until fat granules stars floating on top. The butter is then drained to remove the water and washed several times to remove curd if any. This liquid is known as buttermilk. The acidity formed after fermentation was calculated by titrimetric estimation of buttermilk [22]. The butter was then stored at in a temperature of -16°C for a period of 5 days to observe the free fatty acid development.

2.6 Fat estimation by gerbers method

Estimation of fat is an important step in determining the quality of the milk. The test was conducted by adding 10.75 ml of milk in a Gerber tube, followed by the addition of 10ml of sulphuric acid and 1ml of iso-amyl alcohol in the Gerber tube, permitting the dissolution of protein and release of fat. The tubes are centrifuged and the fat rising into the calibrated part of the tube was measured as a percentage of the fat content of the cream sample [23].

2.7 Free Fatty Acid Estimation

The acidity (free fatty acid content) of fat is normally a measure of the extent to which hydrolysis has liberated the fatty acids from the ester linkage with the parent glyceride molecule. Partly for this reason, acidity of ghee and butter is extensively quoted as a free fatty acid content (% FFA). The FFA content of fresh butter varies from 0.09 to 0.28% with an average of 0.16%. The sensory quality of butter deteriorates with increase in FFA content. As per FSSAI Rules (2011), butter should not contain FFA more than 3%. The FFA present in ghee can be estimated by acid-base titration with alkali (NaOH) using phenolphthalein as an indicator and the end point to pink colour.

About 5g of the butter sample was added in 100 ml conical flask. In an another flask 25ml of ethanol was brought to the boiling point and while still above 70°C, it was neutralized with phenolphthalein. The neutralized alcohol was added to flask containing butter sample and was mixed and further brought to boil. The acidity of butter is frequently expressed as the percentage of free fatty acids in the sample, calculated as oleic acid, using following

$$\text{Free fatty acids (as Oleic acid)} = \frac{\text{Volume of NaOH} \times 28.2 \times 0.1}{\text{Weight of butter (in g)}}$$

Normality of NaOH – 0.1
Normality of oleic acid – 28.2
(- IS 3508 – 1966 (Reaffirmed 1997) Methods of sampling and test for Ghee (Butterfat) Bureau of Indian Standards, New Delhi).

2.8 Moisture Test

Moisture test was done to determine the amount of water content present in butter. The test was done in a hot air oven, electrically or manually done mostly at a temperature of 105±5°C.The final weight after heating determines the amount of moisture loss [24].

A. Moisture Dish: aluminium, nickel or ceramic with a deep neck was preferred.
B. Desiccator: Containing an efficient desiccant.
C. Hot air oven: Electrically heated with thermostatic control.

The test was conducted by accurately weighing about 5±0.5g of the sample in a moisture dish which had been dried previously and weighed. The dish was then placed in a hot air oven for 1 hour at 105 ± 1°C.The dish was removed from the oven, cooled in a desiccator and weighed. The process was repeated by keeping the dish in the oven for 1 hour each time, cooled and weighed till two successive weighing do not exceed 1 mg.

Calculation

$$\text{Moisture and volatile matter \%/w} = \frac{(M1 - M2)}{(M1 - M)} \times 100$$

M1 - Mass in g of dish with butter before drying
M2 - Mass in g of dish with butter after drying
M - Mass in g of empty dish

3. Results and discussion

3.1 Morphological Characters

The gram staining of *Lactobacillus palntarum* (MTCC 9483) revealed that it was a gram positive bacilli and *Streptococcus thermophillus* (MTCC 9450) was observed as a gram positive cocci under 100X magnification. Both the organisms appeared as slimy, colonized and round on MRS media. *Lactobacillus palntarum* showed negative result for catalase and oxidase test whereas, *Streptococcus thermophillus* showed positive result for catalase and oxidase test (Table 1).

Table 1: Morphological Characters

S. No.	Tests	***Lactobacillus palntarum***	***Streptococcus thermophillus***
1	Gram staining	gram positive bacilli	gram positive cocci
2	Catalase	negative	positive
3	Oxidase	negative	positive

3.2 Free fatty acid of fermented cream

Free fatty acid test (FFA) was performed to determine the amount of oleic acid formed in butter, as a part of storage and oxidation. The following results were observed during the test of butter, least FFA value denotes less oxidation and thereby better quality of butter.

At 4°C the control showed FFA of 0.36% (Table 2).

Table 2: Free fatty acid of fermented cream at 4°C

Free fatty acid of fermented cream AT 4°C				
Inoculum concentration in %	**Percentage of free fatty acid in control**	**Percentage of free fatty acid with *L.plantarum***	**Percentage of free fatty acid with *S.thermophilus***	**Percentage of free fatty acid with Mixed Culture**
0.5	0.36	0.48	0.39	0.44
1		0.54	0.41	0.45
1.5		0.49	0.56	0.49
2		0.54	0.56	0.42
2.5		0.53	0.56	0.49
3		0.53	0.56	0.49

The FFA level increased with increase in concentration, the values ranging from 0.36% to 0.56% (Fig1). The highest FFA (0.56%) was shown by butter inoculated with *S.thermophilus* at a concentration of 1.5% to 3% (Fig 1). *Lactobacillus plantarum* showed an increase in FFA ranging from 0.48% - 0.53% (Fig.1). Mixed culture showed an increase in FFA ranging from 0.44%- 0.49% (Fig. 1).

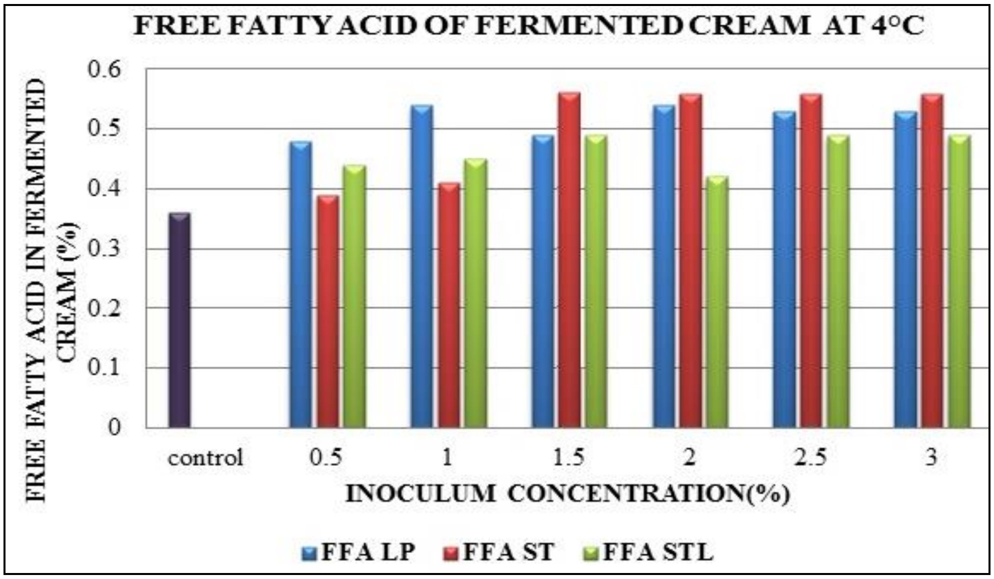

Fig 1: The graphical representation of free fatty acid of fermented cream at 4°C, the minimum being shown by *S. thermophilus* at a concentration of 1.5%.

The cream which was fermented at 18°C had varied result. The control showed an FFA of 0.67% (Table 3) much higher than the butter that was fermented at 4ºC.

Table 3: Free fatty acid of fermented cream at 18°C

Free Fatty Acid Of Butter AT 18°C				
Inoculum concentration in %	**Percentage of free fatty acid in control**	**Percentage of free fatty acid with *L.plantarum***	**Percentage of free fatty acid with *S.thermophilus***	**Percentage of free fatty acid with Mixed Culture**
0.5	0.67	0.71	0.73	0.64
1		0.7	0.71	0.65
1.5		0.72	0.72	0.64
2		0.69	0.73	0.64
2.5		0.73	0.69	0.69
3		0.71	0.7	0.69

Nevertheless there wasn't any significant increase in the values. Notable value of decreased FFA was observed with all the concentrations of mixed cultures. Least value was shown by mixed culture i.e. 0.64% (Fig. 2).

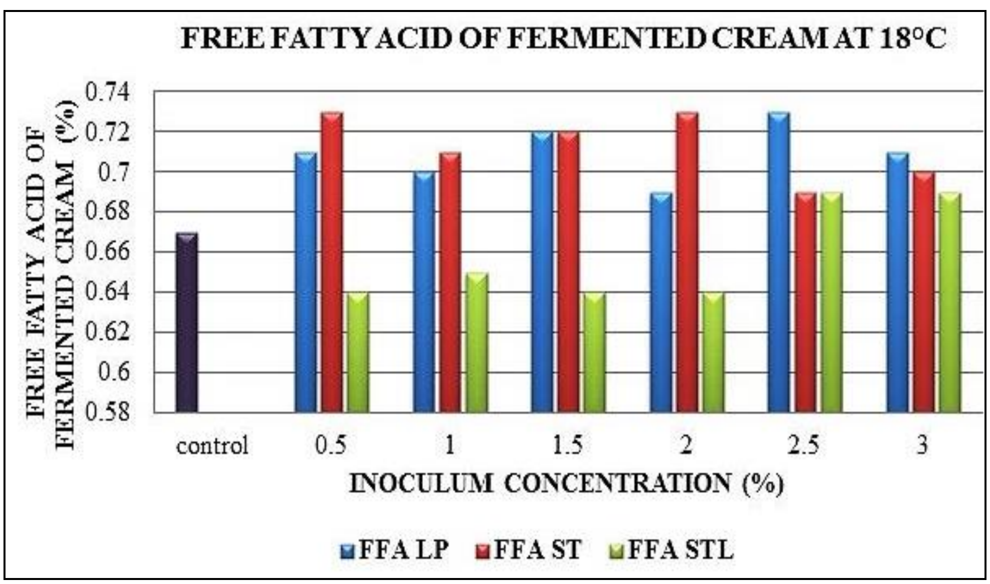

Fig 2: The graphical representation of free fatty acid of fermented cream at 18˚C, the minimum value of free fatty acid is shown by mixed culture in all concentrations.

The butter that was obtained at 25˚C didn't show much variation with increase in concentration. The values almost stood same the least been shown by *S. thermophilus* (0.61% of acidity) at 0.5, 1.0 and 1.5 concentrations (Fig.3). The control value was observed as 0.65% (Table.4). *S. thermophilus* showed higher FFA value (0.81%) at 3% (Fig.3).

Table 4: Free fatty acid of fermented cream at 25°C

Free Fatty Acid Of Fermented Cream AT 25°C				
Inoculum concentration in %	**Percentage of free fatty acid in control**	**Percentage of free fatty acid with *L.plantarum***	**Percentage of free fatty acid with *S.thermophilus***	**Percentage of free fatty acid with Mixed Culture**
0.5	0.65	0.64	0.61	0.67
1		0.66	0.61	0.66
1.5		0.67	0.61	0.66
2		0.73	0.73	0.66
2.5		0.71	0.7	0.67
3		0.69	0.81	0.66

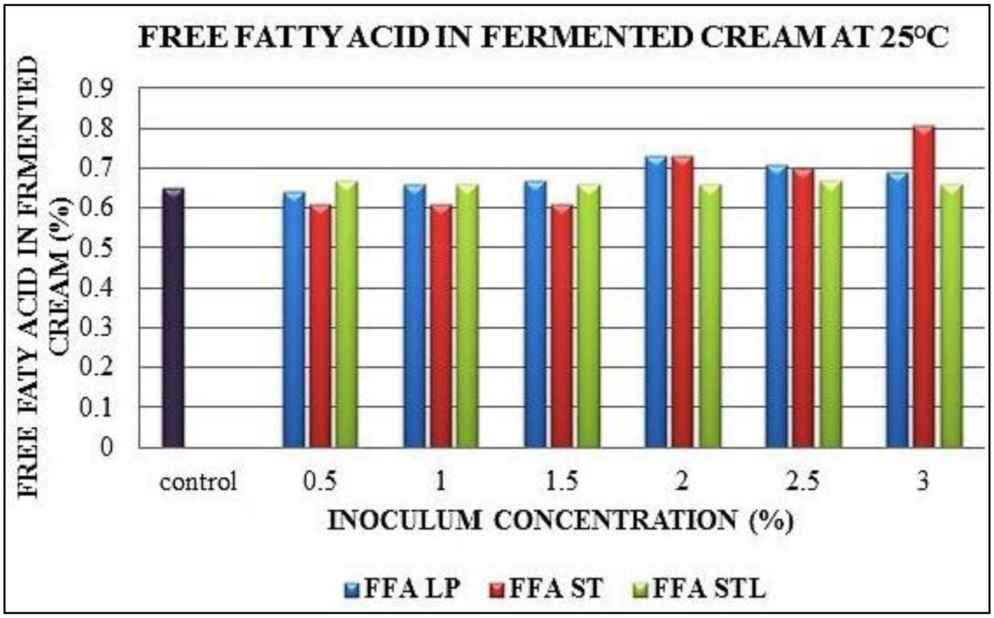

Fig 3: The graphical representation of free fatty acid of fermented cream at 25˚C, the minimum value of free fatty acid is shown by mixed culture in all concentrations.

At 37°C the free fatty acid was almost equal in all concentration in comparison with the control. The control value was observed as 0.72% (Table 5) and other values ranging from 0.78%to 0.89% (Fig.4). The range is less significant.

Table 5: Free fatty acid of fermented cream at 37°C

Free fatty acid of fermented cream AT 37°C				
Inoculum concentration in %	**Percentage of free fatty acid in control**	**Percentage of free fatty acid with *L.plantarum***	**Percentage of free fatty acid with *S.thermophilus***	**Percentage of free fatty acid with Mixed Culture**
0.5	0.72	0.84	0.84	0.89
1		0.83	0.83	0.84
1.5		0.78	0.84	0.84
2		0.84	0.84	0.82
2.5		0.82	0.89	0.81
3		0.87	0.89	0.84

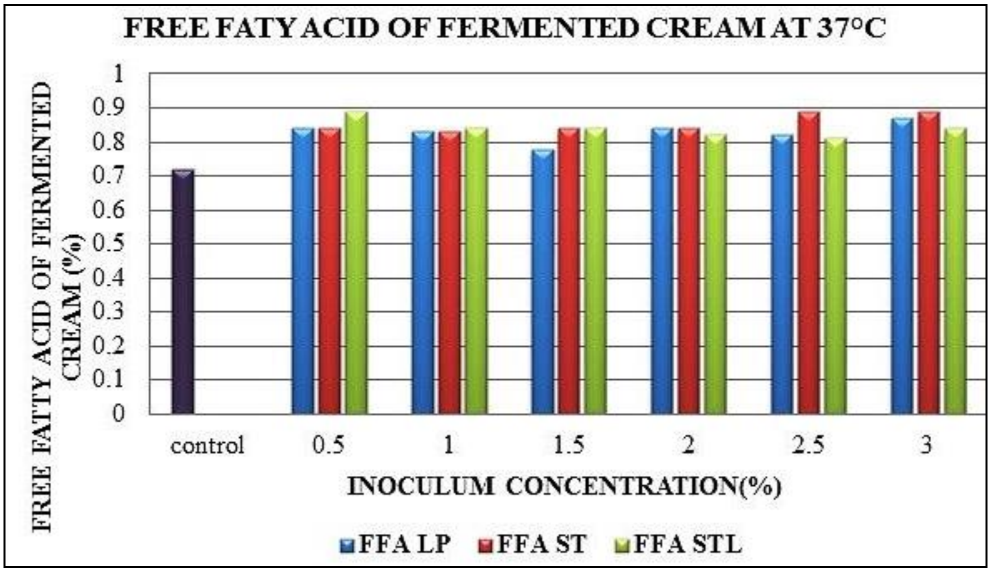

Fig 4: The graphical representation of free fatty acid of fermented cream at 37°C, the minimum value of free fatty acid was shown by *L. Plantarum* in a concentration of 1.5% inoculum.

All the FFA values of butter obtained in fermentation at 45°C showed a lesser FFA with control. Control showed a FFA of 0.84% (Table 6). All the concentration thereby showed a decrease in value with increase in concentration. This varied for *L. plantarum* at 3% concentration with a FFA of 0.95 % (Fig.5), an increased value than the butters in other temperatures.

Table 6: Free fatty acid of fermented cream at45°C

Free fatty acid of fermented cream AT 45°C				
Inoculum concentration in %	**Percentage of free fatty acid in control**	**Percentage of free fatty acid with *L.plantarum***	**Percentage of free fatty acid with *S.thermophilus***	**Percentage of free fatty acid with Mixed Culture**
0.5	0.84	0.62	0.62	0.76
1		0.62	0.73	0.67
1.5		0.67	0.62	0.8
2		0.73	0.67	0.77
2.5		0.73	0.67	0.77
3		0.95	0.71	0.75

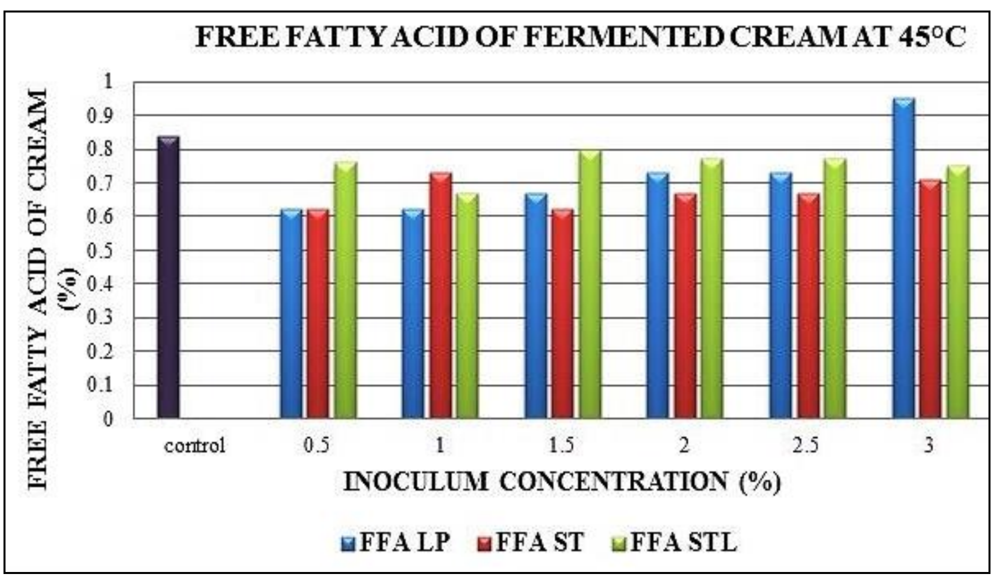

Fig 5: The graphical representation of free fatty acid of fermented cream at 45°C

3.3 Moisture of fermented cream

The amount of moisture should not increase more than 20% to the weight of the butter. In the test it was observed that the moisture of the control at 4° was found to be 21.4% (Table 7).

An increase in moisture was observed for both *L.plantarum* and mixed culture. The least level of moisture was observed for butter by mixed culture at a concentration of 1% moisture being 11.1% (Fig. 6).

Table 7: Moisture of fermented cream at 4°C

Moisture of fermented cream AT 4°C				
Inoculum concentration in %	**Percentage of moisture in control**	**Percentage of moisture with *L.plantarum***	**Percentage of o moisture with *S.thermophilus***	**Percentage of moisture with Mixed Culture**
0.5	21.4	19.7	20.2	24.6
1		19.2	18.2	11.1
1.5		30.6	18.1	31
2		16.9	18	12.9
2.5		15.7	18.1	13.3
3		22	22.2	11.7

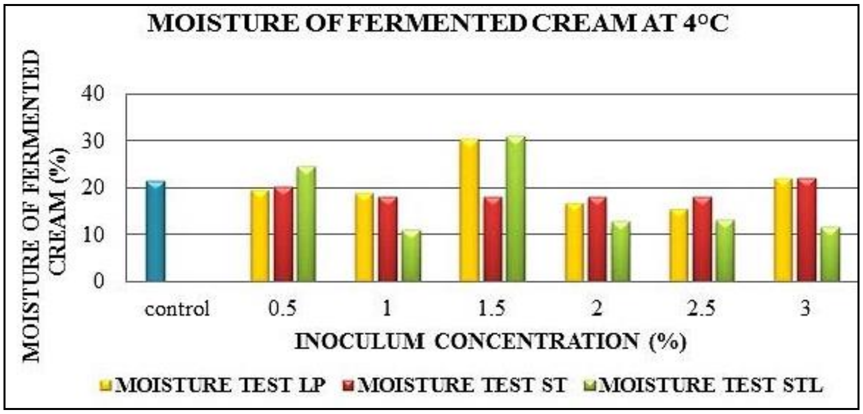

Fig 6: Moisture of fermented cream at 4°C, least moisture is given by mixed culture at an inoculum concentration of 1%.

At 18°C all the butter including the control without any inoculation showed almost same moisture content level of 26.5% all the moisture percentage were within the control value (Fig. 7). The least value of moisture (11.1%) (Table 8) was shown by *S.thermophilus* at a concentration of 1%. The moisture of *S.thermophilus* decreased with increase in concentration till an inoculum concentration of 2.5% and then increased at an inoculum concentration of 3% (Fig. 7).

Table 8: Moisture of fermented cream at 18°C

Moisture of fermented cream AT 18°C				
Inoculum concentration in %	Percentage of moisture in control	Percentage of moisture with *L.plantarum*	Percentage of o moisture with *S.thermophilus*	Percentage of moisture with Mixed Culture
0.5	21.4	19.7	20.2	24.6
1		19.2	18.2	11.1
1.5		30.6	18.1	31
2		16.9	18	12.9
2.5		15.7	18.1	13.3
3		22	22.2	11.7

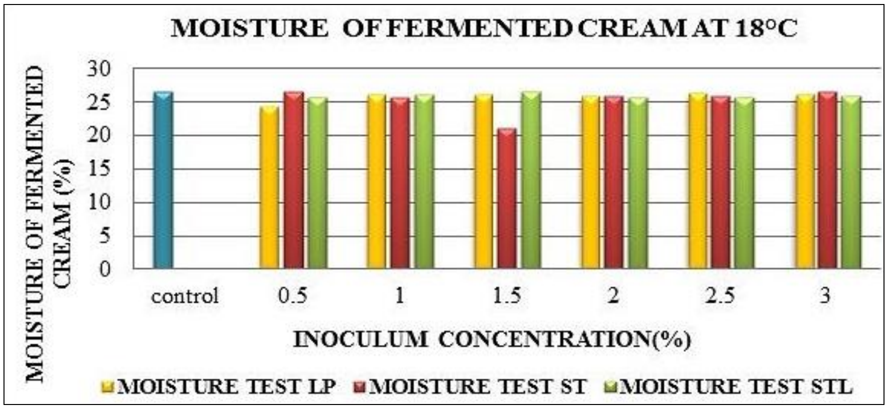

Fig 7: Moisture of fermented cream at 18°c, least moisture is given by mixed culture at an inoculum concentration of 1%.

The moisture content at 25°C was variable. The control showed a moisture level of 22%. There had been variations in the level of moisture with increase in concentration of the cultures. The least value of moisture was observed for mixed culture (14% moisture) (Table 9) at a concentration of 3.0%. Nevertheless the organism then onwards showed an increase in moisture with the concentration but stayed below the control level (Fig. 8).

Table 9: Moisture of fermented cream at 25°C

Moisture of fermented cream AT 25°C				
Inoculum concentration in %	**Percentage of moisture in control**	**Percentage of moisture with *L.plantarum***	**Percentage of o moisture with *S.thermophilus***	**Percentage of moisture with Mixed Culture**
0.5	22.5	24.1	26.7	26.4
1		25.2	28.8	28.4
1.5		26.7	9.28	30.8
2		19.4	15.5	21.2
2.5		24.6	16.1	21.6
3		26.2	17	14.3

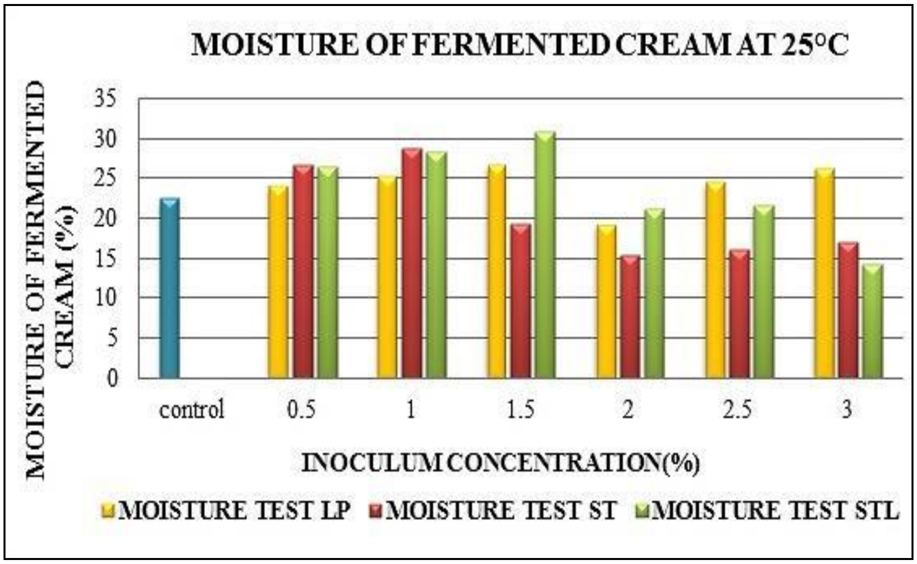

Fig 8: Moisture of fermented cream fermented cream at 25°, minimum moisture shown by *S. thermophilus* at 2% inoculum concentration.

At 37˚C the control showed a moisture level of 28.5% (Table 10), the highest moisture content was observed with *S. thermophilus* at 0.5 and 2% concentration. But all the values were significantly lesser than the control value. A comparative analysis from the graph states that lesser moisture, more favourable to the standard values was observed at 37˚C (Fig 9).

Table 10: Moisture of fermented cream at 37°C

Moisture of fermented cream AT 37°C				
Inoculum concentration in %	**Percentage of moisture in control**	**Percentage of moisture with *L. plantarum***	**Percentage of o moisture with *S. thermophilus***	**Percentage of moisture with Mixed Culture**
0.5	28.5	23.7	32	25.8
1		23.6	18.2	26.1
1.5		24.1	26.1	26.5
2		24	32.2	25.7
2.5		25.1	25.9	25.8
3		24.5	26.5	25.9

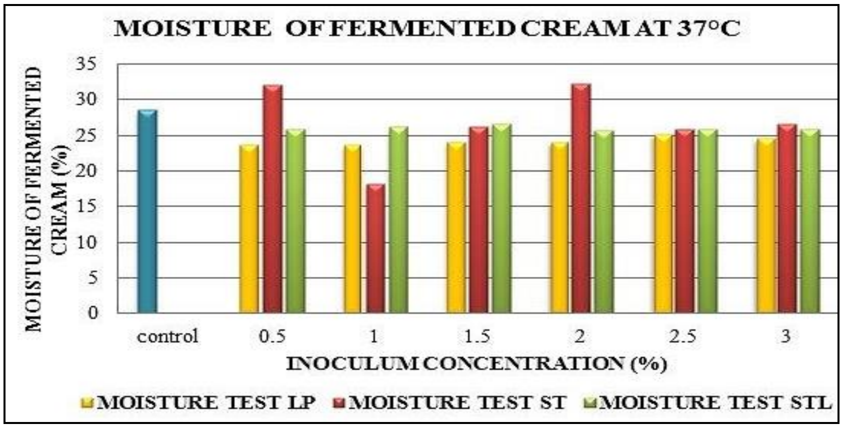

Fig 9: Moisture of fermented cream fermented cream at 37°C

The moisture level there by increased with increase in temperature at 45˚C (Table 11) all the butter showed an increase level of moisture content with increase in concentration and was above the control value. The highest value being 47.2% (Fig10) was shown by *L.plantarum* at 3% concentration.

Table 11: Moisture of fermented cream at 45°C

Moisture of fermented cream AT 45°C				
Inoculum concentration in %	**Percentage of moisture in control**	**Percentage of moisture with *L.plantarum***	**Percentage of o moisture with *S.thermophilus***	**Percentage of moisture with Mixed Culture**
0.5	30.7	41.3	44.75	31
1		43.5	45.01	34.7
1.5		42.1	40.35	30
2		34.8	45.2	17.9
2.5		36.4	42.74	25.3
3		47.2	42.88	31.7

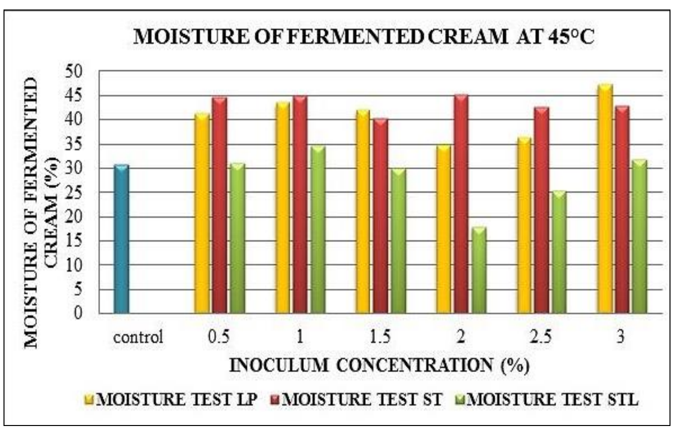

Fig 10: Moisture of fermented cream fermented cream at 45°C, minimum value shown by mixed culture at 2% concentration.

4. Conclusion

The organisms obtained from MTCC (*L. plantarum* and *S. thermophilus)* were used to ferment the milk cream. There was a noticeable change in the acidity of the cream after fermentation. Highest acidity of 0.76% has been shown by *L. plantarum* at a concentration of 3% at 37°C. Both the organism individually as well as in combination showed an increase in acidity at different temperatures and inoculum concentrations. This signifies the ability of the organism to ferment the cream and produce lactic acid.

The quality of butter obtained after churning process were determined by free fatty acid and moisture test, during which it was found that the FFA value increased with increase in temperature of fermentation. Inoculum concentration had less effect in determining FFA. The least value of free fatty acid (0.39%) was shown by butter at 4°C and highest value of free fatty acid (0.95%) was observed for butter at 45°C.

An optimum level of moisture was observed with butter obtained at 18°C, 25°C and 37°C. But there was an increase in moisture content at 45°C irrespective of the inoculum concentration. In conclusion the inoculum concentration and temperature had a role in the acidity formed by the organisms. Moisture and FFA were greatly dependable on temperature than inoculum concentrations. It was also observed that the organisms showed better results in combination than individual. Hence the organisms can potentially be used in the fermentation of cream and hence produce butter of better quality.

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