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ANTIMICROBIAL ACTIVITY OF WHEY POWDER PRODUCED BY NON-THERMAL METHOD

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ABSTRACT

Whey, is a natural product resulting from coagulation of milk. Herein, whey powder prepared by non thermal method, was optimized and investigated for its anti-microbial potential against twelve bacerial isolates belonging to three bacterial species by agar-well diffusion assay. Minimum Inhibitory Concentration values were determined by tissue culture plate method to check the concentration ranges for significant inhibition.From the well diffusion assay, the whey at the concentration of 500mg/ml showed a strong bactericidal activity against all the bacterial isolates, which was almost similar to the commercial antibiotics. The minimum inhibitory concentration for the whey powder was found to be 500 (µg/ml) for nearly six bacterial isolates (*E.coli MTCC 1089, E.coli MTCC 1554, E.coli MTCC 1555, S. typhi S005, S. paratyphi S019* and *S.paratyphi D420*) out of the twelve isolates used in the present study. On the other hand the isolates namely *S.paratyphi S 023, Shigella flexneri MTCC 1457* and *S. typhi UT 3373, S. tyhpi D 23580* were inhibited at the lower dose of 125µg/ml and 250µg/ml respectively. While the strain *E.coli MTCC 82* required about 1000µg/ml of whey powder to attain its Minimum Inhibitory Concentration. The results provide evidence that the whey powder is indeed the potential source of new antibacterial agent which will be helpful for people in the poor community.

KEYWORDS

Agar-well diffusion assay, Minimum Inhibitory Concentration, Non-thermal method, Whey.

INTRODUCTION

A wide variety of antibiotics are commonly used for the treatment of serious infections caused by bacteria[1]. In recent years, multiple drug resistance, a threat to mankind, has developed due to indiscriminate use of existing antimicrobial drugs in the treatment of infectious diseases[2]. In addition to this problem, antibiotics are sometimes associated with adverse effects on the host, including hypersensitivity, immune suppression and allergic reactions[3]. Due to the side effects of conventional medicine, the use of natural products has emerged as an alternative in healing and treatment of various diseases has been on the rise in the last few decades.

Antimicrobial drugs from natural origin are used in medicinal practices for treating food-borne diseases. Over 50% of all modern clinical drugs are of natural product origin [4] and natural products play an important role in drug development programs in the pharmaceutical industry [5]. Since ancient times, gastrointestinal problems caused due to microbes have been treated orally with whey liquid, based on traditional Siddha medicine. Whey is a popular natural dietary protein supplement, resulting from coagulation of milk, purported to provide antimicrobial activity, immune modulation, improved muscle strength and body composition, and prevention of cardiovascular disease and osteoporosis[6]. Intact whey contains several components with broad antimicrobial activity[7].

Whey proteins contain bioactive components including β -lactoglobulin, α -lactalbumin, immunoglobulins, lysosymes and bovine serum albumin. Furthermore, antimicrobial peptides like glycomacropeptides, lactoferrin and lactoperoxidase may be generated from whey protein by proteolysis during gastrointestinal transit [8, 9].

The advantages of whey protein-derived antimicrobial peptides are that they are derived from a safe substance and they may be produced by naturally occurring enzyme activation. Recently protein based edible films coated with active antimicrobials have been shown to be effective at inhibiting growth of pathogenic bacteria, thus increasing food safety[10]. With the above scenario an attempt was made in evaluating the antimicrobial potential of whey powder which was produced by non thermal method.

EXPERIMENTAL METHODS

***1. Media Preparations*:** All media used were reconstituted according to the manufacturer's specifications, sterilized at 121^{0}C.

2. Micro organisms:

The bacterial strains, *E. coli* (MTCC 1089, MTCC 82, MTCC 1554, MTCC 1555), *Salmonella typhi* (MTCC 3917, UT 3373, S 005, D23580), *Salmonella paratyphi* (S 023, S 019, D 420) *Shegilla flexneri* (MTCC 1457), were obtained from the Department of Biotechnology, Dr. M.G.R. University, Tamil Nadu, India. The bacterial strains were maintained with nutrient agar and sub cultured for every three days.

The inoculum of each bacterial strain was suspended in 5ml of Mueller Hinton broth (MHB) and incubated overnight at 37°C. The overnight cultures were diluted with MHB prior to bacterial testing [11].

3. Chemicals and Glass wares:

The Chemicals used for the study are of analytical grade and general glass wares were procured from standard firms.

4. Non-thermal Production of whey powder:

To 1000mL of cow's milk, stored at 4° C for 12 hours, 15mL of 20% food grade lactic acid and 0.001% of rennet powder was added and mixed well. Following a 30 minutes incubation of mixture in a 40°C water bath, casein and whey liquid were separated using a cheese cloth. The whey liquid collected was stored at 4°C until lyophilization. The yield of the lyophilized sample was found to be 6.25% w/v and it was stored at -20°C for further use.

5. Biochemical estimation of whey powder:

Lactose was estimated using a titration method described by Otto Folin and Denis (1918) [12]. Accordingly, the whey sample was titrated against the Benedict's quantitative reagent using standard lactose solution and the amount of lactose present in the whey powder was calculated. The estimation of total protein was performed by Lowry's method[13]. The principle was based on the presence of phenolic group of tyrosine and tryptophan residues (amino acid) in the protein reacting with folin-ciocalteau reagent producing a blue purple color complex, with maximum absorption in the region of 660 nm wavelength.

6. In vitro Antibacterial Study:

Following methods were performed to determine the antimicrobial activity of whey powder.

a) ***The agar-well diffusion method*** of Cappuccino and Sherman (1999) [14] was employed to study the antibacterial activity of the whey powder , 3.7% of Muller Hinton Agar was mixed with hot distilled water and autoclaved at 15 lb pressure for 15 minutes. After autoclaving, it was allowed to cool to 45^{o}C-50^{o}C. Then the medium was poured into sterilized Petri dishes with a uniform depth of approximately 4 mm. The agar medium was allowed to cool to room temperature. For the transformation of bacteria to Petri dish a swab dipped in standard inoculums was used. After dipping, the swab was used to spread the bacteria on the media in a confluent lawn. Then the Petri dishes were left for 3 to 5 minutes. Using cork borer, 6 mm diameter wells were made in all the plates. Different concentrations of whey were added to the groove with one blank of each. Distilled water was used as blank. Plates were incubated for 24 hours at 37^{o}C. After 24 hours the plates were examined. Results were recorded, as the presence or absence of inhibition zone. The inhibitory zone around the well indicated absence of bacterial growth. The diameters of the zones were measured using diameter measurement scale. The effect of whey powder was compared with the standards given by National Committee for Clinical Laboratory Standards (NCCLS). The zone of inhibition of whey as well the standard antibiotics Ampicillin (10µg/ml), Chloramphenicol (65µg/ml) and Norfloxacin (5µg/ml) against E.coli, Salmonella and Shigella respectively [15, 16] was measured.

b) ***The Minimum inhibitory concentration (MIC)*** The MIC concentration of whey powder was evaluated by dilution method[17]. Aliquots of whey powder (100mg/ml to 500mg/ml) were aseptically mixed with liquid media. These media were inoculated with the test bacteria and incubated. The lowest dilution at which there is no bacterial growth is considered significant. The turbidity of the test sample is measured by spectrophotometer with respect to blank which contains only medium.

7. Evaluation of MIC by Tissue Culture Plate:

The Minimum Inhibitory Concentration (MIC) of the whey sample could not be determined by broth dilution method because there was a formation of biofilm in the broth after the incubation period. So we used tissue culture

plate method to evaluate the MIC of the whey sample.

The Minimum Inhibitory Concentration was studied by a tissue culture plate assay method described earlier by [18] Christensen et al., (1985) with slight modifications. All the isolates were inoculated in LB broths separately and were incubated overnight at 37°C. 1 in 100 dilutions of the inoculums of different isolates in fresh LB medium were prepared and 100µl of the same was placed in all the wells marked as "1 to 9". Aliquots of different concentration of the whey powder ranging from 7.80 µg/ml to 2000µg/ml were prepared and 100µl of the whey sample was added to the appropriate wells. The 10th well served as negative control which contained 200 µl of the diluted inoculums. The same concentration of fresh medium was kept as positive control in the 11th well. The 0th hour reading was taken in the ELISA reader at 570 nm. The plate was incubated at 37°C for 18hours in the incubator and final optical densities (OD) were determined with a micro ELISA auto reader at wavelength of 570 nm. These OD values were considered as an index of whey powder showing the Minimum Inhibitory Concentration.

8. Statistical Analysis:

All data was analyzed statistically with Statistica/Macsoftware (Prism, USA). The experimental results were mean ± SE of three parallel measurements.

RESULTS

Considering the possibility of protein turning carcinogenic on prolonged heating in the present study, we standardized a non-thermal method of whey preparation using rennet powder and lactic acid, there by retaining the proteins in native form without denaturation. We were able to optimize our non thermal whey powder preparation using 15mL of 20% food grade lactic acid and 0.001% of rennet powder. The lactose content in whey powder prepared herein was estimated as 6.5% whereas the whey protein content was found to be 0.9%.

Antibacterial Activity by Well Diffusion Method:

The zone of inhibition against all the isolates increased with the increase in the concentration of the whey powder (Table 1). In the present investigation the whey powder could not inhibit the growth of the bacterial isolates at the lower concentration of 100 and 200mg/ml. In higher concentrations the bactericidal activity of the whey powder was quite significant as evidenced by the presence of zone of inhibition surrounding the wells. It was observed that the zone of inhibition produced by all the bacterial isolates as the result of the treatment of 500mg/ml whey powder concentration was almost found to be similar when compared to the standard antibiotics used in the present study.

Evaluation of MIC by Broth Dilution Method:

The Minimum Inhibitory Concentration (MIC) could not be determined by broth dilution method because of the interaction between whey sample microbes and culture media. The solution became turbid and there was appearance of a film revolving around after the incubation period. This indicated the biofilm forming potential of the whey powder. The biofilm formation was observed in concentrations such as 200, 300 mg/ml whereas at higher concentration such as 500mg/ml there appeared a precipitation in the broth due to the dispersion process taking place in the broth.

Table 1
In vitro Antimicrobial Potential Investigation of whey powder

Organisms	No.of isolates	Zone of Inhibition (mm)							
		Concentration of whey powder(mg/ml)					Standard Antibiotic		
		100	200	300	400	500	Amp	Chloramp	Nor
E.coli	04	-	-	11.12±0.15	11.87±0.20	13.97±0.29	14±0.57	-	-
S.typhi	04	-	-	12.23±0.11	12.78±0.33	13.13±0.17	-	13.2±0.57	-
S.paratyphi	03	-	-	11.91±0.52	12.82±0.44	13.4 ± 0.20	-	13.7±0.58	-
Shigella flexneri	01	-	-	12.12±0.17	13.64±0.31	14.13±0.60	-	-	14.30±0.54

All the values are expressed as mean ± SEM (n=3)

Evaluation of MIC by Tissue Culture Plate (TCP) Assay:

As shown in the Table -2, the minimum inhibitory concentration for the whey powder was found to be 500 (µg/ml) for nearly six bacterial isolates (*E.coli MTCC 1089, E.coli MTCC 1554, E.coli MTCC 1555, S. typhi S005, S. paratyphi S019* and *S.paratyphi D420*) out of the twelve isolates used in the present study. On the other hand the isolates namely *S.paratyphi S 023, Shigella flexneri MTCC 1457* and *S. typhi UT 3373* and *S. tyhpi D 23580* was inhibited at the lower dose of 125µg/ml and 250µg/ml respectively. *E.coli MTCC 82* required about 1000µg/ml of whey powder to attain its MIC.

Table 2
MIC Values for Bacterial Isolates against whey Powder Using TCP Assay

Organisms	Whey powder Concentration (µg/ml)								
	7.80	15.60	31.25	62.50	125	250	500	1000	2000
E.coli MTCC1089	-	-	-	-	-	-	β	+	+
E.coli MTCC 82	-	-	+	+	+	+	+	β	+
E.coli MTCC1554	-	-	-	+	+	+	β	+	+
E.coli MTCC1555	-	-	-	-	-	+	β	+	+
S. typhi UT 3373	-	-	-	+	+	β	+	+	+
S. typhi S 005	-	-	+	+	+	+	β	+	+
S. tyhpi D 23580	-	-	+	+	+	β	+	+	+
S.paratyphi S 023	-	-	-	-	β	+	+	+	+
S. paratyphi S019	-	-	-	+	+	+	β	+	+
S.paratyphi D420	-	-	-	+	+	+	β	+	+
Shigella flexneri MTCC 1457	-	-	-	-	β	+	+	+	+

- = Resistance (Growth of bacteria i.e turbid)
+ = Concentration showing no turbidity (Inhibition of bacterial growth)
β = Least Concentration showing no turbidity. (MIC)

DISCUSSION

Whey is a by-product in cheese manufacturing process and is usually prepared by a thermal process, wherein alteration in protein structure might occur by the heating as observed by aggregation and gelling of whey protein[19]. In general, protein profiles for heat-treated skimmed milk indicate that the denaturation of the total proteins begins at 40°C, accelerates with increasing temperature, and becomes 95% complete at 85°C [20].The presence of considerable quantities lactose and total protein of whey powder and its antibacterial potential shows the presence of biomolecules intact. The biological components of whey and whey products depend on the methods of production, purification and concentration of lactic acid and rennet. Hence this method can be adapted for commercial preparations of whey powder.

In developing country like India, majority of people living in rural areas almost depend on the use natural biomolecules to treat their various health problems because of their cost effective nature. So an attempt was made to evaluate the antimicrobial activity of the whey powder which can be utilized in an effective manner as a therapeutic agent against the Gastro intestinal bacterial infection. In the present investigation the whey powder showed a significant antimicrobial activity against the pathogenic intestinal bacterial strains. The antimicrobial activity of whey powder was almost similar to the commercially available antibiotics used in the study. According to De Wit (1989) [21], whey power is predominantly made of up of whey proteins namely lactoferrin (Lf), lactotperoxidase (Lp), glymacropeptide (GMP), immunoglobulins (Ig) etc. The bactericidal activity of the whey powder may be due to the presence of these protein fractions.

Sicairos et al., (2006)[22] have reported that the iron binding protein lactoferrin which is one of the component of whey powder, is able to kill the pathogenic Gram positive bacteria by sequestering iron from bacteria there by inhibiting its growth and metabolism[23] . This may be one of the mechanisms of action for its antimicrobial activity. The antimicrobial activity of whey protein can also be accounted for the presence of lactoperoxidase. Lp catalyse the oxidation of thiocyanite into hypothiocyanate ion, a strong oxidizing agent which causes damage to bacterial cells[24].The antimicrobial action might also be due to different types of Immunoglobulins (IGS) present in whey powder. IGS from milk were used for effective treatment of various infections in new born infants according to Reiter (1978)[25]. The presence of IGS, Lf, Lp and GMP in whey powder are cumulatively responsible for its antimicrobial activity which act in different manner on different pathogenic strains of bacteria. In addition, the antimicrobial potency was found to be almost similar to the commercially antibiotics ampicillin, chloramphenicol, norfloxacin used in our present study. Even though we could not succeed to find out the MIC of whey powder by broth dilution method, we were able to identify the biofilm forming tendency of whey in interaction with microbes at certain concentrations which can also accounted for the antimicrobial activity. This is because according to Kinesella (1984)[26] most abundant and important component for film formation in whey powder is Beta- lactoglobulin. Finally the antimicrobial activity may be due to the bio-film formation by the interaction of whey proteins with that of bacterial cells or the bacterial cells might get trapped in the biofilm which is formed by means of whey power alone.

CONCLUSION

Due to the population explosion and occurrence of unhygienic conditions, the incidence of bacterial infection is more common in a developing country like India. The whey

protein, one of the by-products of milk industry is not properly utilized in spite of its various important bio molecules. In the present investigation it has been shown that whey powder can be utilized in an effective manner as antimicrobial agent which will be helpful for the people in the poor community.

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