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Analysis of *Moringa oleifera* Seeds to *Lactobacillus* Extracts for the Production of Prato Cheese Using Aerobic Bioreactor

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Abstract

Queijo Prato ("plate shaped cheese") named after the shape it was originally made by immigrants, and it is a Brazilian soft cheese. The objective of this project is to compare the government authorized milk to privatized milk which gives the better yield and quality of Prato cheese. Moreover, the anticoagulants i.e. the clotting agent used for the cheese production is taken from different sources i.e. one from plant source *Moringa oleifera* seeds which has high milk clotting activity and another one from microbial source *Lactobacillus acidophilus* has been chosen for the study. After the production of cheese from different sources of milk using different milk clotting agents the cheese produced will be analyzed for testing the efficacy of the microbial source and the plant source used and the physical characteristics of the cheese produced. Then the better yielded cheese will be scaled up using Aerobic bioreactor.

Keywords: Queijo Prato, *Lactobacillus acidophilus, Moringa oleifera,* Aerobic bioreactor

Foods are not only to satisfy starving people and also to provide essential nutrients to humans but also helps in prevention of nutrition related diseases. It also helps in improving consumers' physical and mental health. Many surveys have been conducted in 1990's and 2000's which states that there is upward growth on overweight and obesity mainly in school going children and teenagers at world level. Due to increase in the world population and the constant rise in obesity and other nutritional diseases people are concerned to have proteins and low fat dairy products from all over the world. (Munoz *et al*., 2017)

About Cheese

Cheese is the most commonly consumed dairy products, involving biochemical, chemical and microbiological processes. The steps in all cheese making include milk acidification, milk coagulation, whey removal, packaging and storage. Most cheese making also includes heating the cheese curd and salting the curd. Even slight changes in these processes can lead to significant differences in final cheese. Cheddar, Mozzarella, Gouda and Egmont are the cheese varieties made in greatest quantity and make up the bulk of our export cheese. (S. Neelakantan *et al*., 1999)

From 6000-7000 BC manufacture of cheese is one of the classical example of food preservation. Preservation of the most important constituents of milk (i.e. fat and protein) as cheese exploits two of the classical principles of food preservation, i.e. lactic acid fermentation reaction and water activity reduction through removal of water and addition of salt (NaCl). The establishment of a low redox potential as a result of bacterial growth contributes to the storage stability of cheese. (Christina Coker *et al*., 2005)

About Prato Cheese

Typical from Brazil Prato cheese is produced by enzymatic coagulation and partially cooked followed by the addition of hot water or by heating of the vat. It must be ripened for 25 days minimum. Proteolysis is the main indicator of ripening, usually expressed by the extent and depth of proteolysis indexes (EPI and DPI). EPI is the percent of decomposed protein molecules, mostly to large peptides, and DPI the percent to which these large peptides are degraded into smaller molecules. (Spadoti *et al.,* 2005)

Clotting Agents

About Microbial Sources

At present, microbial rennet is used for one-third of all the cheese produced worldwide. There are two stages in the action of renin on the milk, one by enzymatic and another one by non-enzymatic action, resulting in coagulation of milk. In the enzymatic phase, the resultant milk becomes a gel due to the influence of calcium ions and the temperature used in the process. Several microorganisms can produce rennet like proteinases which is able to substitute the calf rennet micro-organisms like *Rhizomucor pusillus, R. miehei, Endothia parasitica, Aspergillus oryzae*, and *Irpez lactis* are used extensively for the production of rennet in the manufacture of cheese. In bacterial source the most commonly used bacterias are *Lactobacillus rhamnosus*, *Lactobacillus paracasei, Lactobacillus acidophilus* and *Lactococcus lactis*. (Barbara Turchi *et al.,* 2011). Lactic acid bacteria producing bacteriocins including Lactobacillus acidophilus and other types of Lactobacillus species can be used as biological preservatives in order to inhibit growth of L. monocytes in various kinds of foods especially fermentative dairy products like yogurt and cheese. (Razzaqh. M *et al.,* 2012)

Enzymes

Chymosin: The most commonly used enzyme in any type of cheese production is Recombinant bovine chymosin. But a recent study has claimed that recombinant camel chymosin (Chy-Max M) has been developed and also it is commercially available as milk coagulant for natural cheese manufacture.

A typical Brazilian variety cheese obtained by enzymatic curdling is called Prato cheese. It is prepared from a half-cooked, washed and pressed mass which is then kept for aging 25 days. The traditional method for using the coagulant obtained in laboratory are protease from different sources like *Thermomucor indicae-seudaticae* N31, which has been recently isolated in Brazil and known as Thermomucor cheese. And one more commercial coagulant from *Rhizomucor* sp. (Alternative, Bela Vista) which has been used as control cheese. The milk clotting will be achieved in approximately 35min. Then the milk, cheese and whey were evaluated and finally cheese yield was calculated. (Biswas *et al.,* 2013)

Protease: It has been claimed that commercially available plant proteases**,** such asbromelain and papain is capable of clotting milk and helps to form curd. (Munoz *et al.,* 2017).

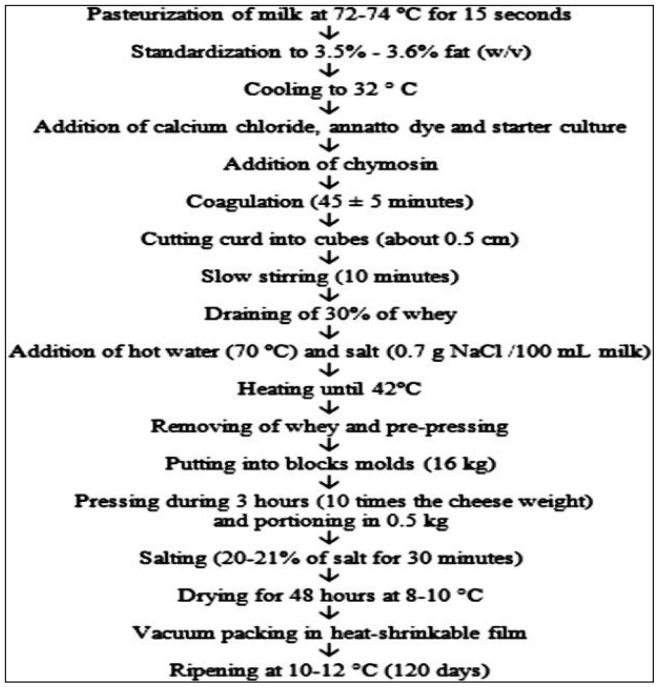
The proteolytic activity was determined by the casein digestion method at pH 6.5 and it is expressed as absorbance (optical density) at 660 nm. The milk clotting units (SU) is determined by the ratio of milk clotting activity to protolytic activity versus the OD at 660 nm obtained in the proteolytic measurements. (Chen-Wu, 2013)

Rennets: Plant rennet playsan importantrole in several coagulants used in cheese production. Suitable plant coagulant is very important due to increase in global demands of cheese because of decrease in supply of calf rennet. Many efforts had been taken to distinguish certain rheological and sensory properties of cheese products obtained from plant ans animal based rennets. Some of the plant coagulants also produce cheese with sensory qualities similar to those produced by animal rennet. Few examples include ginger, cucumisin or hieronymain proteases. (Amira *et al.,* 2017)

Moringa oleifera

It is grown in different rural regions of Mexico and different parts of this plant such as leaves, flowers

and seeds are edible. It has a big source of protein, calcium, carotenoids, iron and phytochemicals, and it has several applications in many developing countries. Moringa oleifera seeds is rich in proteases (Munoz *et al.,* 2017). Plant proteases is an enzymes that catalyse the hydrolysis of peptide bonds which is responsible for several biological processes, which includes mobilisation of storage proteins, light-damaged chloroplast proteins degradation, defense against phytopathogen attack, floral senescence and tissue differentiation.(Pontual *et al.,* 2012)



Flow Chart of Prato Cheese Production

MATERIALS AND METHODS

Sample collection

Moringa oleifera seeds were collected freshly and dried in hot air oven for about 2hrs at 70°C and crushed at room temperature and stored in air tight container. The fresh buffalo milk is collected directly from the farm and pasteurized at 72°C for 10min and stored at 10°C for further use.

SEM analysis

It is a type of electron microscopewhich focuses on giving high resolution images by focused beam of high energy electron beam of the sample.

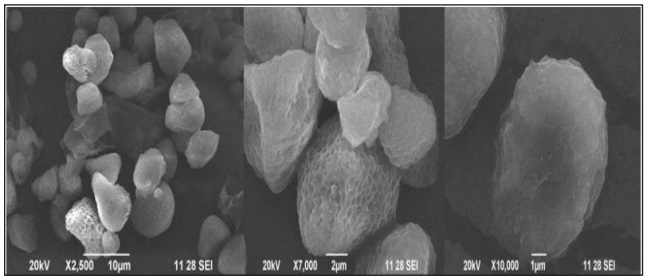


Fig. 1: SEM image of *Moringa oleifera* seeds

Enzyme Extraction

Collect 2gm of seed powder and was immersed in 10ml of 50mM phosphate buffer and the pH 7.0 was maintained at the ratio of 1:10 (w/v). The mixture was macerated by traditional stirring using magnetic stirrer for 4hrs at room temperature. Then the extracts were centrifuged at 3500 rpm for 10 min. And finally the supernatant was stored at 4°C for further use.

Protein estimation of milk

The concentration of protein in the milk was determined by Lowry's method. A standard curve was plotted using bovine serum albumin as 10-500µg/ml in concentration as the standard. Alkaline copper sulphate reagent was added to different dilutions in the sample solution and were incubated at room temperature for 10 min. then the commercially available Folin & Ciocalteu's reagent was added to each tube and incubated for 30 min. Then the mixture was measured using spectrophotometer at 600 nm.

Fat Estimation

The fat estimation of the buffalo milk was done by Rose Gottleib method. The milk sample is treated with 10 ml of ammonia sp.gr.0.8794 and mixed well. Then 10 ml of ethyl alcohol was added and mixed well, then the mixture is allowed to cool at room temperature. Then 25 ml of diethyl ether was added and shaked vigorously for about 1 minute, to this mixture 25ml of light petroleum ether was added and again shaked vigorously for 1 minute. Then the tube

was kept undisturbed until the ethereal layer is clear and separated from the aqueous layer for 30 minutes and the aqueous layer was discarded.

Then the mixture was transferred to petri dish to dry and it was dried and weighed. Then that petri dish was washed and weighed again. The amount of fat extracted was calculated through the difference between the petri dish having the fat and the petri dish without fat.

Isolation of *Lactobacillus* strain

Freshly prepared curd made from buffalo milk was taken and serial dilution was done. In serial dilution, 9 test tubes were taken and to that each test tube 9 ml of distilled water was added and marked as concentrations 10^{-1}, 10^{-2},….. and 10^{-9}. Then 1ml of sample curd was added to 1st test tube and 1ml of solution was taken and added to 2nd test tube and this process was repeated till 9th test tube. After this plates having MRS agar was prepared and the concentrations 10^{-1}, 10^{-3} and 10^{-5} was swabbed using L-rod and plates were kept for incubation at 37°C for 24 hours. Then for confirmation gram staining was done.

Protease Assay

A protease is any enzyme that conducts proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain. 0.2, 0.4, 0.6, 0.8 and 1 ml of protease enzyme was taken and 0.7 ml of skim milk powder solution and the tube was marked as T. Then 0.7 ml of skim milk powder solution and the tube was marked as Sub blank. Then the contents of tube was equalised with 2 ml with phosphate buffer and this tube was labelled as Blank. Then 6 ml of distilled water was added and absorbance was taken at 660 nm. Finally, a standard curve was plotted using concentration of protein in X axis and absorbance in Y axis. Then the absorbance of test was subtracted from absorbance of sub blank. The subtracted absorbance was plotted in the standard curve and protein was found out.

Milk clotting Activity

The milk clotting activity was determined by following the procedure described by (Arima *et al.,* 1967) with some modifications. A suspension of buffalo milk was used as a substrate by adding 10mM $CaCl_2$ and pH was maintained at 6.5. Then the milk was incubated at 35°C for 5min. Then the milk clotting enzyme was added at a ratio of 1:10 (v/v). One unit of milk clotting activity (MCA) was defined as the amount of enzyme to clot 1 ml of a solution containing 0.1 gm of milk in 40 min at 35°C.

The time between the addition and the appearance of clots was noted and the total MCA was calculated as:

MCA (SU/ ml) = (2400/ coagulation time) × dilution factor

Design of impeller

An impeller was designed which has the following features:

1. Length of the shaft = 300mm
2. Diameter of the shaft = 32mm
3. Description of the blade = (260 × 40 × 3) mm
4. Motor volt = 25 volt
5. Torque of the motor = 120 Nm (Newton metre)
6. Rpm of the blade = 8 rotations/ 15 seconds i.e. 32 rotations / 1 minute

Cheese Production

3 litres of freshly collected buffalo milk was taken and pasteurised at 72°C for about 10 minutes. Then the pasteurized milk was poured in the fermenter and then calcium chloride, starter culture and turmeric was added and was stirred for about 15 minutes. Then after sometime milk clotting enzyme were added and again stirred for about 5 min. After this the mixture was taken out and again heated at 72°C for about 10 min. The clotted cheese were collected and the whey was drained and salting was done in order to enhance the taste and texture of the cheese.

RESULTS AND DISCUSSION

Analysis of normal milk and buffalo milk

Protein estimation has been done in commercially available milk and freshly collected buffalo milk. It has been concluded that buffalo milk has high amount of protein present in it compared to normal commercially available milk.

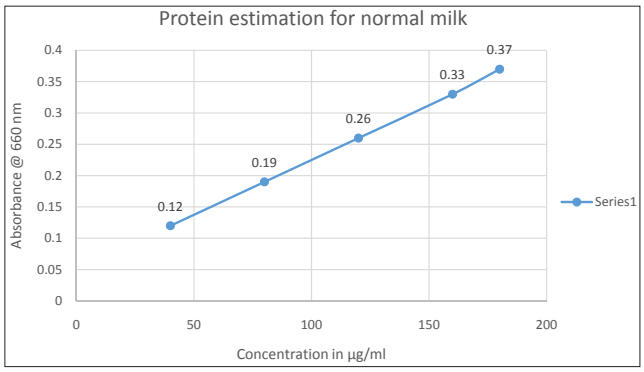


Fig. 2. Graph between Concentration and Absorbance

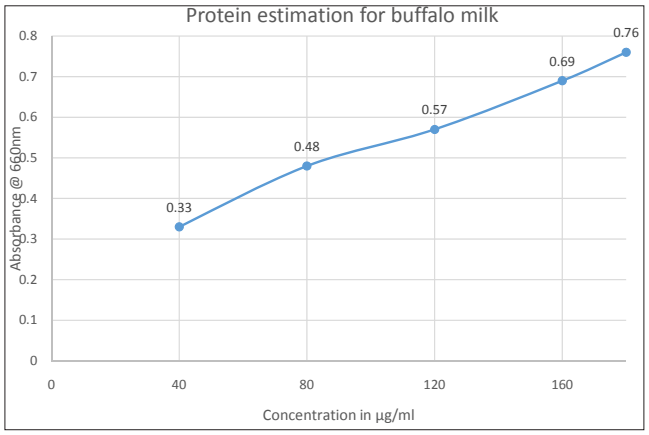


Fig. 3: Graph between Concentration and Absorbance

Calculation

1. **For normal milk**:

   1.0 OD of test corresponds to 40µg of protein

   i.e. 0.4ml of test contains 40µg of protein

   Therefore, 100ml of test contains = (40 × 100)/0.4 = 10000µg of protein

2. **For buffalo milk**

   1.0 OD of test corresponds to 60µg of protein

   i.e. 0.4ml of test contains 60µg of protein

   Therefore, 100ml of test contains = (60 × 100)/0.4 = 15000µg of protein

It has been concluded that the buffalo milk 15000 µg/ml of protein whereas normal milk has 10000 µg/ml of protein which shows that buffalo milk has higher amount of protein.

Fat Extraction

It has been done by Rose Gottleib method which shows that buffalo milk has 10% of fat in 100ml of milk which is a bit higher than cow's milk as it contains only 4.2% - 4.5%

Purpose of chemicals used

- Ammonia (10ml) – dissolve protein
- Ethyl alcohol (95%-95% v/v) -precipitation of protein
- Diethyl ether (10ml) - fat extraction
- Petroleum ether (25ml)- fat extraction

The precipitated protein is extracted into a petriplate.

The petriplate is dried in hot air oven for 2 hours at 102 degree celcius

Calculation

Weight of petri plate = 54.6g

Weight of petri plate with fat = 55.6g

The content of fat in 10 ml of Buffalo milk = 55.6 – 54.6 = 1g

$$\text{Fat \%} = \frac{\text{weight of extracted fat}}{\text{Volume of milk}} \times 100$$

The percentage of fat in milk

$$= \frac{1}{10} \times 100$$

= 10% of fat in 100 ml

Isolation of *Lactobacillus* strain

Lactobacillus strain was isolated from freshly prepared curd made from buffalo milk in MRS agar and the result shows the isolation of *Lactobacillus* present in the curd. To confirm it gram staining was done and it shows gram positive, non-motile and rod shaped bacteria which confirms it is *Lactobacillus* and finally sub-culture was done.

Protease Assay

It has been reported that *Moringa olifera* seeds have higher amount of protease enzyme than any other parts of the plant like flower and leaves. (Munoz *et al.*, 2017). Therefore, protease amount was calculated.

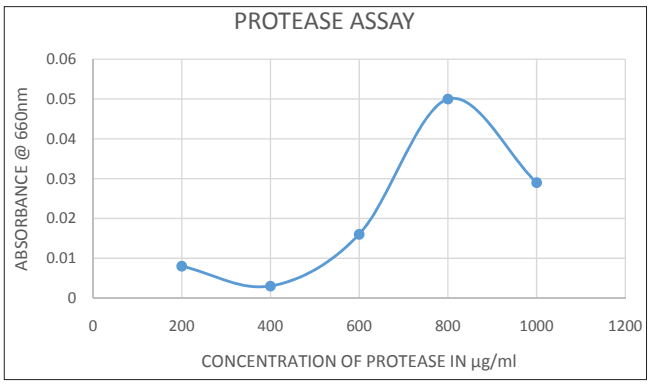


Fig. 4: Graph between concentration of Protease and Absorbance

Calculation

Absorbance value of Blank = 0.055

Absorbance value of Enzyme = 0.076

Amount of protease present = 0.076-0.055

= 0.021

From graph,

The amount of protease present in 1ml of enzyme was 601 µg/ml

Lab scale production of Prato cheese

it has been claimed that milk clotting activity in *M. oleifera* seeds are higher than other parts of the plants. (Munoz *et al.*, 2017). The coagulant activity of seed extract is shown in fig. using whole buffalo milk as the substrate.

Milk Clotting Activity

The milk clotting activity was done in 3 test tubes in which 1st test tube has taken as positive control in which *Lactobacillus* strain was added, in 2nd tube enzyme kept for 3hrs stirring and in 3rd tube enzyme kept for 4hrs stirring. The total milk clotting activity of the 1st tube was 6838 SU/ml, in 2nd tube the total milk clotting activity was 2051.40 SU/ml and in 3rd test tube it was 3419 SU/ml.

	Samples	Milk clotting activity value	%age value
1	Test tube 1	6838 SU/ml	100
2	Test tube 2	2051.4 SU/ml	30
3	Test tube 3	3491 SU/ml	50

This shows that the positive control i.e. *Lactobacillus* strain has higher milk clotting activity as it was known but in test tube 2 had enzyme 3hrs stirring shows 30% activity whereas test tube 3 had enzyme shows 50% activity compared to test tube 1.

Large Scale Production of Prato cheese

As it was described in the materials and methods the cheese was produced using the protocol.

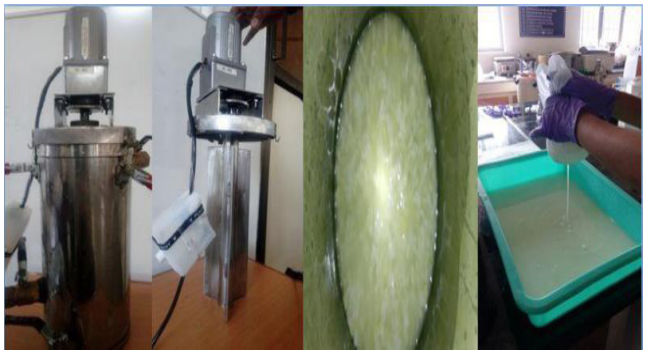


Fig. 5: Process of Prato cheese production

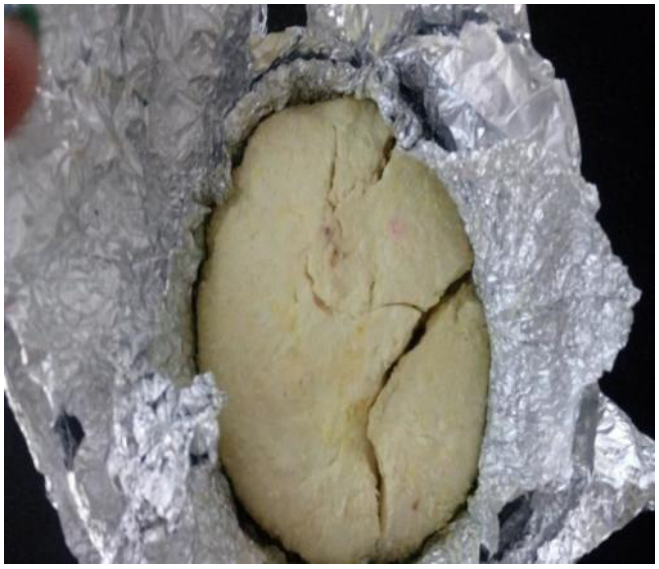


Fig. 6: Prato cheese after 10 days of ripening

CONCLUSION

From this research we can conclude that we can produce better yielding Prato cheese can be produced not only from microbial clotting agent but also from plant source i.e. *Moringa oleifera* seeds extract which is rich in protease enzyme responsible for milk clotting activity. And also a new design of impeller can be made with low rpm which might be used only for cheese production. And finally if the product is successful we can commercialize. And also a new design of impeller can be made with low rpm which might be used only for cheese production. The power number needed to produce Prato cheese from 3 liters of buffalo milk is 2.2 × 10-6 which is optimum when compared to the Reynold's Number with respect to power number of plain water. The cheese was milkier and was a bit sour initially and after an aging of 10 days the sourness also slightly reduced.

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