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Original Research Article

Optimization of conditions for production of bioactive peptides showing antimicrobial effect on bacterial pathogens during fermentation of bovine milk with Lactic Acid Bacteria

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Abstract

Antimicrobial peptides (AMPs) are small molecular weight proteins with broad spectrum antimicrobial activity against bacteria, viruses, and fungi. There are varieties of antibacterial peptides encrypted within the sequence of milk proteins that are released upon suitable hydrolysis of the precursor protein. AMPs kill microbial cells by interaction with molecules. Present study carried out for optimization of conditions to produce bioactive peptides with antimicrobial activity during fermentation of bovine milk with Lactic Acid Bacteria. We used three different cultures of Lactic Acid Bacteria for the fermentation of bovine milk from different sources. Out of three one was pure culture of Lactobacillus helveticus, second one was curd with unknown culture and third was combination of Lactococcus and Lactobacilli. Culture was inoculated @ 1.0%, 2.0%, 3.0%, 4.0%, and 5.0%, and incubated at 37℃, 40℃ and 44℃ for a period of 16h, 20h, 24h, and 28h respectively in bovine milk. Bioactive peptides generated during fermentation were separated using different techniques and tested for antimicrobial activity. These peptides first purified with gel filtration technique and then subjected to reverse phase high performance liquid chromatography. Different fractions were collected at different time interval and were subjected to antimicrobial activity against Gram's positive and Gram's negative bacterial pathogen. Different peptides showed different antimicrobial effect on the inhibition of pathogen. This study demonstrated that bioactive peptides generated during fermentation with L. helveticus showed a good degree of inhibition including zone of inhibition -17mm against Gram's negative bacteria and 15mm for Gram's positive bacteria at 5.1 pH for 20 hours of incubation and combination of Lactococcus and Lactobacilli at 4% inoculation showed a good degree of inhibition zone 15.6 against Gram's positive bacteria and 12mm for Gram's negative bacterial when kept at 3.2 pH for 28 hours of incubation.

Keywords: *Antimicrobial peptide, bovine milk peptides, fermented milk peptides.*

1. Introduction

In the industrial countries malnutrition is hardly a problem anymore, so that concern increasingly focuses on the quality rather than quantity of food. Bioactive peptides have a positive impact on body functions or conditions and may ultimately influence health. Many food proteins can exert a physiological action either directly or after degradation (Bhagat Singh *et al*, 2011, Butikofer, U. *et al*, 2007, Minervini, F. *et al* 2003). Peptides represent a quite heterogeneous class of compounds and their characteristics deeply depend on the amino acid composition and on the length of the chain (Flavio Tidona *et al*. 2009, Joachim Molkentin., 2000, Meisel, H. 1997). Additionally and synergistically to peptide hydrolysates, some intact milk proteins can participate in the host defense (Lahov, E. and Regelson, W., 1996). Lysozyme content is particularly rich in the milk of humans. It work by peptidoglycan hydrolysis causing the lysis of the bacterial cell wall, although an increasing body of evidence supports the existence of a non- enzymatic and/or non lytic mode of action. Common feature of antimicrobial peptides is their net positive charge & properties for forming highly ordered amphipathic conformation, such as helices or β -sheets upon interaction with the negatively charged phospho-lipids of the bacterial cell membrane (Tomita, M., *et al*., 1991).

The first antimicrobial peptides have been derived from the whey protein lactoferrin. The peptide derived from casein also have antimicrobial activity Casocidin released by chymosin digestion of casein at neutral pH, was the first defensive peptide actually purified and exhibited activity in vitro against *Staphylococcus aurius*, *Serracia marcecens*, *Bacillus subtilis Diplococcus pneumonniae* & *Streptococcus pyogens*. Casocidin I a cationic αs_2 casein derived peptide inhibited growth of *E. coli* and *Staphylococcus*, Isracidin, a N- terminal segment of α-s_1 – casein has been reported to protect mice against S*taphylococcus aureus* and *Candida albicans* (Yamauchi, K., *et al*., (1993). These peptides also protect sheep and

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cow from mastitis. Dionysius and Milne have identified two peptides from the N- terminal of lactoferrin which displayed antimicrobial activity towards a number of pathogenic and food spoilage micro-organisms. Strains of *Lactobacillus helveticus* were screened for antimicrobial activity exerted by the bovine skim milk whey supernatant. The cell-free extract of different strains of *L. helveticus* showed inhibition for both Gram -positive and -negative pathogens (Bhagat Singh *et. al*, 2012). Some AMPs exhibit unique mechanism for killing bacteria compared with current antibiotics. These AMPs selectively bind to the outer lipid membrane of the bacterium and form blisters and pores, which eventually result in lyses of the cell and cellular death. AMPs also have the ability to stimulate the production of II-Iβ. The stimulation of IL-Iβ would create an increase in chemo taxis of the neutrophils to that area. These neutrophils contain AMPs produced from the animal, which would serve as a secondary source of AMPs for the host. These peptides may be beneficial for treatment against microbial infection (Yamauchi, K., *et al*., 1993).

2. Materials and Methods

2.1 Fermentation condition

Activation of *Lactobacillus helveticus* culture was done using MRS broth which is specific for Lactic Acid Bacteria. Five ml of MRS broth was taken in 15 ml test tube in duplicate. After sterilization, it was cooled at room temperature. The lyophilized ampoule of *L. helveticus* was broken aseptically in laminar air flow and small amount (one loop full) of the dried culture was transferred into tube containing MRS broth. It was mixed properly using vortex shaker and then incubated at 30°C in an incubator for 24 to 48 hours. After 48 hours the tubes were observed for the growth and purity of culture was tested, other inoculums were prepared by using equal amount of *Lactococcus* and *Lactobacillus*. The skimmed buffalo milk was prepared in lab. The milk was autoclaved at 121°C for 15 minutes and was cooled to room temperature. And the flask was inoculated with the three combinations respectively. Three different cultures of Lactic Acid Bacteria for the fermentation of bovine milk were used. Out of three one was pure culture of *Lactobacillus helveticus*, second one was local curd with unknown culture and third was combination of *Lactococcus* and *Lactobacilli*. Culture was inoculated @ 1%, 2%, 3%, 4% and 5%, at 37°C, 40°C and 44°C for a period of 16h, 20h, 24h, and 28h respectively. Then the curd was mixed properly to break large curd particles and centrifuge at 10,000 rpm for 10min at 4°C using Kubota high speed centrifuge (Japan). The supernatant was carefully decanted and used for further analysis. The supernatant obtained after centrifugation and filtration was lyophilized and used when required.

2.2. Purification condition

Gel filtration column with 5ml capacity was purchased from Banglore Geni. Lyophilized sample was reconstituted in double distilled water and used for filtration through Gel filtration column. Reconstituted sample 2-5% of bed volume was loaded and eluted with double glass-distilled water. Double the bed volume number of fractions (1ml each) was collected the first 5ml were discarded. The presence of peptide was determined by taking the absorbance at 340nm. The peaks obtained in G-25 chromatogram were again lyophilized and dissolved in minimal amount of double glass distilled water and antibacterial activity was determined. Reverse Phase High Performance Liquid Chromatography Spherisorb C-18 5µm column (4.6 X 250mm) with 20µl loop (Waters, USA) was used for the separation of the peptides. Gradient solvent delivery was achieved using two Water's pumps at the flow rate of 0.75 ml/min. Solvent A was 0.1% trifluoroacetic acid (TFA) in HPLC (Milli-Q) grade water. Solvent B was 0.09% TFA, 90% acetonitrile. Both solvents were filtered using 0.45µm membrane filters and degassed before use. The C-18 column was thoroughly washed with solvent until the base line was obtained. Twenty µl of the sample were injected. Detection was monitored with (Water's dual detector) at 220 nm and 280 nm for all the fractions. The fractions of the respective peaks were pooled and lyophilized. These fractions were again tested for the anti-bacterial activity.

2.3. Antimicrobial activity using spot on lawn assay

Inhibition of pathogens was initially observed using spot on lawn assay. In this method pure culture of pathogen was used to make a lawn on agar plate it was allowed to dry for five to ten minutes then a drop of peptide solution collected from different fermentation conditions was kept over this lawn at equal distance. These plates were incubated for 24-48hr at 37^0C and plate was observed for inhibition of pathogens.

2.4. Antimicrobial activity using Agar well assay

Test bacterial culture was grown for 16 h at 37ºC. 100 µl of the culture was grown in nutrient broth medium so that all the cells should be in the log phase. About 50µl of a bacterial suspension containing test bacterium was transferred to soft agar plates already prepared 24h old nutrient agar plates and allowed for solidification for half an hour. The plates were punched out of the agar with the cork borer, 100µl of the supernatant was transferred to a plates previously spread with the test bacterial culture. The plates were incubated at 37ºC for 24 h. Appearance of clear zone around bacterial culture after incubation was considered as positive and absence of zone was considered as negative.

2.5. Antimicrobial activity using disc diffusion assay

In disc diffusion assay method, it was performed by uniformly coating an agar plate with the organism being tested and a paper disc was soaked in a suitable concentration of the supernatant solution. It was placed on the surface of the plate. Since the growth of the organism and the introduction of the supernatant start at the same

Table 1 Effect of change in time and temperature of culturing conditions for release of peptide and inhibition of bacterial pathogen by *Lactobacillus helveticus* NCDC-292 at 4% inoculation

Incubation temp.	Incubation period	pH	Curd cfu/g	Whey Peptide content mg/ml	Zone of Inhibition Gram +ve	Zone of Inhibition Gram -ve
37^0C	16h	5.6	$4x10^9$	1.07	Nil	Nil
	20h	5.4	$8x10^{11}$	1.15	Nil	Nil
	24h	4.9	$9x10^{12}$	1.11	2.5mm	Nil
	28h	4.0	$8x10^{10}$	1.10	4.2mm	2mm
40^0C	16h	5.7	$6x10^{10}$	1.40	10mm	4mm
	20h	5.1	$8x10^{13}$	1.14	15mm	17mm
	24h	4.7	$5x10^{15}$	1.09	13mm	07mm
	28h	3.8	$4x10^{14}$	1.00	11mm	6.5mm
44^0C	16h	6.4	$6x10^{14}$	1.27	9mm	Nil
	20h	5.2	$7x10^{12}$	1.01	10mm	9mm
	24h	4.8	$9x10^{15}$	1.06	14mm	12mm
	28h	4.4	$8x10^{13}$	1.05	12mm	11mm

Note-The values are averages of three replicates

Table 2 Effect of change in time and temperature of culturing conditions for release of peptide and inhibition of bacterial pathogen by combination of *Lactococcus* and *Lactobacilli* at 4% inoculation

Incubation temperature	Incubation period	pH	Curd cfu/g	Whey Peptide content mg/ml	Zone of Inhibition Gram +ve	Zone of Inhibition Gram -ve
37^0C	16h	5.5	$5x10^8$	1.04	Nil	Nil
	20h	4.3	$7x10^{10}$	1.07	Nil	Nil
	24h	3.7	$8x10^{11}$	1.25	5mm	2mm
	28h	3.4	$8\ x10^{12}$	1.40	6mm	3.5mm
40^0C	16h	5.1	$4x10^{10}$	1.09	07mm	10mm
	20h	4.8	$8x10^{11}$	1.27	12mm	07mm
	24h	3.5	$6x10^{12}$	1.80	14mm	11mm
	28h	3.2	$7x10^{14}$	1.75	15.6mm	12mm
44^0C	16h	4.9	$5x\ 10^{10}$	1.05	5.5mm	6mm
	20h	4.2	$3x10^{14}$	1.27	13mm	10mm
	24h	3.9	$8x10^{15}$	1.44	9mm	6mm
	28h	3.6	$30x10^{11}$	1.35	11mm	9mm

Note-The values are averages of three replicates

time, it creates a circular zone of inhibition around the disc where the supernatant stops the growth of the organism. The diameter of this zone depends on the concentration of the supernatant in the disc and its effect on the growth of the test microorganism.

3. Results

Recently, *in vitro* natural proteins upon degradation by digestive or microbial enzymes have been identified that possess the antimicrobial properties Based on data it is hypothesized that the feeding of these natural proteins results in the production of AMPs, which function as effecting antibiotics via the direct antimicrobial activity of the peptides. In present study we have used different combination of temperature and duration of incubation at fixed amount of culture for fermentation of bovine milk. After completion of fermentation process at desired temperature pH of the product was measured and it showed that as period of incubation increases pH value decreases. The no of colony forming unit was increased as the incubation period increases. Peptide content of the whey supernatant was also measured and we observed that at 37°C the peptide content was increased as incubation period increased while at higher temperatures peptide content get reduced this is because of production of more peptidases at higher temperature i.e. 40°C and 44°C. The antimicrobial activity of cell free extract was observed using different methods including spot on lawn assay, agar well assay and disc diffusion assay. Bacterial pathogens used for the study was *Staphylococcus aureus* and *Escherichia coli*. Inhibition of these pathogens was found less effective at lower incubation temperatures it did not result in antimicrobial activity as effectively as at high temperature. On the other hand cell free extract of whey supernatant at higher incubation temperature showed good antibacterial activity, thus indicating that it is having peptides with antibacterial activity. This indicates that temperature has a very influential effect in the release of peptides with anti-microbial activity by the specific enzymes. The result (Table 1) showed that the lowest peptide content was obtained when the milk was

fermented with the culture at 40°C for 28 h (1.00 mg/ml) resulting in the complete digestion of protein and showing antibacterial activity for both pathogen used. It may be due to the release of highly proteolytic enzymes by the culture at this temperature.

In overall picture, it can be said the peptide content obtained at different combination was obtained variable after incubation at 37ºC, 40ºC and 44ºC at 4% inoculation around. The supernatant obtained with 4% inoculums at 40ºC showed good degree of inhibition at the four different time intervals for both Gram's negative bacteria and Gram's positive bacteria. Hence by comparing Table 1 and table2 this study demonstrated that *L. helveticus* showed a good degree of inhibition zone (17mm) against gram negative bacteria and also for Gram's positive bacteria showing inhibition zone (15mm) for 20 hours of incubation and combination of *Lactococcus* and *Lactobacilli* at 4% inoculation showed a good degree of

inhibition zone (15.6mm) against gram positive bacteria when kept for 28 hr of incubation. Thus the acitimicobial acitivity was found higher with the *Lactobacillus heleveticus* in comparision to the combination of *Lactococcus* and *Lactobacilli*

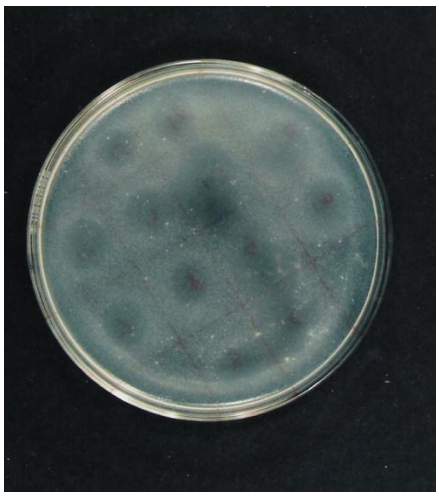

Figure: 1 Showing inhibition of *E. coli* by *Lactobacillus helveticus* NCDC-292 at 4% inoculation

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