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Evaluation of antimicrobial and antioxidant activity of fermented whey and its different molecular weight fractions

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Abstract
Whey is a major By Product of channa or paneer or cheese making dairy industry where its utilization and proper disposal is a major problem. This study aimed at evaluation of antimicrobial and antioxidant activity of whey and its partial characterization. The obtained whey was divided into three treatments, un-supplemented whey, whey supplemented with 1% soya flour and whey supplemented with 2% soya flour and all the whey treatments were fermented with 2% LAB culture at 37ºC for 48 hrs. All the whey treatments were subjected to high speed centrifugation followed by filtration, concentration and then reconstitution with sterile distilled water and stepwise ultra-filtration. The antioxidant activity of whey supplemented with 2% soya flour and its fractions (>30 kDa, <30kDa, >10 kDa, <10 kDa) was determined by DPPH free radical scavenging activity method and antimicrobial activity was tested against *Bacillus cereus, Listeria monocytogenes, Salmonella* Typhimurium and, *Escherichia coli* by agar well diffusion method. There were significant differences ($P< .05$) in ZOI and antioxidant activity of whey supplemented with 2% soya flour than other whey treatments. The ZOI was maximum against *Listeria monocytogenes* and there was significant ($P< .05$) difference in ZOI against *Listeria monocytogenes* and *Salmonella* Typhimurium.

Keywords: Antimicrobial, antioxidant, fermented whey, ultra-filtration, mw fractions

1. Introduction
Now a day consumers are very conscious and concerned about food safety issues and adverse effects of chemical food preservatives. Especially food spoilage is now a major concern of the society (Soomro *et al.*, 2002) [16]. Chemical preservatives are being used from decades and there are various scientific data that are showing intolerance of chemical food additives and their negative effects on health (Kregiel, 2015; Sweis and Cressey, 2018) [17]. Lipid oxidation is one of the reasons for deterioration of food quality which leads to undesirable changes in flavour, texture, and nutritional value. From a technical point of view, antioxidants are intended to prevent food spoilage by oxidation, thereby reducing nutrient loss and preserving texture, colour, taste, freshness, functionality and aroma of food. Antioxidants are, therefore, an essential group of food preservatives. By the use of antioxidants, rate of lipid oxidation can be retarded effectively. Synthetic antioxidants were widely used in food industry, but consumer concerns over safety and toxicity forced the food industry to find natural resources (Coronado *et al.*, 2002) [2]. There is a need of preservative which contain natural antimicrobial and antioxidant properties and for this bio-preservation is the only solution for both, the consumer concerns regarding food safety and chemical preservatives. Traditional fermented foods (dahi, yoghurt etc.) contain starter cultures preparations of one or several systems of microorganisms that can be applied to initiate the process of fermentation during food manufacture (Wigley, 1999) [19]. The most common bacteria are lactic acid bacteria that can ferment carbohydrates (lactose) to form chiefly lactic acid. These bacteria generally recognized as health enhancing bacteria. Whey used to be considered as the most significant pollutant of the dairy business, because of its high organic (lactose) burden, yet additionally because of its high volume (Walzem *et al*., 2002) [18]. Liquid whey is composed of more than 50% of whole milk solids including lactose (5%), water (93%), protein (0.85%), water soluble vitamins and minerals (0.53%), fat (0.36%) (Pescuma *et al*., 2008) [8]. There are two ways for valorisation of whey, one is recovery of valuable constituents like protein and lactose by implementation of technologies (ultrafiltration, nanofiltration or reverse osmosis etc.) and

another way is fermentation of whey to get value added products like single cell proteins (SCP), different acids (Lactic acid, succinic acid, propionic acid etc), biopolymers and bacteriocins (Prazeres, 2012) [19]. The aim of this study was to evaluate potential of fermented whey to utilize as natural bio-preservative in food system.

2. Materials and Methods

2.1 Preparation of whey

Whey was prepared by heating the skimmed milk in a stainless steel vessel up to a temperature of 90 ºC and then milk was cooled to a temperature of 72 ºC. The hot milk was acidified by using 2% citric acid solution with continuous stirring action resulting in coagulation of casein protein of milk and remaining liquid whey was filtered with double layered muslin cloth and cooled to a temperature that is adequate for the growth of starter culture.

2.2 Analysis of growth of revived culture in whey

Freeze dried cultures of lactic acid bacilli were revived following standard methodology according to NDRI, NCDC or ATCC catalogue. The growth of LAB in different whey treatments at different time intervals was determined by measuring the change in pH and acidity of whey.

2.3 Fermentation of whey

Here collected whey was divided into three treatments, treatment first was fresh un-supplemented whey, second treatment, whey supplemented with 1% soya flour and in third treatment, whey supplemented with 2% soya flour. Each treatment was inoculated with LAB culture @ 2% and allowed to ferment for 48 hours at 37 ºC under micro-aerophilic conditions in incubator.

2.4 Clarification of fermented whey

Clarification of different whey treatments was performed according to the method of Gobetti *et al*. (2004) and Samlesh *et al*. (2015) [14] with slight modifications. After 48hours of fermentation, each treatment was subjected to centrifugation at 8500rpm at 4 ºC for 20 minutes in a refrigerated centrifuge (Hermle Z32HK, Germany) and supernatant was collected and filtered with Whatman no.1 filter paper.

2.5 Concentration and reconstitution of supernatants

Concentration of obtained supernatants was done by drying in air incubator at 37 ºC for 48 hours by transferring supernatants into crucibles or petri plates. Reconstitution was done by adding sterile distilled water in appropriate ratio to each batch of dried supernatant. After reconstitution, each treatment was filtered with 0.22 µ sterile syringe filter to make all the treatments sterile and cell free.

2.6 Fractionation of clarified fermented whey and determination of protein content of different fractions

Clarified fermented whey treatments were fractionated by successive ultra-filtration steps using ultrafiltration spin columns of molecular weight cut off (MWCO) membrane filters of size 30 kDa, 10 kDa (Amicon Ultra Milipore, USA) by centrifugation at 8000 rpm for 15minutes at 4oC in refrigerated centrifuge (Hermle Z32HK, Germany). Permeates and retentates were collected after centrifugation. Different fractions (>30 kDa, <30 kDa, >10 kDa, <10 kDa) were passed through 0.22 micron syringe filters to make them sterile. The protein content (mg/ml) of each fraction was determined by using UV spectrophotometer method by using Take 3 plate at 280 nm (Biotek, USA).

2.7 Determination of antioxidant and antimicrobial activity of clarified fermented whey treatments and its fractions

Antioxidant activity of different clarified fermented whey treatments and its MW fractions was determined by 2, 2'–diphenyl-1-picrylhydrazyl (DPPH) assay with slight modification in method of Brand-Williams *et al*. (1995) [1]. Free radical scavenging activity was measured by visible light spectrophotometer (Genesys, 10S UV-Vis spectrophotometer) at 517 nm. Distilled water and BHT were used for blank value and as positive control respectively.

Antimicrobial activity of different clarified fermented whey treatments and its MW fractions was determined against two pathogenic Gram positive organisms, (*Listeria monocytogenes*, *Bacillus cereus*) and two Gram negative organisms (*Salmonella* Typhimurium, *Escherichia coli*) according to the method of Perez *et al*. (1990) [7] with some modification. On the MHA plates, 100 µl of the culture was spread uniformly with L- shaped spreader by preventing the entry of culture into the wells and 200 µl volume of each sterilized clarified fermented whey treatment and its fractions was placed into each well. Normal un-supplemented whey was taken as a negative control. Ampicillin (100mg/ml) solution was used as positive control.

2.8 Optimization of processing/fermentation conditions for maximum antimicrobial activity

2.8.1 Effect of inoculum level

The supplemented and un-supplemented whey treatments were inoculated with LAB culture @ 1%, 2% and 3% and incubated at 37 °C for 48 hours. All the treatments were subjected to centrifugation, concentration, reconstitution and filtration then finally evaluated for antimicrobial activity against two pathogenic indicator microorganisms by well diffusion method.

2.8.2 Effect of incubation time

Fermentation of whey treatments was performed from 12 to 48 hrs with selected inoculation level (@2%) of LAB culture. Antimicrobial activity of fermentates of different incubation time was determined against two pathogenic indicator organisms by well diffusion method.

3. Results and Discussions

3.1 Analysis of growth of revived culture in whey

Table 1. Represents significant ($P < .05$) reduction in pH and significant ($P < .05$) increment in titrable acidity with the progress in fermentation time showed a fast growth of the selected LAB strain. The maximum reduction in mean value of pH was observed in treatment C which reduced to 4.45 from an initial value of 6.63. The highest increase in T.A. was observed in treatment C from an initial acidity of 0.253% to final value of 1.032%. The obtained values can be compared with the findings of Shukla *et al*. (2013) [15] who had reported pH decline and rise in acidity during fermentation of whey ranging from 4.82- 3.30 and 0.394% to 1.353% respectively.

Table 1: Change in pH and acidity of whey samples with duration of fermentation

pH					
Samples	**0 hour**	**12 hour**	**24 hour**	**36 hour**	**48 hour**
A	5.96 ± 0.019^{Ca}	5.78 ± 0.078^{Ab}	5.14 ± 0.012^{Ac}	4.74 ± 0.012^{d}	4.64 ± 0.013^{Ad}
B	6.37 ± 0.020^{Ba}	5.47 ± 0.016^{Bb}	5.05 ± 0.014^{Bc}	4.65 ± 0.011^{d}	4.55 ± 0.015^{Be}
C	6.63 ± 0.012^{Aa}	5.11 ± 0.011^{Cb}	4.75 ± 0.014^{Cc}	4.56 ± 0.015^{d}	4.45 ± 0.009^{Ce}
Titrable acidity (%)					
Samples	**0 hour**	**12 hour**	**24 hour**	**36 hour**	**48 hour**
A	0.280 ± 0.02^{e}	0.339 ± 0.002^{Cd}	0.513 ± 0.003^{Cc}	0.672 ± 0.005^{Cb}	0.795 ± 0.020^{Ca}
B	0.212 ± 0.00^{e}	0.379 ± 0.003^{Bd}	0.611 ± 0.002^{Bc}	0.865 ± 0.003^{Bb}	0.987 ± 0.003^{Ba}
C	0.253 ± 0.14^{c}	0.554 ± 0.004^{Ab}	0.868 ± 0.002^{Aa}	0.915 ± 0.004^{Aa}	1.032 ± 0.004^{Aa}

n=6; Mean+S.E. with different superscripts differ significantly ($P< 0.05$); Capital alphabets show significant difference column wise and small alphabets show significant difference row wise ($P< 0.05$), A: Fermented un-supplemented whey with 2% inoculum level, B: Fermented whey supplemented with 1% soya flour with 2% inoculum level, C: Fermented whey supplemented with 2% soya flour with 2% inoculum level

3.2 Determination of protein content of fractions of different MW

There existed significant ($P< 0.05$) difference between protein content of unfermented un-supplemented whey, whey supplemented with 2% soya flour and its fractions. From the findings it was clear that high molecular weight fractions contained more protein than lower molecular weight fractions. The ultra-filter membrane concentrates the protein fraction in retentate, ultimately increasing the protein content in high molecular retentate fraction.

Table 2: Protein concentration of whey supplemented with 2% soya flour and its fractions of different MW

Samples	Protein (mg/ml)
C	74.06 ± 0.64^{A}
F_1 (<30kDa)	58.92 ± 1.48^{B}
F_2 (>30kDa)	72.91 ± 1.46^{A}
F_3 (<10kDa)	70.56 ± 2.06^{A}
F_4 (>10kDa)	71.43 ± 1.27^{A}
Control (whey)	8.58 ± 0.31^{C}

n=6; Mean+S.E. with different superscripts in a column differ significantly ($P< 0.05$), C: Fermented whey supplemented with 2% soya flour with 2% inoculum level, Control: Unfermented un-supplemented whey

3.3 Determination of antioxidant and antimicrobial activity of clarified fermented whey and its fractions

Table 3 Represents that average % free radical scavenging activity for treatment A, B and C was 61.49, 49.61 and 56.96 respectively, while that of positive control (BHT) at same concentration was 76.86%. Although, the antioxidant activity of fermented whey treatment was significantly ($P< 0.05$) lower than the BHT, however, these showed good antioxidant activity, significantly ($P< 0.05$) higher than unfermented whey. The DPPH radical-scavenging activity of all the three fermented whey treatments in the present study was much higher than the activity reported for hydrolysate of whey protein isolate (WPI) (31.48%) by Zhidong *et al.* (2013) [20]. However the activity was lower than the value reported for whey protein hydrolysates produced by *Bacillus clausii* (80%) in a study by Rochin *et al.* (2018) [12].

Table 3: Antioxidant activity of different whey samples

Samples	DPPH radical scavenging activity (%)
A	61.4933 ± 0.50^{B}
B	49.6100 ± 0.29^{C}
C	56.9600 ± 0.77^{B}
Control	1.3067 ± 0.30^{D}
BHT	76.8683 ± 0.19^{A}

n=6; Mean±S.E. with different superscripts in a column differ significantly ($P< 0.05$), A: Fermented un-supplemented whey with 2% inoculum level, B: Fermented whey supplemented with 1% soya flour with 2% inoculum level, C: Fermented whey supplemented with 2% soya flour with 2% inoculum level, Control: Unfermented un-supplemented whey.

Table 4 Represents that BHT exhibited maximum antioxidant activity followed by treatment C, >30 kDa, <30 kDa, >10kDa and <10 kDa. However, the significant ($P< 0.05$) difference was observed between whey and >10 kDa and <10 kDa. Among all the test samples, whey supplemented with 2% soya flour exhibited maximum % inhibition as compared to its other fractions, which may be indicative of the fact that larger peptides have greater free radical scavenging activity. The results were in tune with the various studies conducted on similar aspects as mentioned here. Das *et al.* (2021) [3] reported that crude extract of Indian curd exhibited higher antioxidant activity than its fractions. In study of Pritchard *et al.* (2010) [10] >10 kDa size fractions of cheddar cheese extract exhibited maximum inhibition of DPPH. Kumar *et al.* (2016) [5] observed higher antioxidant activity in camel milk casein hydrolysates than its fractions in terms of DPPH reduction.

Table 4: Antioxidant activity of whey supplemented with 2% soya flour and its different fractions

Samples	DPPH radical scavenging activity (%)
C	56.96 ± 0.77^{B}
F_1 (<30kDa)	51.59 ± 1.46^{BC}
F_2 (>30kDa)	51.98 ± 2.96^{BC}
F_3 (<10kDa)	46.49 ± 0.89^{C}
F_4 (>10kDa)	46.94 ± 0.76^{C}
BHT	76.86 ± 0.19^{A}

n=6; Mean+S.E. with different superscripts in a column differ significantly ($P< 0.05$), C: Fermented whey supplemented with 2% soya flour with 2% inoculums level

Table 5 Represents the ZOI of treatment A, B and unfermented whey, against all the four pathogens was found to be as zero or in single digit, showing non availability of antimicrobial activity. However, the treatment C, i.e. whey with 2% soya flour showed antimicrobial activity against all the four pathogens under study in terms of mean ZOI generated as 20.34, 23.17, 14.00 and 14.34 mm against *Bacillus cereus, Listeria monocytogenes, Salmonella* Typhimurium and *E. coli* respectively. Further, this sample showed a significantly ($P< 0.05$) higher ZOI against *Listeria monocytogenes*. The ZOI against *Salmonella* Typhimurium and *E. coli* were not differ significantly however, ZOI against *Bacillus cereus* was significantly ($P< 0.05$) higher than these two. The results were compared with the study of Sabo *et al.*

(2019) [13] who used supplemented cheese whey formulations along with soybean extract and fermented with *Lb. plantarum* ST16Pa and obtained cell free supernatant exhibited a ZOI of 13.23 mm against *Listeria innocua* 6a CLIST 2865, while cheese whey inoculated with same strain did not exhibited any inhibition activity.

Table 5: Antimicrobial activity of different whey samples, ZOI (mm)

Samples	*Bacillus cereus*	*Listeria monocytogenes*	*Salmonella typhimurium*	*E.coli*
A	NA	NA	NA	NA
B	NA	NA	NA	NA
C	20.34±0.76^{b}	23.17±0.65^{a}	14.0±0.36^{c}	14.34±0.56^{c}
Control (whey)	NA	NA	NA	NA

n=6; Mean+S.E. with different superscripts in a row differ significantly ($P< 0.05$), A: Fermented un-supplemented whey with 2% inoculum level,
B: Fermented whey supplemented with 1% soya flour with 2% inoculum level, C: Fermented whey supplemented with 2% soya flour with 2% inoculum level, Control: Unfermented un-supplemented whey, NA: Not available

The results showed that ZOI created by whey supplemented with 2% soya flour was the highest than its all fractions, however the difference was significant ($P< 0.05$) only with F3 treatment in case of *Bacillus cereus*, *Salmonella* Typhimurium and *E. coli* and with F3 and F4 in case of *Listeria monocytogenes*. The fraction of MW <10 kDa had shown minimum antimicrobial activity against all selected pathogens. All the samples showed maximum ZOI against *Listeria monocytogenes*, followed by *Bacillus cereus*, *Salmonella* Typhimurium and *E.coli*. However, this trend was significant at some places and non significant at some places as depicted in Table 6. These results may be supported by some studies as Kumar *et al*. (2016) [5] observed higher antimicrobial activity in camel milk casein hydrolysates than its fractions. Pritchard *et al*. (2010) [10] reported inhibition of *Bacillus cereus* by peptides of size greater than 10 kDa of cheddar cheese. Das *et al*. (2020) [3] reported that crude extract of Indian curd exhibited higher antimicrobial activity than its fractions by agar well diffusion assay.

Table 6: Antimicrobial activity of whey supplemented with 2% soya flour and its fractions of different MW, ZOI (mm)

Samples	*Bacillus cereus*	*Listeria monocytogenes*	*Salmonella typhimurium*	*E.coli*
C	21.17±0.65^{Ab}	25.50±0.67^{Aa}	18.67±0.71^{Abc}	15.00±1.46^{Ac}
F_1 (<30kDa)	20.34±0.49^{Aa}	22.67±0.66 ^{ABa}	17.50±0.42^{Ab}	13.50±0.99 ^{ABc}
F_2 (>30kDa)	20.50±0.42^{Aa}	23.34±0.61 ^{ABa}	16.50±0.99 ^{Ab}	14.00±1.21 ^{ABb}
F_3 (<10kDa)	14.50±0.56^{Bb}	20.50±0.76^{Ba}	13.34±0.76 ^{Bb}	10.34±0.42^{Bc}
F_4 (>10kDa)	19.16±0.60^{Aab}	21.67±0.80^{Ba}	16.67±0.55 ^{Ab}	12.17±0.60 ^{ABc}

n=6; Mean+S.E. with different superscripts differ significantly ($P< 0.05$); Capital alphabets show significant difference column wise and small alphabets show significant difference row wise ($P< 0.05$), C: Fermented whey supplemented with 2% soya flour with 2% inoculum level

3.4 Optimization of processing/fermentation conditions for maximum antimicrobial activity

3.4.1 Effect of inoculum level

It was observed that whey supplemented with 2% soya flour showed maximum inhibition as compared to the other treatments. The average mean values of zone of inhibitions against *Listeria monocytogenes* for whey supplemented with 2% soya flour were 20.34, 24.34 and 26 mm and against *Salmonella* Typhimurium were 14, 14.34 and 15.67 mm at 1%, 2% and 3% inoculum level respectively (Table 7). There was no significant ($P< 0.05$) difference between ZOI received for whey supplemented with 2% soya flour at 2% and 3% levels against *L. monocytogenes* and at 1%, 2% and 3% level against *S.* Typhimurium. The fermented un-supplemented whey and whey supplemented with 1% soya flour did not exhibit any significant inhibition at different levels of inoculation except whey supplemented with 1% soya flour had shown zone of inhibition of size 13mm at 3% inoculum levels against *Listeria monocytogenes.* Pourahmad *et al*. (2011) [11] reported effect of inoculum level on microbial population in kefir soymilk. Kumari *et al*. (2015) [6] reported effect of inoculum level on antimicrobial activity of *Lactobacillus rhamnosus* C6 fermented whey and soy milk with maximum acceptability at 2% inoculum level. Thus, on the basis of findings of this experiment, 2% inoculum level was found as optimum.

Table 7: Effect of inoculum level on antimicrobial activity of different whey samples, ZOI (mm)

Listeria monocytogenes			
Samples	**Inoculum level**		
	1%	**2%**	**3%**
A	NA	NA	NA
B	NA	NA	13.00±0.57
C	20.34±0.88^{b}	24.34±0.67^{a}	26.00±0.58^{a}
***Salmonella* Typhimurium**			
Samples	**Inoculum level**		
	1%	**2%**	**3%**
A	NA	NA	NA
B	NA	NA	NA
C	14.00±0.57	14.34±0.34	15.67±0.34

n=6; Mean±S.E. with different superscripts in a row differ significantly ($P< 0.05$), A: Fermented un-supplemented whey with 2% inoculum level,
B: Fermented whey supplemented with 1% soya flour with 2% inoculum level, C: Fermented whey supplemented with 2% soya flour with 2% inoculum level, NA: Not available

3.4.2 Effect of incubation time

It was observed that whey supplemented with 2% soya flour exhibited larger zone of inhibition at different time intervals than other whey treatments against both *L. monocytogenes* and *S.* Typhimurium. The whey supplemented with 2% soya flour did not exhibit any zone of inhibition at 12 hours but it was clear from Table 8. that mean ZOI was increased significantly ($P< 0.05$) after 24 hours to 48 hours ranging from 10.84-22.34 mm against *L. monocytogenes* and 10.34-14.10 mm against *S.* Typhimurium. ZOI against *L. monocytogenes* was observed to be significantly ($P< 0.05$) higher at 48hrs of incubation than at 24 and 36hrs while ZOI against *S.* Typhimurium was significantly higher ($P< 0.05$) at

48hrs than at 24hrs and comparable at 36 hrs. The maximum mean ZOI was observed at 48 hours, so the optimum time for fermentation was selected as 48 hours. A study by Kumari *et al*. (2015) [6] also reported the effect of incubation time on antimicrobial activity of whey and soy milk derived peptides with maximum acceptability at 48 hours of fermentation.

Table 8: Effect of incubation time on antimicrobial activity of different whey samples, ZOI (mm)

Listeria monocytogenes				
Samples	**Incubation time**			
	12 hour	**24 hour**	**36 hour**	**48 hour**
A	NA	NA	NA	NA
B	NA	NA	NA	NA
C	NA	10.84±3.45^{c}	16.50±1.34^{b}	22.34±1.40^{a}
***Salmonella* Typhimurium**				
Samples	**Incubation time**			
	12 hour	**24 hour**	**36 hour**	**48 hour**
A	NA	NA	NA	NA
B	NA	NA	NA	NA
C	NA	10.34±0.49^{b}	12.84±0.40^{a}	14.0±0.36^{a}

n=6; Mean+S.E. with different superscripts in a row differ significantly (P<0.05), A: Fermented un-supplemented whey with 2% inoculum level

B: Fermented whey supplemented with 1% soya flour with 2% inoculum level, C: Fermented whey supplemented with 2% soya flour with 2% inoculum level, NA: Not available

4. Conclusion

Fermented whey supplemented with 2% soya flour gave good antimicrobial activity than unfermented un-supplemented whey. The antioxidant activity of fermented whey supplemented with 2% soya flour was higher than unfermented un-supplemented whey by DPPH free radical scavenging activity method. Antioxidant and antimicrobial activity of whey supplemented with 2% soya flour was better than its fractions of different molecular weight. This study was a trial for the utilization of whey as a bio-preservative. Thereby whey supplemented with 2% soya flour can be explored for further work like shelf life trials in food systems, use of fermented whey in neutraceuticals etc.

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