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Full Length Research paper

Extraction and Characterization of Casein Phosphopeptides from Fermented Milk

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The fermented milk peptides have a varied composition and they contain CPP (casein phosphopeptides) which has a wide array of uses. The study was undertaken to isolate CPP from fermented milk and it was then characterised using appropriate techniques. The 3 parameters of viscosity, titrable acidity and pH were standardised before commencing the studies. The antimicrobial activity of the CPP was tested against a number of clinical pathogens and it gave positive answer for *ESCHERICHIA COLI* and *PSEUDOMONAS* species with 14 and 16 mm of zone of inhibitions, respectively. The molecular weight range of the CPP was determined by gel electrophoresis and it was found to be around 3.5- 4.0 KD (KiloDaltons). FTIR analysis of the CPP done along with non-fermented milk as the control revealed the constituents. The characterisation of the CPP provided detailed insights of its nature and potential uses across numerous fields.

Key words: CPP, fermented milk, antimicrobial activity, FTIR.

INTRODUCTION

CPP (casein phosphopeptides) are an important kind of peptide group present in milk (Otani, 2000). Milk itself is a unique food item comprising of proteins, fats and carbohydrates (Nudda, 2006). Fermented milk is commercially sold in various forms across developed countries in the name of yoghurt, flavoured yoghurt, cheese, butter etc. Fermented milk develops various new constituents while undergoing fermentation and that's what causes a marked difference between fermented and non-fermented milk.

Although many peptides are present in the fermented milk, we concentrated on CPP due to their multiple uses and potential in various fields. The peptides are basically classified in to 3 types based on their charge, positive, negative and neutral peptides (Anderson, 2002). The classification of peptides present in fermented and non-fermented peptides is a very broad topic and the classification is effected based on physical, chemical properties. CPP in a way have been dealt by Indian researchers in a relatively smaller way taking in to consideration the amount of literature available. CPP has always been a widely studied peptide group in dentistry (Mazzouaki, 2003). CPP also has been researched in the areas of sports medicine, anti-hypertensive medicine, remineralisation, immunoenhancement and immuno-modulation (David, 2002; Tobita, 2006). The concept of food based nutrition has been practised and advocated in India from the time immemorial and this has again gained momentum in the recent past due to social trends such as globalisation, booming economy, growing purchase power of Indian middle class. CPP has the potential of becoming a food based nutritional item and boosting the immune system of humans (Cross, 2005).

Virtual problem associated with any food based item is that it has less shelf life, chances of infection by micro organism become quite high and dissipation rate also increases considerably. If CPP is isolated from the fermented milk, then it reduces the dissipation rate, increases shelf life and the risk involved with the micro organisms decreases considerably. Various parameters are important to be studied and standardised before commencing the work like viscosity, titrable acidity and pH. Viscosity of fermented milk that is prepared in a do-mestic environment will be lower than the fermented milk which is produced in a commercial firm (Vesa, 1997).

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Table 1. Arokya milk after being added with Dodla dairy curd.

Time	pH (Day 1)			pH (Day 2)			Mean ± S.D
11:40 am	6.03	6.14	5.93	6.18	6.16	6.24	6.028 ± 0.108
12:40 pm	5.98	6.17	6.17	5.94	5.95	6.18	6.053 ± 0.112
01:40 pm	6.06	6.14	6.16	5.88	6.05	6.07	6.008 ± 0.135
02:40 pm	5.90	6.05	6.08	5.85	5.86	5.98	5.91 ± 0.133
03:40 pm	5.80	5.93	5.94	5.79	5.75	5.89	5.733 ± 0.191
04:40 pm	5.50	5.56	5.68	5.67	5.46	5.60	5.42 ± 0.212
05:40 pm	5.22	5.25	5.31	5.16	5.17	5.45	5.297 ± 0.091

In the case of titrable acidity, it is bound to have differences with commercial curd due to preservatives used and the multiple cultures of Lactobacillus, Bifidobacterium which are used to ferment the milk. pH is an important property for fermented milk as it indirectly shows the level of acidity in that fermented milk. Even a slight alteration in the pH can drastically affect the properties of the fermented milk resulting in the physical and chemical changes.

CPP has a proven role as an anti-hypertensive enhancing agent and it also has a remarkable anti-microbial activity (David, 2002). Although the anti-microbial potential of fermented milk has been rarely dealt with by the researchers, they have considerable anti-microbial activity against well known clinical pathogens like *Escherichia coli* and *Pseudomonas* species. Indian people have been using various forms of fermented milk products such as curd, buttermilk in their daily diet due to their anti-microbial potential. The fermented milk products can be used against a wide range of microbes including various classes of bacterium.

The molecular weight of the CPP becomes relevant in the context that molecular weight directly correlates to SAR (structure activity relationship). The gel electrophoresis is a known and most common method to find out the molecular weight range of fermented milk products. The structure of CPP is quite unique in structure due to the linkages which are present in them (Emmanuelle, 1992). Resonance also plays an important role in increasing the stability of CPP. There are a few exceptional cases in which high temperature affects the resonance process thereby decreasing the stability of CPP.

FTIR (Fourier trans-infra red) spectroscopy is an important instrumentation technique which has wide application in various fields including stereo chemistry, analytical chemistry, biophysics etc. (Scheineder, 1985). It is chosen over other types of spectroscopy techniques such as UV-spectroscopy, visible spectroscopy and even finer types of infra red spectroscopy due to its high precision, reliability and flexibility to analyse an array of samples having different characteristics. Analysis by FTIR at a defined wavelength gives a graph which can be deciphered to gain an insight in to the composition and constituents of various fermented milk products and CPP in particular. The study was aimed to successfully isolate CPP from fermented milk and characterise the CPP using relevant techniques.

MATERIALS AND METHODS

Milk fermentation

Two litres of milk from the local brand Arokya having 4% fat was purchased and was autoclaved at 121°C for 15 min. It was then divided in to 4 equal proportions containing 500ml each. Then about 5ml of 10^8-10^9 CFU/ml *Lactobacillus acidophilus*, (obtained from IMTECH, Chandigarh) designated as Culture A was added to all the 4 portions overnight. 4 portions are done so that we can get a concordant value and arithmetic errors can be avoided. Another batch of 2 litres Arokya milk was divided in to 4 portions containing 500ml. Then about 5ml of 10^8 - 10^9 CFU/ml *Lactobacillus bulgaricus*, (obtained from IMTECH, Chandigarh) designated as Culture B was added to all the 4 portions overnight. Another 2 batches were repeated using the same procedure with fermentation done by commercially available curd brands, Dodla and Aavin. Same 5 ml was added with the microbes concentration being steadied at 10^8 - 10^9 CFU/ml. The fermentation was allowed to take place over night and the experiments were carried out on the subsequent day morning.

Initial standardisation

The parameters which were taken in to consideration were pH, titratable acidity and viscosity. All these parameters are vital for the effective fermentation of the milk and have drastic impact on the result, if altered.

pH

The pH of the fermented milk samples was recorded using a pH meter (obtained from Mettler Toledo, 2006 model) in a time interval of 1 hour and the values were taken arithmetic mean. The Standard Deviation was also calculated based on the mean value. Table 1 shows the change in pH after Dodla curd was added to the Arokya milk. Table 2 depicts the change in pH value of the Arokya milk after being added with Aavin curd for milk fermentation. Table 3 shows the change in pH values after the culture *Lactobacillus acidophilus* was added to the Arokya milk. Table 4 depicts the pH value change in the Arokya milk after being added with *Lactobacillus bulgaricus.* The same brand of milk, Arokya was used throughout the studies. Table 5 is the summary of all these 4 table results in terms of pH value changes. Figure 1 depicts the summary of pH values plotted against time for milk fermented using commercial curd and the bacterial cultures (*Lactobacillus acidophilus, Lactobacillus bulgaricus*).

Table 2. Arokya milk after being added with Aavin curd.

Time	pH (Day 1)			pH (Day 2)			Mean ± S.D
11:40 am	6.45	6.48	6.37	6.55	6.47	6.32	6.38±0.092
12:40 pm	6.53	6.60	6.55	6.31	6.43	6.24	6.447±0.151
01:40 pm	6.25	6.34	6.23	6.36	6.45	6.19	6.237±0.108
02:40 pm	6.12	6.22	6.08	6.23	6.33	6.04	6.117±0.106
03:40 pm	5.87	5.85	5.60	6.07	6.25	5.96	5.792±0.155
04:40 pm	5.40	5.50	5.32	5.81	6.00	5.62	5.46±0.107
05:40 pm	5.06	5.18	5.12	5.45	5.64	5.45	5.282±0.185

Table 3. Arokya milk after being added with culture A*.

Time	pH (Day 1)			pH (Day 2)			Mean ± S.D
11:40 am	6.23	6.34	5.98	6.21	6.30	5.99	6.175 ± 0.154
12:40 pm	6.12	6.21	5.79	6.10	6.18	5.83	6.038 ± 0.182
01:40 pm	6.04	6.15	5.67	5.98	6.02	5.70	5.927 ± 0.196
02:40 pm	5.90	6.08	5.51	5.80	5.89	5.56	5.79 ± 0.218
03:40 pm	5.86	5.73	5.42	5.72	5.77	5.44	5.657 ± 0.183
04:40 pm	5.71	5.46	5.38	5.54	5.48	5.21	5.463 ± 0.166
05:40 pm	5.42	5.35	5.11	5.22	5.31	5.12	5.255 ± 0.126

* - *Lactobacillus acidophilus.*

Table 4. Arokya milk after being added with culture B*.

Time	pH (Day 1)			pH (Day 2)			Mean ± S.D
11:40 am	6.26	6.29	6.07	6.22	6.34	6.10	6.213 ± 0.107
12:40 pm	6.01	5.99	5.88	6.04	6.05	5.92	5.982 ± 0.068
01:40 pm	5.72	5.74	5.56	5.77	5.78	5.66	5.705 ± 0.083
02:40 pm	5.56	5.58	5.40	5.59	5.62	5.52	5.705 ± 0.083
03:40 pm	5.34	5.44	5.23	5.35	5.49	5.29	5.357 ± 0.095
04:40 pm	5.27	5.30	5.14	5.21	5.35	5.16	5.238 ± 0.082
05:40 pm	5.15	5.21	5.03	5.18	5.29	5.07	5.155 ± 0.095

* - *Lactobacillus bulgaricus.*

Table 5. Summary of pH values for milk fermented using commercial curd and bacterial cultures.

Time	Dodla daily curd	Aavin curd	Culture A	Culture B	Control
11:40	6.028 ± 0.108	6.38 ± 0.092	6.175 ± 0.154	6.213 ± 0.107	7.05
12:40	6.053 ± 0.112	6.447 ± 0.151	6.038 ± 0.182	5.982 ± 0.068	7.05
13:40	6.008 ± 0.135	6.237 ± 0.108	5.927 ± 0.196	5.705 ± 0.083	7.05
14:40	5.91 ± 0.133	6.117 ± 0.106	5.79 ± 0.218	5.705 ± 0.083	7.05
15:40	5.733 ± 0.191	5.792 ± 0.155	5.657 ± 0.183	5.357 ± 0.095	7.05
16:40	5.42 ± 0.212	5.46 ± 0.107	5.463 ± 0.166	5.238 ± 0.082	7.05
17:40	5.297 ± 0.091	5.282 ± 0.185	5.255 ± 0.126	5.155 ± 0.095	7.05

Titratable acidity

The titratable acidity was determined titrimetrically by a 0.1 M NaOH with phenolphthalein as an indicator and it represented the used up millilitres of a 0.1M NaOH for neutralization of the acid in 10 ml of the product expressed in Toerner's degree (ºT). Table 6 gives the value of titratable acidity for milk fermented using commercial curd and the bacterial cultures (*Lactobacillus acidophilus, Lactobacillus bulgaricus*). Figure 2 depicts the summary of titratable acidity values for milk fermented using commercial curd and the

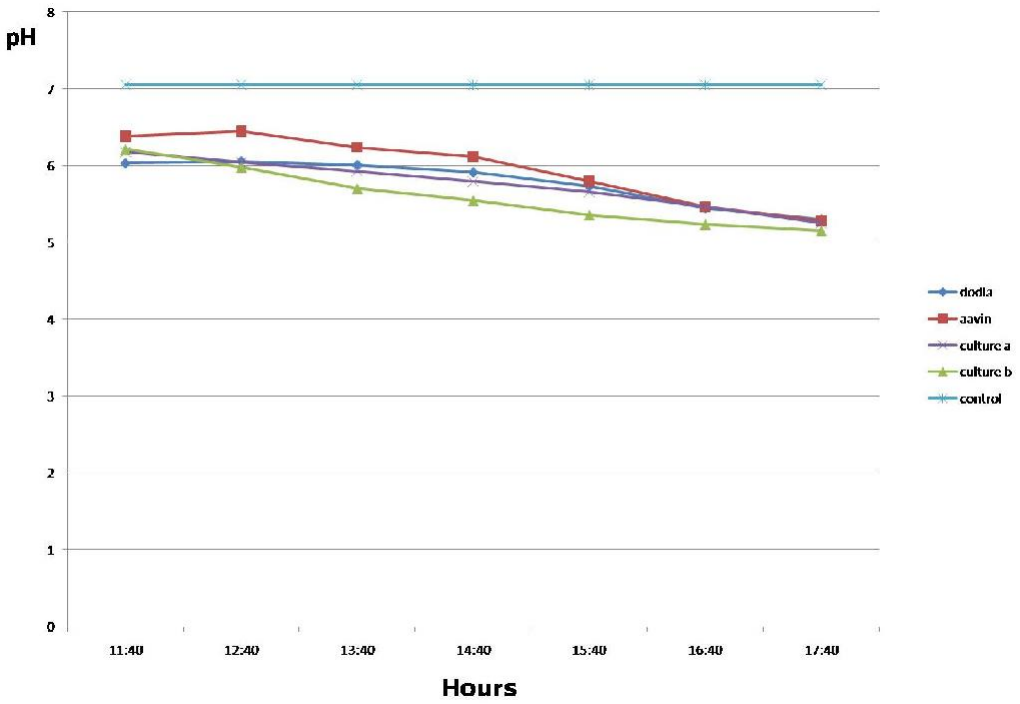

Figure 1. Time Vs mean +- S.D for pH values obtained for milk fermented using commercial curd and bacterial cultures.

Table 6. Titratable acidity values obtained for milk fermented using commercial curd and bacterial cultures.

Dodla curd	Aavin curd	Culture A	Culture B	Control
98.34	95.43	92.67	90.78	50.78
99.69	97.32	91.55	88.41	50.78
97.54	96.05	92.12	89.30	50.78
Mean				
98.52	96.27	92.11	89.49	50.78

Table 7. Viscosity values for pH values obtained for milk fermented using commercial curd and bacterial cultures.

Dodla curd	Aavin curd	Culture A	Culture B	Control
7.98	7.65	7.12	7.09	7.52
8.04	7.74	7.05	7.20	7.52
8.01	7.78	7.18	7.11	7.52
Mean				
8.01	7.72	7.12	7.13	7.52

bacterial cultures (*L. acidophilus, L. bulgaricus*).

Viscosity

Viscosity was measured using a viscometer (SVM 3000, Stabinger, manufactured by Antony paar) at 25°C and was expressed as mPas. Table 7 gives the value of viscosity for milk fermented using commercial curd and the bacterial cultures (*L. acidophilus, L. bulgaricus*). Figure 3 depicts the summary of viscosity plotted for milk fermented using commercial curd and the bacterial cultures (*L. acidophilus, L. bulgaricus).*

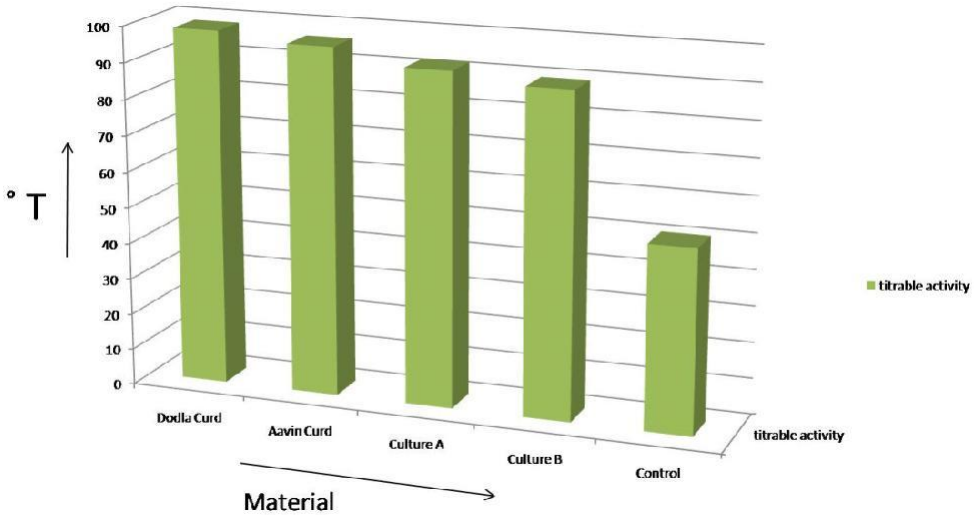

Figure 2. Time vs. mean ± S.D for titratable acidity values obtained for milk fermented using commercial curd and bacterial cultures.

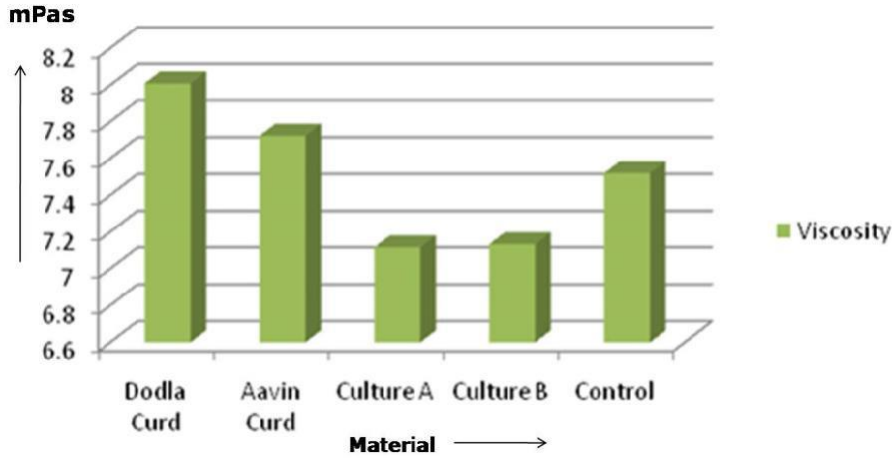

Figure 3. Time vs. mean ± S.D for viscosity values obtained for milk fermented using commercial curd and bacterial cultures.

Microbiological analysis of fermented milk

Commercial curds contained *Lactobacillus* which was identified by gram's staining and microscopic analysis. LB agar medium plates were used for this purpose. The streaked plates were kept for 48 hours at 37°C after which the microbial colonies were assessed. Figure 4 depicts the result of curd after streaked in a LB agar medium Petri plate for 48 hours. Gram's staining was performed (Figure 5) using gram's staining kit (Medox suppliers, India). Figure 6 illustrates the milk after fermentation by commercial curd and Figure 7 illustrates the milk after fermentation by bacterial cultures. Figure 8 shows the bacterial cultures used for the study, *Lactobacillus acidophilus, Lactobacillus bulgaricus*.

The mass of a 125 ml Erlenmeyer flask was determined. 50 ml of milk was added to the flask and re-weighed to determine the mass of the milk. A water bath was prepared by placing 200 ml of water in a 600 ml beaker and heated to 40°C. The flask containing the milk was placed into the water bath. 10 drops of glacial acetic acid was slowly added to the milk while stirring with a glass rod. Acetic acid was added drop wise until no more precipitate was formed when a drop of acid is added. The mixture was allowed to cool. The mixture was filtered into a 250 ml beaker by pouring it through cheesecloth that has been fastened to the beaker with a rubber band. Liquid was squeezed as much as possible from the solid. The solid was scraped into a 100 ml beaker. To remove the fat from the curd, 25 ml of 95% ethanol was added to the solid in the 100ml beaker. The mixture was stirred for about 5 minutes; then the solid was allowed to settle. The liquid was decanted into another beaker.

Isolation of caseinophosphopeptides (CPP)

Prepare 5 % casein suspension by mixing casein on a magnetic

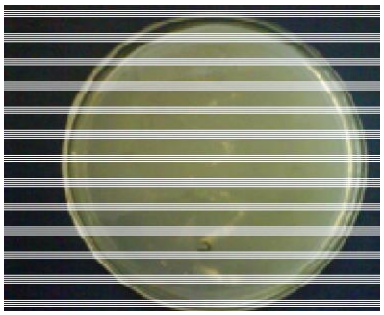

Figure 4. Curd streaked on a Petri plate containing LB agar medium.

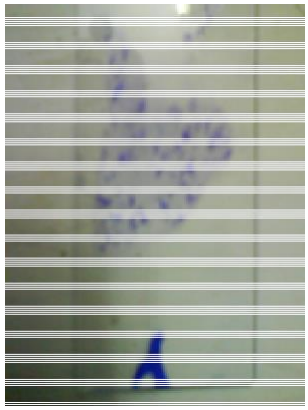

Figure 5. Gram's staining.

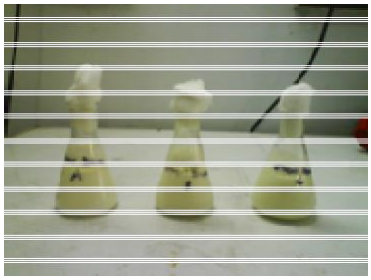

Figure 6. Fermented milk after the addition of curd.

Figure 7. Fermented milk after the addition of culture.

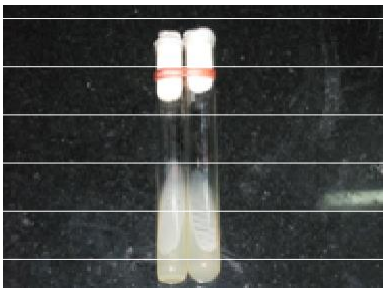

Figure 8. Cultures of *Lactobacillus* used.

Figure 9. Isolation of casein.

stirrer. Adjust the pH to 7 using 0.5 N NaOH. Add enzyme trypsin (obtained from himedia, Mumbai) at Enzyme: substrate ratio of 1:100. Hydrolysis is carried out by mixing the suspension using electric stirrer in water bath at 37ºC for 30 min. The pH of solution is kept constant at 7.0 by addition of 0.1N NaOH solution. After complete hydrolysis remove the mixture from water bath. Adjust the pH of casein hydrolysate to 4.6 using 2M HCl. Centrifuge at 3000 rpm (Alfa Laval manufactured from Houston, Texas, USA) for 10 min to remove the non-phosphorylated peptides. Collect the supernatant and adjust pH to 7.0 using 2.0 M NaOH. Add calcium chloride at 1% level to the supernatant and allow it for 10 minutes at room temperature. Add Ethanol 50% (V/V). The precipitate is collected by centrifugation at 6000 rpm for 10 min. The CPP thus obtained (Figure 9) was lyophilized.

Antimicrobial activity determination

The standard antibacterial assay used *Escherichia coli* (MTCC Number 443) *and Pseudomonas* sp (MTCC Number 1194). Bacteria were grown in LB broth representing 109 colony-forming units/ml, and 106 bacteria were added to 8 ml of 0.7% agarose in LB broth and poured over a 150-mm Petri dish containing 50 ml of 1.5% agarose in LB broth. Standard LB broth was prepared as described. Antibacterial activity was assayed by suppression of bacterial growth dependent on application of fractions to the top agar surface. For assays in liquid culture, fractions were added to a suspension of the organisms diluted from a mid-logarithmic phase liquid culture to a concentration of 105 cells per ml, in standard TSB broth. TSB was prepared from premixed components as described by the manufacturer (Hi-Media Laboratory) and adjusted to pH 7.5 with NaOH prior to autoclaving.

Molecular weight determination by gel electrophoresis

Polyacrylamide gels containing 2.0 - 2.6% of acrylamide (recrystallized from chloroform) were prepared as previously described by Loening (1967, 1968a). The concentration of

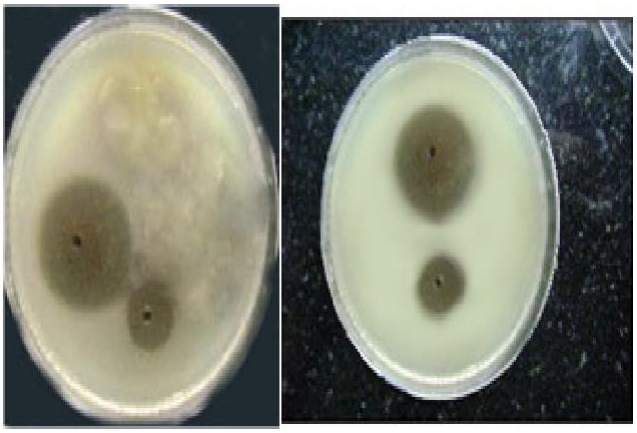

Figure 10. Zone of inhibition in case of (a) *E. coli* and (b) *Pseudomonas.*

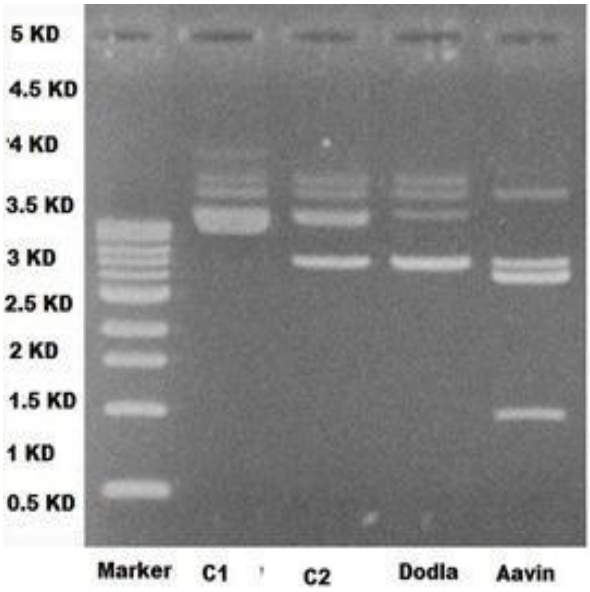

Figure 11. Gel electrophoresis for molecular weight determination.

bisacrylamide was 5% of that of the acrylamide throughout. Three buffers were used, E buffer contained 36 mM-tris, 30 mM-NaH_2PO_4 and 1 mm-EDTA (disodium salt), pH 7.7 - 7.8 at room temperature. The running buffer in the buffer compartments also contained SDS (0 - 2%). This buffer has a greater buffering capacity and a lower UV absorption than the tris-acetate buffer. Mg buffer was the same as the low-salt buffer but with magnesium acetate (2 mM) added.

FTIR analysis

FTIR analysis of the milk fermented by a commercially available curd brand, Dodla and the milk fermented by bacterial culture *Lactobacillus bulgaricus* were done. The interference pattern obtained from a two beam interferometer as the path difference between the two beams is altered, when Fourier transformed, gives rise to the spectrum. The transformation of the interferogram into spectrum is carried out mathematically with a dedicated on-line computer.

The Bruker IFS66v FT-IR instrument (VERTEX model, Bruker optics, Germany) consists of globar and mercury vapour lamp as sources, an interferometer chamber comprising of KBr and Mylar beam splitters followed by a sample chamber and detector. Entire region of 10-10000 cm^{-1} is covered by this instrument. The spectrometer works under vacuum conditions. Solid samples are dispersed in KBr or polyethylene pellets depending on the region of interest. This instrument has a resolution of 0.1 cm^{-1}. Signal averaging, signal enhancement, base line correction and other spectral manipulations are possible with multitasking OPUS software on the dedicated PC/AT 486. Spectra are plotted on a HP plotter (19 inch plotter, HP, USA) and data was printed.

RESULTS AND DISCUSSION

The pH of all the 4 separate samples which were taken in equal proportion was noted. There was a steady downfall in the pH as the time increased due to the fermentation process which was taking place. The milk samples fermented by cultures A and B that is *L. acidophilus* and *L. bulgaricus* showed lower values of pH when compared with the milk samples fermented by commercially sold curd. The lowest pH reached in case of microbial cultures was 5.155 ± 0.095 and the lowest value reached in case of milk samples fermented using commercially available curd was around 5.282 ± 0.185.

The titrable acidity was found to be the highest in case of the commercially sold curd brand, Dodla and the lowest was in the case of Culture B (*L. bulgaricus*). The control had the value at around 50.

The viscosity was highest for Dodla, commercially available curd brand and the lowest was in the case of another commercially available curd, Aavin.

Anti-microbial activity of the CPP was proven against *E. coli* and *Pseudomonas* sp. The zone of inhibition in case of *E. coli* was 14 mm and it was 16 mm in the case of *Pseudomonas* sp (Figure 10). These results establish the antimicrobial potential of the CPP against common clinical pathogens.

The molecular weight of the CPP was found to be in the range of 3.5 - 4 KD (Figure 11). The cultures were run along with two commercially available curd brands. A standard marker was made to run from the 1^{st} well.

FTIR analysis of the milk fermented by a commercially available curd brand, Dodla (Figure 13) and the milk fermented by bacterial culture *L. bulgaricus* were done. The peak was observed at 3411 cm^{-1} (Figure 12).

The CPP have got immunomodulatory activity and they have antihypertensive potential too. CPP are used in sports medicine and their role in that field also can be widened. The fermented milk peptides play a natural role in many biochemical and immunological mechanisms inside the human body and they can be formulated in to effective oral medicine for all age groups.

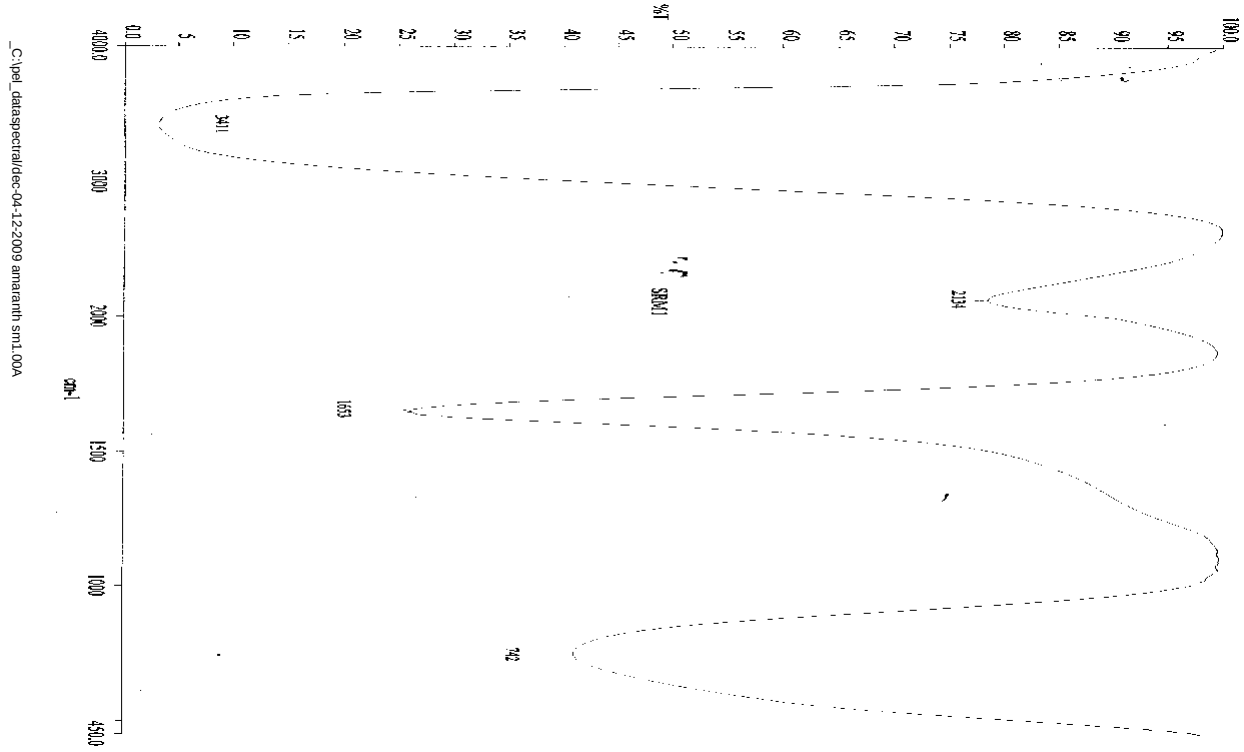

Figure 12. FTIR analysis of milk fermented by bacterial culture *Lactobacillus bulgaricus*.

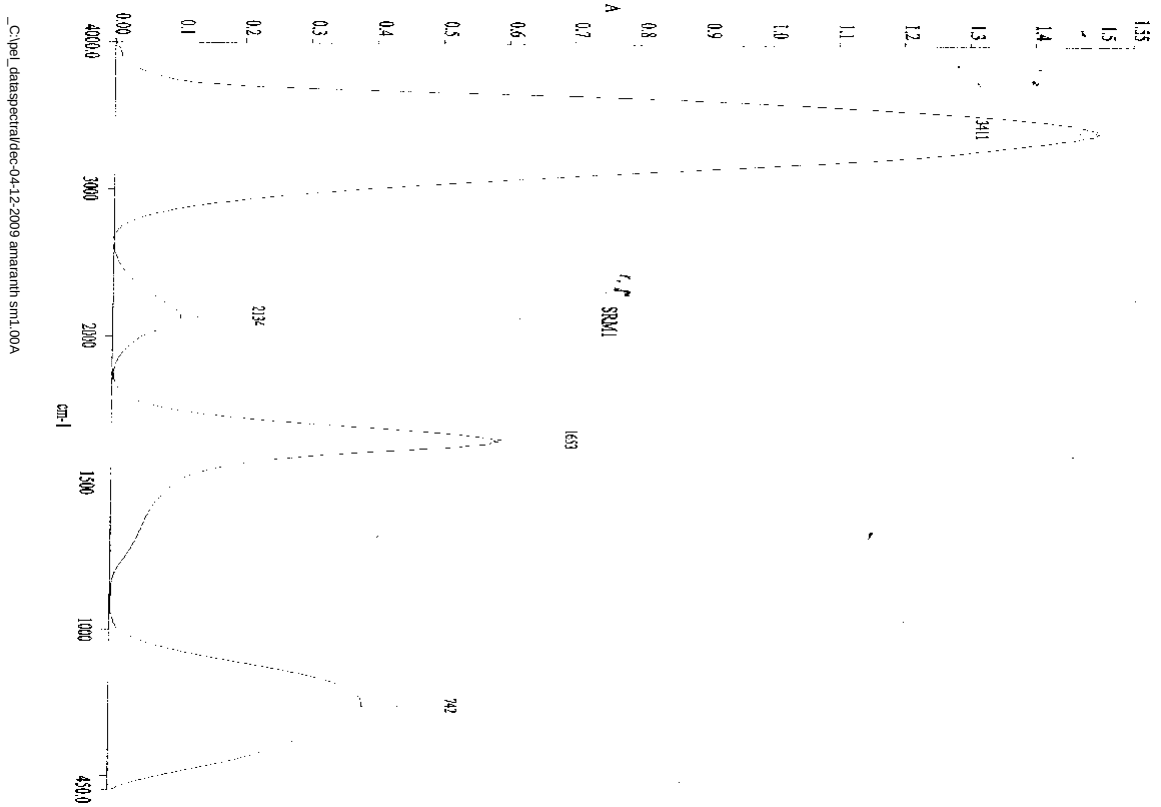

Figure 13. FTIR analysis of milk fermented by commercial curd brand Dodla.

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