

Full Length Research Paper

# Optimization of conditions for generation of antimicrobial peptides from milk proteins by *Lactobacillus* spp.

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The batch growth conditions for the generation of antimicrobial peptides from skim milk were optimized by using *Lactobacillus* spp. Proteolysis being important factor for peptide production, 4 *Lactobacillus* strains were screened for proteolytic activity, two strains each from *Lactobacillus acidophilus* and *Lactobacillus delbrueckii*. *L. acidophilus* NCDC 14 and *L. delbrueckii* NCDC 09 with higher activity were selected to produce antimicrobial peptides. Incubation period (12-72 h), inoculum level (1-3%) and incubation temperature (37 and 42°C) had an individual effect on antimicrobial activity of 10KDa Ultrafiltered skim milk hydrolysates checked against pathogenic *E. coli* ATCC 25922. Optimal conditions for generation of antimicrobial peptides include incubation period of 48 and 12-24 h, inoculums level of 2 and 1% and incubation temperature of 37°C for *L. acidophilus* NCDC 14 and *L. delbrueckii* NCDC 09 with proteolysis in range of 7.34-8.51 and 20-27.2 mMLeucine, respectively. Antimicrobial activity increased with proteolysis (>8.51 and >11 mMLeucine) but upto a certain extent after which it decreased for both strains. Maximum antimicrobial zone diameter obtained was 20 and 16.5 mm for *L. acidophilus* NCDC 14 and *L. delbrueckii* NCDC 09, respectively. The variables studied were very relevant due to their significance in improving the peptide production from both microorganisms and likely economic optimum conditions.

**Key words:** Antimicrobial peptides, fermentation, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii*, optimization, proteolytic activity.

## INTRODUCTION

The advanced development in the field of health and nutrition has directed focus towards 'food-derived bioactive peptides' that have regulatory functions in the human system beyond normal and adequate nutrition. Research in the field of bioactive peptides has intensified during the past two decades and it has been recognized

that dietary proteins especially milk proteins are a rich source of biologically active peptides. Dziuba and Darewicz (2007) defined such peptides as inactive sequence of amino acids that are encrypted within the sequence of parent protein requiring proteolysis for their release from precursor. These are released during

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enzymatic digestion *in vitro* or *in vivo* (Pihlanto et al., 2010). Beneficial health effects of these peptides are due to their antimicrobial, antioxidative, antithrombotic, antihypertensive and immunomodulatory activities (Muller et al., 2008). All these biological peptides has its own physiological role but antimicrobial peptide has gained wide importance as emerging pathogens causing illness poses a threat to human health. Antimicrobial peptides are important component of innate immunity. Till date more than 880 such peptides are well recognised in international database. They can rapidly kill a broad range of microbes and have additional activities which impact on the quality and effectiveness of innate responses and inflammation (Rydengerd et al., 2008). Milk-derived antimicrobial peptides have high potential as supplements in functional foods or as food-grade biopreservatives or in medicinal use (Benkerroum, 2010).

Fermentation of milk using the proteolytic systems of lactic acid bacteria is an attractive approach for generation of bioactive peptides with low cost (Philanto, 2010). Such bioactive peptides are expected to be fundamentally different from those released by digestive proteinases which differ from microbial proteinases in specificity and mode of action (Benkerroum, 2010). Lactic acid bacteria (LAB) possess cell envelope-associated proteinases (CEP) and intracellular peptidases, which release bioactive health-beneficial peptides during food fermentation. Proteinases of lactic acid bacteria may hydrolyse more than 40% of the peptide bonds of bovine  $\alpha_{S1}$  casein and  $\beta$ -caseins, producing oligopeptides of 4 to 40 amino acids residues (Kunji et al., 1996). The use of LAB for release of bioactive peptides encrypted in primary sequence of milk proteins is a promising strategy as it is already benefitted from "generally recognized as safe" status (Benkerroum, 2010). Among the *Lactobacillus* species, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus rhamnosus* and *Lactobacillus helveticus* are most extensively studied for release of bioactive peptides during fermentation.

Fermentation temperature significantly influences the bacterial growth, proteolytic activity and bioactivity. Otte et al., (2011) reported fermentation rate of *L. helveticus* MI1198 and *L. helveticus* CHCC 4080 increased with increasing temperature from 33°C to 40°C and the final pH decreased after 24 h suggesting that the optimum fermentation temperature for these strains may be higher than 40°C. Higher ace-inhibitory activity of *L. helveticus* strains at higher temperature point to the optimal growth temperature as the most efficient due to the largest number of bacterial cells and higher level of cell wall proteinase activity. The cell envelope proteinase, and aminopeptidase and X-prolyldipeptidyl aminopeptidase activity was maximum at 37- 40°C in another *L. helveticus* LB 10 strain (Pan and Guo, 2010). The good proteolytic activities with intra- and extracellular specific peptidases including X-prolyl-dipeptidyl aminopeptidase

was also reported in different *Lactobacillus* strains that is *L. acidophilus*, *L. casei*, *L. rhamnosus*, *L. reutri*, *L. delbrueckii* and *Bifidobacterium* spp. at 37°C incubation temperature (Kholif et al., 2011). So in present study two fermentation temperatures [37°C (below 40°C) and 42°C (above 40°C)] were selected to check temperature effect on proteolytic activity which further can affect antimicrobial activity.

To date, only a few studies (Hayes et al., 2006; Tadesse et al., 2006; Lopez-Exposito et al., 2007) have reported antimicrobial peptides from fermented dairy products. However, no information is available on generation of antimicrobial peptides from skim milk by extended fermentation and varying growth conditions with *Lactobacillus* spp. Proteolytic system of LAB provide transport systems specific for amino acids, di- and tripeptides and oligopeptides of up to 18 amino acids. Longer oligopeptides which are not transported into the cells can be a source for the liberation of bioactive peptides in fermented milk products when further degraded by intracellular peptidases after cell lysis. Furthermore growth conditions affect the activity of proteolytic system of bacteria. Therefore by extending the fermentation period and varying the growth conditions peptide content as well as their activity can be enhanced. Hence, the present investigation was proposed with the view of exploring the proteolytic capability of lactobacilli to optimize the generation of antimicrobial peptides from skim milk.

## MATERIALS AND METHODS

### Bacterial strains and growth conditions

Two strains of *L. acidophilus* NCDC 14, NCDC 15 and *L. delbrueckii* NCDC 08, 09 were procured from National Collection of Dairy Cultures (NCDC), National Dairy Research Institute (NDRI), Karnal and revived by propagating twice in de Man-Rogosa-Sharpe (MRS) broth at 37°C. One set of cultures was stored at -80°C in MRS broth containing 20% glycerol. Revived cultures were used to inoculate fresh MRS broth for experiment.

### Growth media

For optimum cell envelope proteinase activity three different test media MRS, Minimal defined media (MDM) and bovine Skim milk were screened. On the basis of nutritional requirements of respective strains (Morishita et al., 1981) the minimal defined media was prepared from concentrated individual stock solutions of constituents stored at 4°C after filtration, except for the cysteine solution, which was freshly prepared. Stock solutions were composed of 100-fold-concentrated solutions of each amino acid, base, and vitamins. All amino acids, vitamins, purines, pyrimidines, and inorganic salts were of analytical grade (Sigma Chemical Co., S.D Fine, Himedia., Fischer scientific). Media and stock solutions were sterilized by filtration through a Durapore PVDF membrane (0.22- $\mu$ m-pore size; Millipore Ireland Ltd.).

Bovine milk was collected from dairy farm, NDRI and fat fraction was removed in skim milk separator. 25 ml of skim milk was added in test tubes (50 ml volume) and sterilized in autoclave at 121°C

for 15 min and stored at 37°C for 24 h for sterility test.

Working culture was propagated in MRS broth at 37°C for 16 h. To eliminate carryover nutrients, the cells were harvested by centrifugation at 8,000 g for 15 min, washed twice in sterile 50 mM sodium phosphate (pH 7.0) and resuspended in buffer to the original volume. This cell suspension was used to inoculate the different media at an initial optical density OD<sub>580</sub> of 0.07 whereas in skim milk inoculation it was on percent basis and incubated for overnight. Bacterial growth was monitored by measuring the OD<sub>580</sub>. In the case of skim milk growth was monitored by diluting incubated SM media by a factor of 3.5 with 35 mM ethylene diaminetetraacetic acid adjusted to pH 12 (Kanasaki et al., 1975).

Cells grown in the MRS and MDM were harvested by centrifugation (10,000 g/10 min/4°C) at the exponential growth phase (OD<sub>580</sub> 0.67) and washed twice with 0.85% (w/v) saline supplemented with 10 mM CaCl<sub>2</sub>, and resuspended to final OD<sub>580</sub> of approximately 10 in 100 mM sodium phosphate (pH 7.0). To harvest cells from skim milk 5 ml of 20% trisodium citrate/100 ml of inoculated skim milk was added, pH was adjusted to 7.0 with 10M NaOH and centrifuged (6000 rpm, 15 min, 4°C). Cell pellets thus obtained was washed twice with 50 mM Tris –HCl containing 1% trisodium citrate (pH 7.0) and further processed as described above.

### Enzyme assay

The proteinase activity of whole-cell suspensions resuspended in 100 mM sodium phosphate buffer (pH 7.0) at 37°C was measured with the chromogenic substrate succinyl-alanyl-alanyl-prolyl-phenylalanine-p-nitroaniline (Sigma) as described by Hebert et al. (2008) with slight modifications. The assay mixture, containing 200 µl of 50 mM sodium phosphate buffer (pH 7.0), 100 µl of 5 M NaCl (final concentration, 1.5 M), 20 µl of 20 mM substrate, and 60 µl of the cell suspension, was incubated at 37°C for 10 min. The reaction was stopped by heating at 90°C, and mixture was centrifuged at 10,000 g for 5 min. The released nitroaniline was measured at 410 nm by using a microplate reader (Infinite F200 PRO). One unit of proteinase was defined as the amount required to liberate 1 nmol of nitroaniline per minute. Specific activity was expressed as units of proteinase per mg protein. Milk itself contains principal protease plasmin with other including cathepsins and elastase which can hydrolyse milk proteins (Kelly et al., 2006). So to get the net proteolysis carried out by harvested bacterial cells from milk, MRS and MDM endogenous activity of unfermented control milk as well as of MRS and MDM control was deducted.

### Effect of process variables on proteolysis and antimicrobial activity by selected *Lactobacillus* spp.

Inoculated Skim milk media was used further to inoculate skim milk. Different process variables incubation time, inoculation level and incubation temperature were studied to investigate their effect on antimicrobial activity of hydrolysates.

A volume of 25 ml of sterilised bovine skim milk was inoculated on 2% basis with 12 h inoculated skim milk (exponential phase culture) and incubated at incubation temperature of 37°C. Samples were removed periodically at an interval of 12 h up to 72 h. Similarly different inocula sizes of 1, 2 and 3% (v/v) were studied only at 37°C up to 24 h. To check the effect of temperature, skim milk inoculated at 2% level was incubated at two different temperatures (37 and 42°C) for 24 h. Samples were stored at -20°C till further study.

### Proteolytic activity

Proteolytic activity of fermented milk samples was measured using

Ortho-phthaldehyde (OPA) method (Church et al., 1983). To 2.5 mL fermented milk, 0.5 mL distilled water and 0.5 mL of 0.72 mol.L<sup>-1</sup> trichloroacetic acid (TCA) were added and solution was filtered after 10 min. To 3 mL OPA reagent, 150 µL of sample (TCA filtrate) was added and incubated for 2 min at room temperature; the absorbance was measured at 340 nm using dual beam spectrophotometer (Specord 200, Analytik Jena AG, Germany). A calibration curve of leucine (0.25 - 2.5 mmol.L<sup>-1</sup>) was prepared and the results were expressed as mmol L<sup>-1</sup> leucine. One control sample without inoculation was also included in each experiment. For net proteolytic activity of inoculated sample, control sample's activity was deducted. *L. helveticus* NCDC 288 was taken as positive control as many previous studies revealed higher proteolytic activity of *L. helveticus* (Gandhi and Shah, 2014; Ramesh, 2011).

### Antimicrobial activity

pH of the fermented milk sample was adjusted to 3.8 with 50% lactic acid and centrifuged at 10000 g /15 min at 4°C. pH of supernatant obtained was adjusted to 7 by 10 M NaOH, centrifuged and ultrafiltered through 10 KDa membrane (vivaspin). Hydrolysate thus obtained was used to check antimicrobial activity.

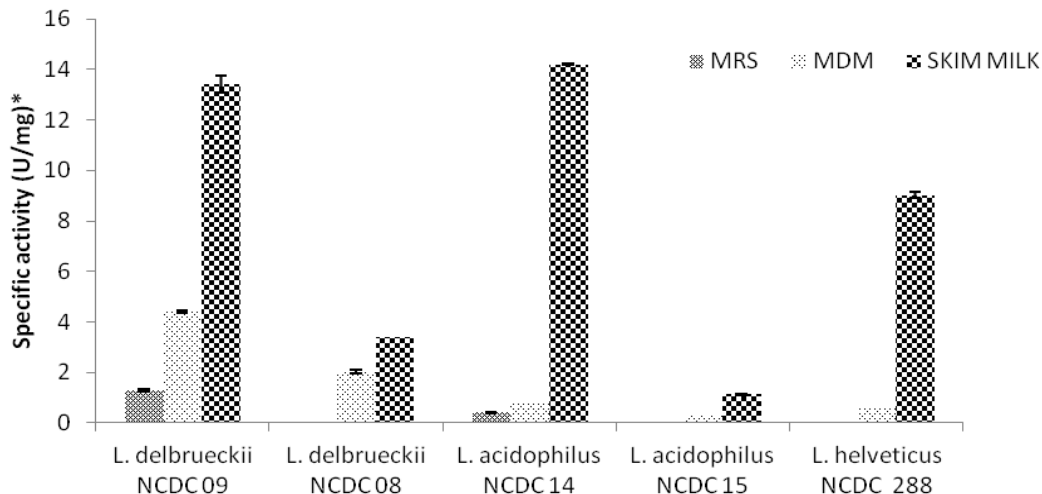
A well diffusion assay was performed using the indicator strain *E. coli* ATCC 25922 to screen antimicrobial activity of hydrolysates. The sensitivity of a strain was scored according to the diameter of the zone. The assay was carried out in the nutrient agar media (2% w/v) and 5 ml of soft agar media containing 10<sup>4</sup> CFU of an overnight culture of the indicator strain. Wells, 17 mm in diameter, were cut into solidified agar plates and 10 µl of soft agar was added to seal wells. Then 100 µl of the sterile 0.22 µm filtered samples were placed into each well. Plates were stored at 4°C for 1 h to permit radial diffusion of hydrolysates, incubated at 37°C for overnight, and subsequently examined for zones of inhibition. One control sample without inoculation was also included in each experiment. The net antimicrobial activity was obtained by subtracting inhibition zone of control milk from inoculated sample.

The effect of different fermentation conditions on bioactive peptide production from skim milk fermented by *L. acidophilus* NCDC 14 and *L. delbreuckii* NCDC 09 was studied and results are given below. The results were statistically analyzed by two way ANOVA. All three process parameters had a significant effect on bioactivity and peptide production. The study undertaken for the production of antimicrobial peptides by exploiting the proteolytic activity of Lactobacilli represents a novel and promising strategy and to best of our knowledge no study have been undertaken before.

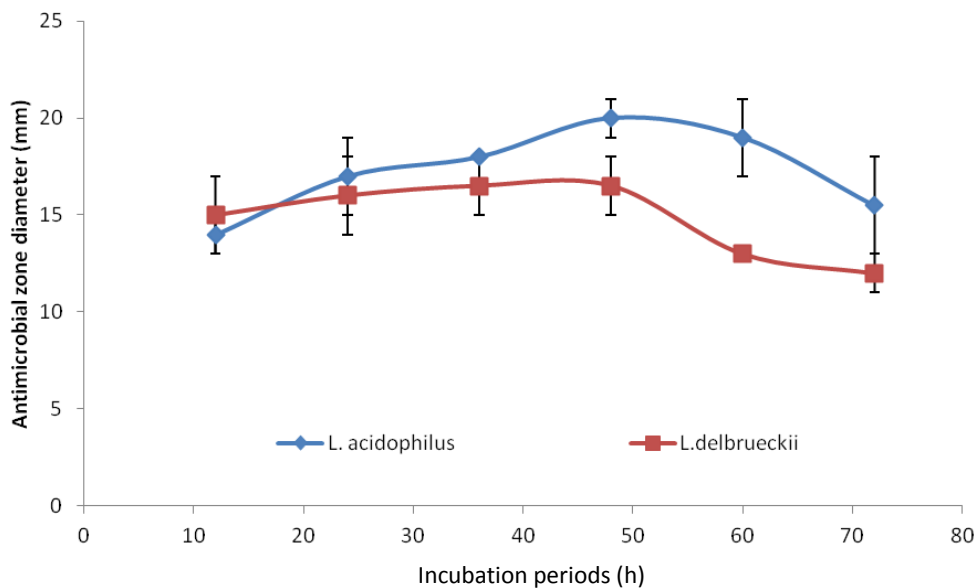
## RESULTS

### Influence of growth media on CEP activity

The proteinase activity of *Lactobacillus* grown in MRS, MDM and Skim milk were compared. Among three media higher activity was observed in case of skim milk for *L. acidophilus* NCDC 14 (14.2 U/mg protein) followed by *L. delbreuckii* NCDC 09 (13.42 U/mg protein) being twenty fold higher than synthetic media MDM. However, the extent of increase in proteolytic activity in skim milk was different for each strain. In case of MRS broth proteinase activity was almost negligible as compared to MDM (Figure 1). On the higher proteinase activity basis, skim milk was selected as media to grow cells for antimicrobial peptide production from skim milk. *L. acidophilus* NCDC



**Figure 1.** Effect of growth media on cell envelope proteinase specific activity of *L. acidophilus* NCDC 14 and *L. delbrueckii* NCDC 09. \*One unit of enzyme (UE) was defined as the amount required to liberate 1  $\mu\text{mol}$  of nitroaniline per minute. Specific activity was expressed as units of proteinase per mg protein. values are the mean  $\pm$  standard deviation (error bars) of three independent experiments.



**Figure 2.** Antimicrobial activity (zone diameter) of skim milk hydrolysates obtained by fermentation at different incubation periods with *L. acidophilus* NCDC 14 and *L. delbrueckii* NCDC 09 against *E. Coli* ATCC 25922. Error bars show standard deviation.

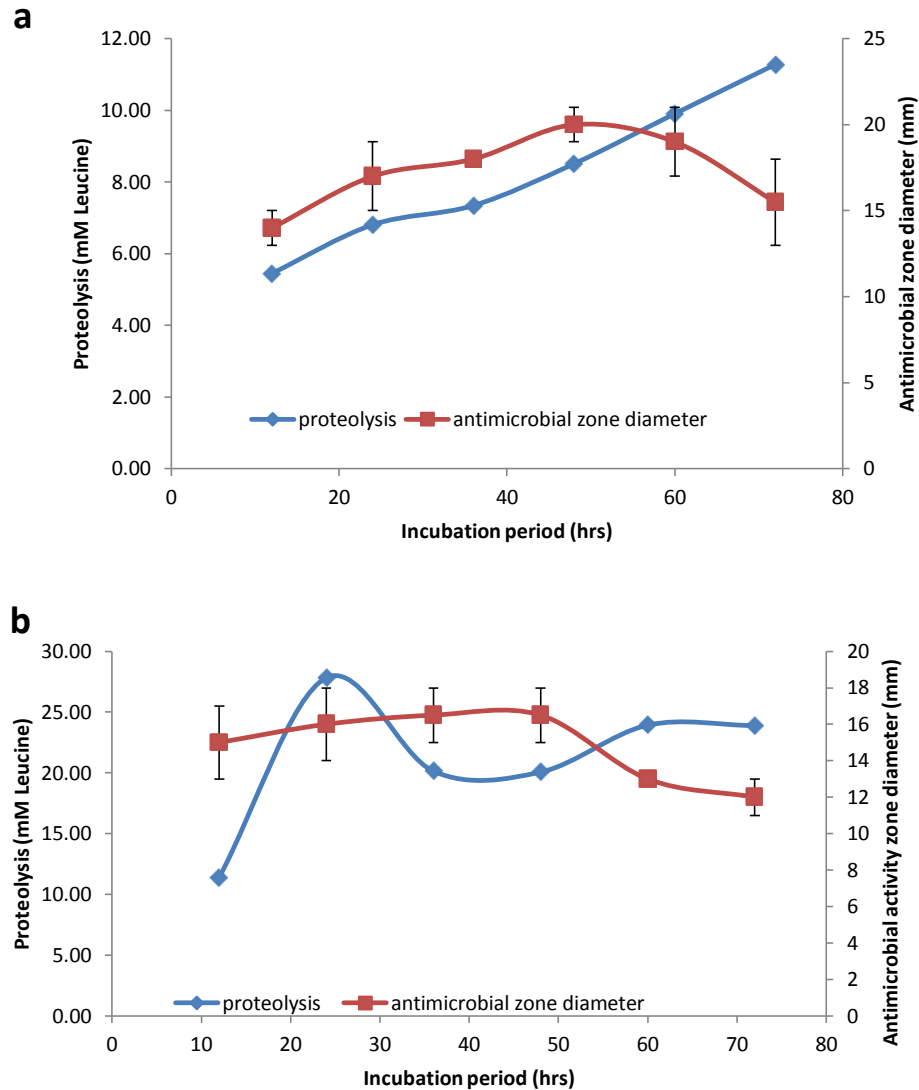
14 and *L. delbrueckii* NCDC 09 showed higher proteinase activity in skim milk as compared to positive control *L. helveticus* NCDC 288 (9.02 U/mg protein).

#### Effect of incubation period on antimicrobial and proteolytic activity

For production of antimicrobial peptides three important

fermentation variables i.e., incubation period, inoculation level and incubation temperature were optimized by three independent experiments by keeping other two variables constant.

In the current study, Figure 2, depicts the antimicrobial activity of skim milk fermented by *L. acidophilus* NCDC 14 and *L. delbrueckii* NCDC 09 at various time intervals (0, 12, 24, 36, 48, 60 or 72 h) during incubation at 37°C. The measured net inhibitory activities varied from zone

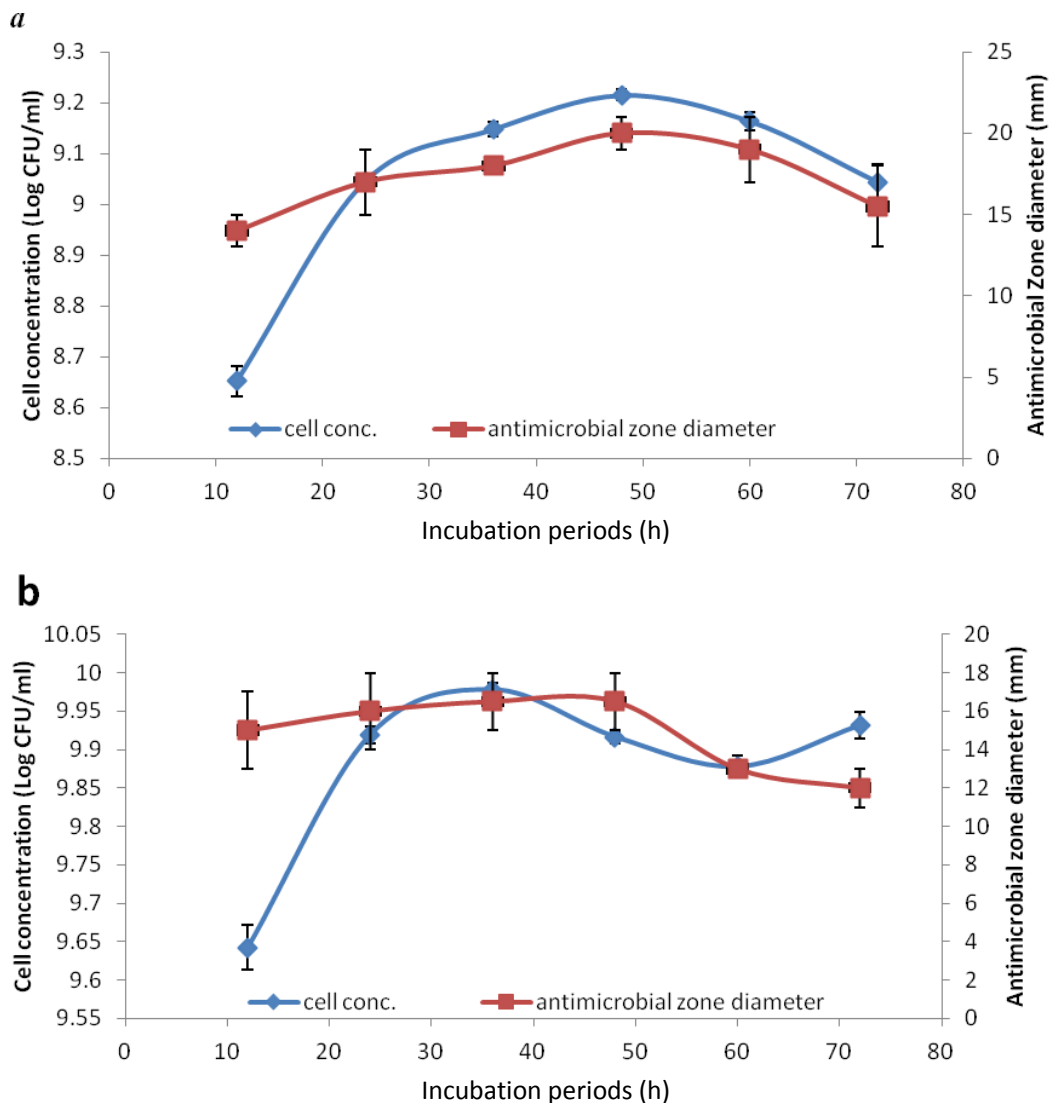


**Figure 3.** Effect of proteolysis on antimicrobial activity at different incubation periods of fermentation by *L. acidophilus* NCDC 14 (a) and *L. delbrueckii* NCDC 09 (b). Error bars show standard deviation.

diameter of 13 to 20 mm after deducting endogenous antibacterial activity of control milk during the fermentation time. There was no significant difference between the strains and reached a similar or relatively higher level of antimicrobial activity. The fluctuations were observed in antimicrobial activity at different incubation periods. In *L. acidophilus* NCDC 14 fermented milk antimicrobial zone diameter (14 mm) at 12 h of fermentation period increased to 20 mm at 48 h after which it decreased significantly ( $P < 0.05$ ) at the end of fermentation that is 60-72 h. Although, for *L. delbrueckii* NCDC 09 no significant difference was observed up to 48 h after which it decreased significantly.

The antimicrobial activity pattern observed for both strain could be associated with the proteolytic activity of strains. As depicted in Figure 3a and b the amount of

liberated amino groups and peptides increased significantly ( $P < 0.05$ ) during fermentation from 12 to 72h for *L. acidophilus* while for *L. delbrueckii* upto only 24 h with higher degree of proteolysis within 12 h. Further in 24 h proteolysis increased at a higher rate and decreased thereafter up to 48 h with small increase again at the end of fermentation period. However for *L. acidophilus* NCDC 14 continuous and steady increase in proteolysis was observed. Peptide content for *L. acidophilus* NCDC 14 fermented skim milk increased significantly up to 36 h while insignificant differences were observed up to 60 h and increased again significantly at the end of fermentation period which is directly related to proteolysis. With increasing proteolysis peptide content also increased and was higher in *L. delbrueckii* NCDC 09.



**Figure 4.** Effect of cell concentration on antimicrobial activity at different incubation periods of fermentation by *L. acidophilus* NCDC 14 (a) and *L. delbrueckii* NCDC 09 (b). Error bars show standard deviation.

#### Effect of incubation period on growth performance

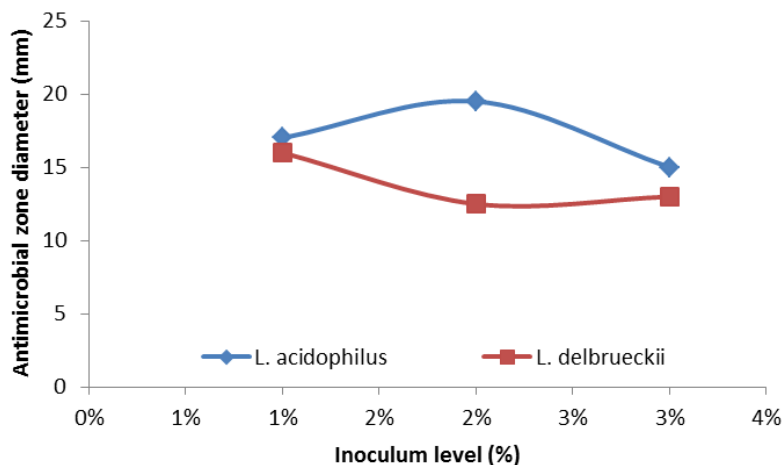
The proteolysis further may be dependent on growth performance of the strains which may also affect the antimicrobial peptide production. Further positive correlation coefficient ( $r^2= 0.94, 0.77$ ) among proteolysis and cell concentration for both strains also suggest this. Changes in growth and pH of all organisms at 12 h interval during growth in sterile skim milk at 37°C are shown in Figure 4a and b. The variation in initial count of different organisms is due to variation in growing ability of these organisms. There were greater differences in initial counts of *L. delbrueckii* NCDC 09 (7.73 log CFU) as compared to *L. acidophilus* NCDC 14 (6.4 log CFU). Both organisms showed increase in log counts (9.12-9.92) up to 36-48 h with corresponding decrease in pH (3.67). For

*L. delbrueckii* NCDC 09 the drop of pH was much faster reaching 3.9 within 12 h of incubation with corresponding growth rate reaching to 10 log CFU/ml.

Cell concentration has great effect on proteolysis as well as antimicrobial activity. However at higher cell concentration antimicrobial activity may not be higher as in case of *L. delbrueckii* NCDC 09, as with higher proteolysis antimicrobial zone diameter decreased. At lower proteolysis, antimicrobial activity was higher with insignificant difference at higher proteolysis.

#### Effect of inoculum size on antimicrobial and proteolytic activity

In this study, inoculum size resulted in a significant effect



**Figure 5.** Antimicrobial activity (zone diameter) of skim milk hydrolysates obtained by fermentation at different inoculums levels with *L. acidophilus* NCDC 14 and *L. delbrueckii* NCDC 09 against *E. Coli* ATCC 25922.

on antimicrobial peptide production (Figure 5). At the given inoculum size range (1% to 3%, v/v), antimicrobial activity for *L. acidophilus* NCDC 14 increased with increasing size of the inoculum, from 1 (17 mm) to 2% (19.5 mm) and then decreased for 3% inocula size (15 mm) (Figure 6a). Instead, *L. delbrueckii* NCDC 09 (Figure 6b) showed higher zone diameter (16 mm) at 1% (v/v) and activity decreased further at 2-3% inoculum levels (12.5-13 mm). Based on these results, the optimal inoculum size for antimicrobial peptide production was determined to be 2 and 1% (v/v) for *L. acidophilus* NCDC 14 and *L. delbrueckii* NCDC 09, respectively of exponential phase culture, which gave the highest antimicrobial zone diameter (20 and 16 mm) against pathogenic *E. coli* ATCC 25922.

The inoculum size resulted in significant effect on proteolysis which varied among both strains also. The amount of liberated amino groups and peptides increased significantly ( $p < 0.05$ ) during fermentation at 1-3% inoculums levels for both strains. Higher degree of proteolysis and peptide content was observed at 3% level for *L. delbrueckii* NCDC 09 (23.9 mM Leucine and 6.23 mg/ml) followed by *L. acidophilus* NCDC 14 (4.21 mM Leucine and 2.43 mg/ml) with significant differences among 2 and 3% inoculums levels. With increasing proteolysis peptide content also increased and was higher in *L. delbrueckii* NCDC 09 (6.23 mg/ml) followed by *L. acidophilus* NCDC 14 (4.21 mg/ml).

#### Effect of Inoculum Size on growth performance

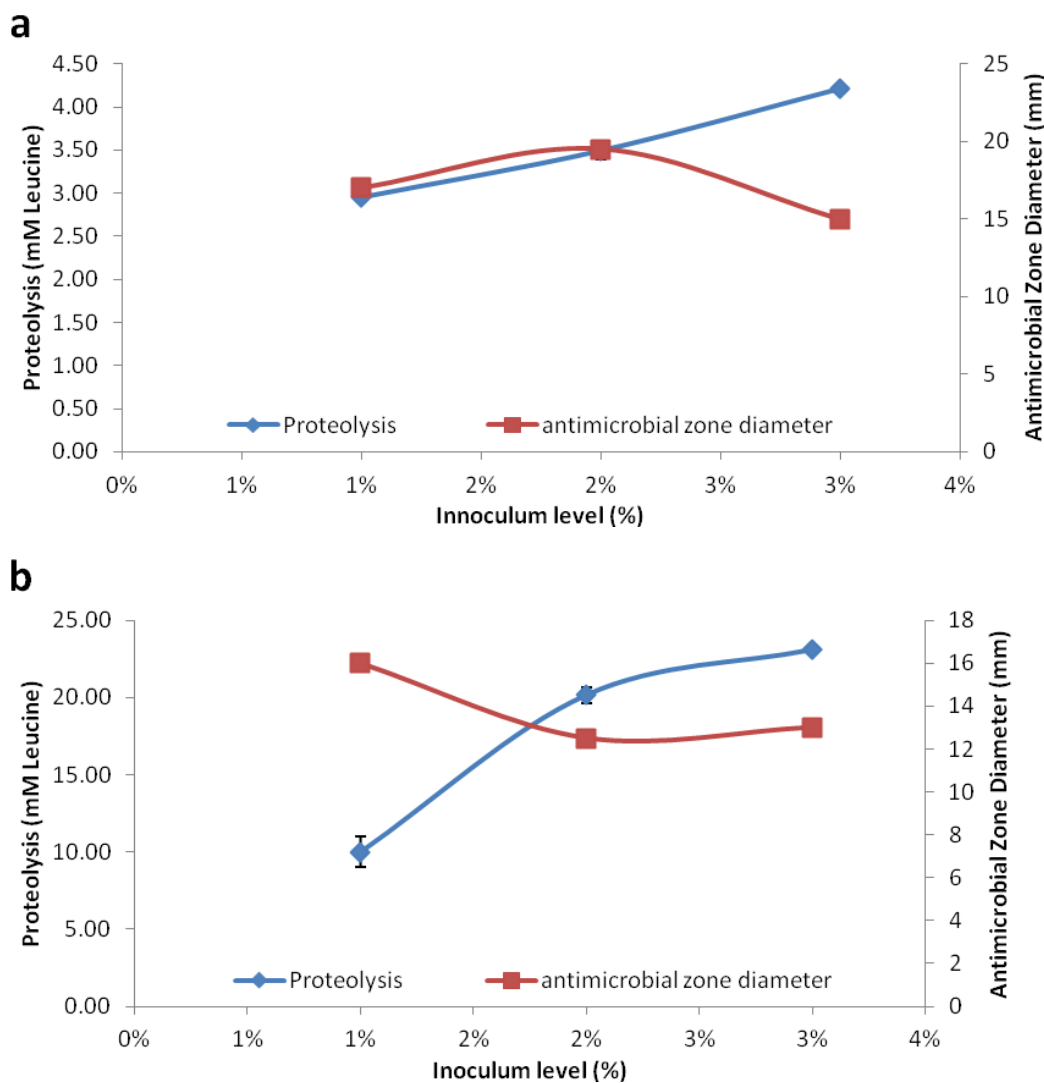
Both lactobacilli used at inoculation levels 1-3% successfully achieved the desired level and reached 9.07 and 9.12 log cfu/ml, respectively at the end of 24 h incubation period. *L. delbrueckii* NCDC 09 reached

highest growth level with significant differences ( $p < 0.05$ ) among inoculation levels 1%-3% (9.73, 9.96 and 10 log cfu/ml respectively) at the end of incubation period. *L. acidophilus* NCDC 14 also showed significant differences ( $p < 0.05$ ) in cell concentration and decrease in pH at all inoculation levels. Highest pH decrease was observed for *L. delbrueckii* NCDC 09 (3.67) followed by *L. acidophilus* NCDC 14 (4.43). Comparison of pH values of fermented milks revealed that at 1 and 3% inoculation levels pH values were significantly different from each other being lowest at 3% for *L. delbrueckii* (data not shown). As shown in Figure 7a and b, higher cell concentration was observed at higher inoculums level for both *Lactobacillus* strains. Both strains at 3% (v/v) observed highest pH decrease and proteolytic activity but decreased antimicrobial activity.

#### Effect of Incubation temperature on antimicrobial and proteolytic activity

Incubation temperature was observed to have a significant effect on antimicrobial activity. Generally for both *Lactobacillus* strains evaluated, antimicrobial activity of skim milk hydrolysates (10 KDa) obtained by fermentation with *L. acidophilus* NCDC 14 at incubation temperatures of 37 and 42°C was found to be significantly different and was higher at 37°C as shown in Figure 8. Although *L. delbrueckii* NCDC 09 was highly proteolytic but the peptides released by *L. acidophilus* NCDC 14 fermentation had higher antimicrobial activity.

In this study hydrolysis parameters affecting antimicrobial activity that is, proteolytic activity decreased significantly with increased incubation temperature (37 and 42°C) studied (Figure 9). The incubation temperature resulted in significant effect on proteolysis which varied



**Figure 6.** Effect of proteolysis on antimicrobial activity at different inoculum levels of *L. acidophilus* NCDC 14 (6a) and *L. delbrueckii* NCDC 09 (6b). Error bars show standard deviation.

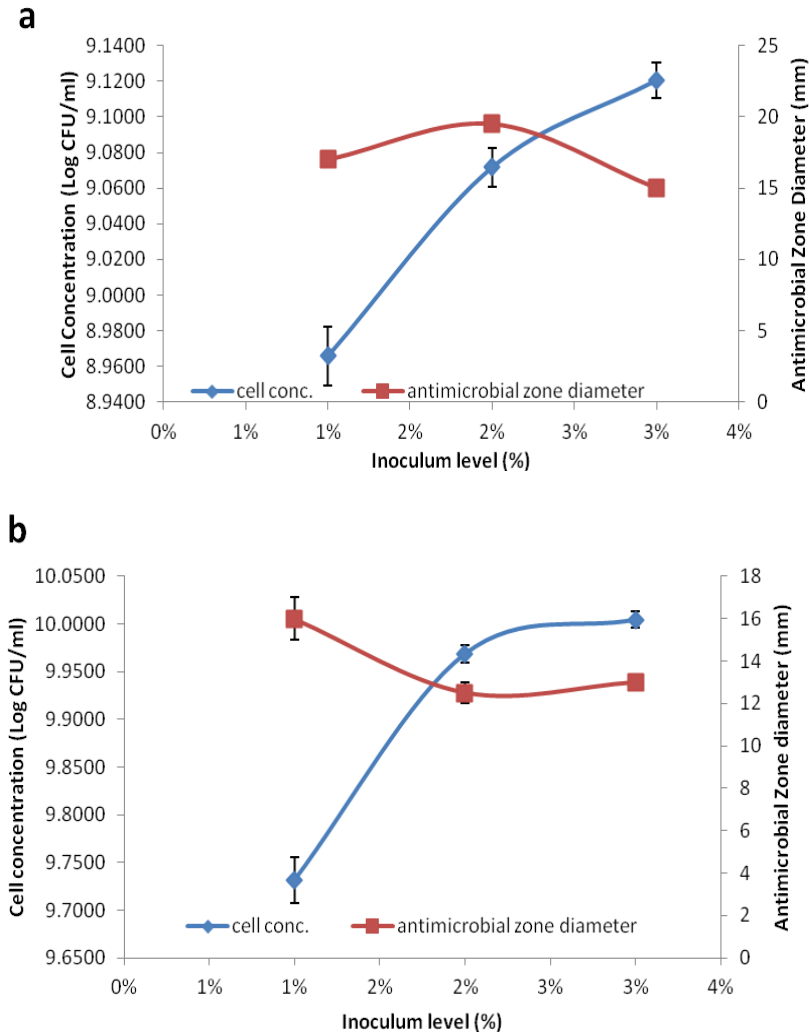
among both strains also. The amount of liberated amino groups and peptides vary significantly ( $P < 0.05$ ) during fermentation at two different incubation temperatures. The peptide content was higher than 1 mg/ml at both temperatures but higher degree of proteolysis and peptide content was observed at 37°C for both strains. Like other fermentation variables at two different incubation temperatures, higher proteolysis and peptide content was observed for *L. delbrueckii* NCDC 09 (27 mM Leucine and 5.2 mg/ml) followed by *L. acidophilus* NCDC 14 (6.7 mM Leucine and 2.03 mg/ml) at 37°C followed by 42°C with significant differences ( $p < 0.05$ ). Optimum peptide production for both strains was obtained at 37°C. Significant differences were observed in cell concentration and pH decreased at both temperatures. Cell concentration was higher at 37°C and decreased at 42°C for both strains.

## DISCUSSION

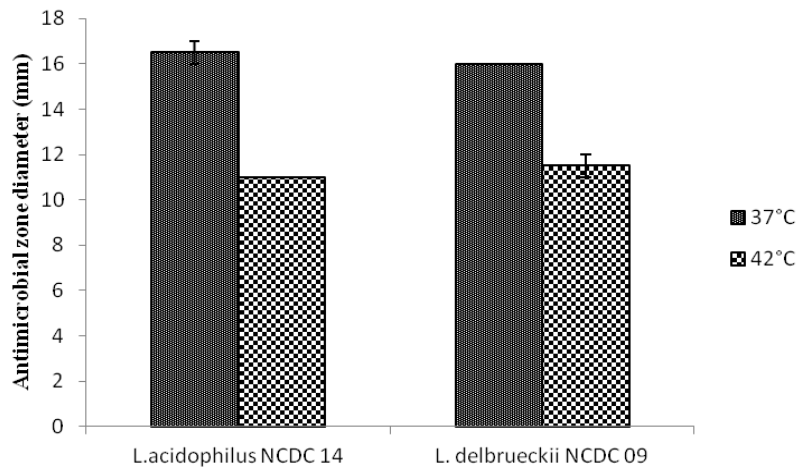
Results showed that proteinase activity of *Lactobacillus* strains is enhanced in skim milk which is in accordance to previous findings. Hebert et al. (1997) compared proteinase activity of *L. helveticus* CRL 581 cells grown in reconstituted skim milk, MRS and casein-yeast extract glucose (CYG) and reported nine times increase in enzyme activity in milk and about two times in CYG as compared to MRS. Decrease in proteolytic activity might be due to the presence of an available nitrogen source leading to decrease in proteinase activity. As in Lactococci, the production of CEP of *Lactobacillus* was influenced by composition of culture medium; which is in line with the present study (Hebert et al., 2000).

In a number of studies proteinases of *Lactobacillus* species responsible for proteolytic digestion were

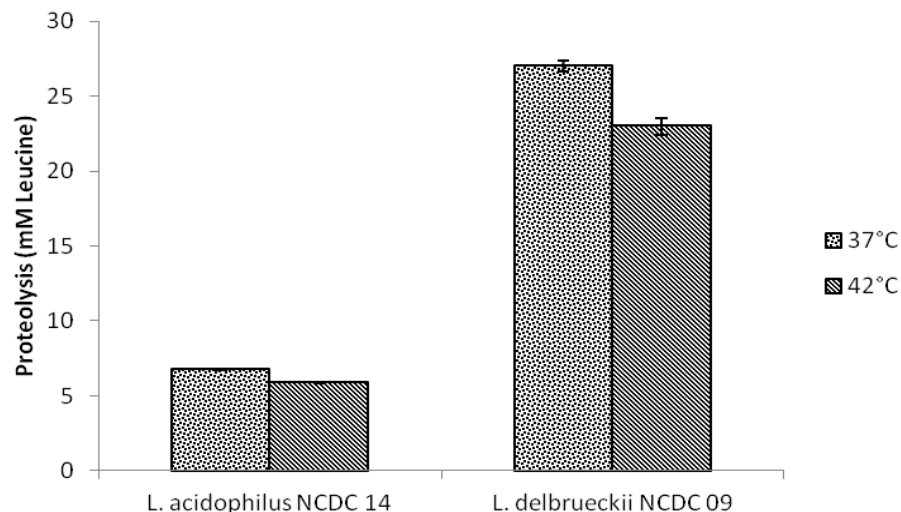




**Figure 7.** Effect of cell concentration on antimicrobial activity at different inoculum level of *L. acidophilus* NCDC 14 (7a) and *L. delbrueckii* NCDC 09 (7b). Error bars show standard deviation.



**Figure 8.** Antimicrobial activity of skim milk hydrolysates fermented by *Lactobacillus* strains at 37 and 42°C. values are the mean±standard deviation (error bars) of three independent experiments.



**Figure 9.** Proteolytic activity of *Lactobacillus* strains at 37 and 42°C. Values are the mean±standard deviation (error bars) of three independent experiments.

studied. In general the degradation of casein is initiated by an extracellular proteinase prtP. Five different types of these enzymes were cloned and characterized from LAB, including PrtP from *L. lactis* and *L. paracasei*, PrtH from *L. helveticus*, PrtR from *L. rhamnosus*, PrtS from *S. thermophilus*, and PrtB from *L. bulgaricus*. LAB typically possesses only one CEP but the presence of two CEPs was reported in *L. bulgaricus*. After the casein-derived peptides are taken up by the LAB cells, they are degraded by a concerted action of peptidases with differing and partly overlapping specificities. The intracellular endopeptidases, general aminopeptidases (PepN and PepC), and the X-prolyl dipeptidyl aminopeptidase (PepX) are the first enzymes to act on oligopeptides. Other peptidases capable of acting on oligopeptides are the broad-specificity metallopeptidase PepN and cysteine peptidase PepC proteins that were characterized from diverse LAB strains. An enzyme possessing specificity toward di/tripeptides with N-terminal leucine residues and dipeptides containing proline was biochemically characterized from *L. bulgaricus* (Savijoki et al., 2006).

The above stated proteinase and peptidases may produce a number of bioactive peptides among which some may be multifunctional peptides. To date, a repertoire of more than 880 antimicrobial peptides derived from different sources exists in international database (Rydengard et al., 2008). Antibacterial peptides have been derived from milk proteins, lactoferrin,  $\alpha_{S1}$ ,  $\alpha_{S2}$ , k-casein,  $\alpha$  lactalbumin,  $\beta$ -lactoglobulin, and lysozyme. Caseicins A, B and C, isracidin  $\alpha_{S1}$ f(1-23), Casocidin-I  $\alpha_{S2}$ -CN(150-188), CMP k-casein-A, f(138-158), k-casecidin k-CN f(17-21), Lactoferricin-H lf(1-47), lactoferrampin lf(268-284) are different known antimicrobial peptides. Along with these peptides another novel

peptides may also appear in the present study depending on specificities of proteinases and peptidases of both strains.

The ability of *Lactobacillus* strains to generate bioactive peptides during milk fermentation was observed to be a strain specific characteristic (Pihlanto et al., 2010) which might be connected to many factors such as bacterial growth, organic acid production and proteolytic activity of these strains. Furthermore, the time-dependent release of various peptides observed in our study might have important consequences on the extent of *in vitro* antimicrobial activity in fermented milk, which deserves further elaboration. The antimicrobial activity pattern observed for both strain could be associated with the proteolytic activity of strains and its ability to produce antimicrobial peptides stronger than others (Fuglsang et al., 2003). Our results are in accordance with Anas et al. (2008) who evaluated antimicrobial activity of 8 *Lactobacillus* species of three genera *L. plantarum*, *L. casei* and *L. rhamnosus* which increased with the incubation period and reduced the growth of *Staphylococcus aureus* by 1.6 log phase within 24 h and no bacteria was found after 72 h.

During fermentation, milk proteins were hydrolysed by LAB proteinases and peptidases resulting in an enhanced amount of free amino groups and peptides. The extent of proteolysis varied among both strains and appeared to be time dependant. Leclerc et al. (2002) demonstrated a linear increase in extent of proteolysis with fermentation time for *L. helveticus* as observed in present study. In a different study protease activity increased with incubation period up to 36 h in MRS and decreased thereafter being highest for *L. rhamnosus* followed by *L. delbrueckii*, *L. helveticus* and *L. plantarum* (Kholif et al., 2011). Haq and Muktar (2009) reported

maximum proteolytic activity of 15 *Lactobacillus* strains after 48 h of cultivation which corresponded to logarithmic growth phase of the bacterium and thereafter steady decrease in amount of protease.

The proteolysis further may be dependent on growth performance of the strains which may also affect the antimicrobial peptide production. For *L. delbrueckii* NCDC 09 the drop of pH was much faster which is in concordance to the Heller (2008) that *L. delbrueckii* ssp. *bulgaricus* leads to higher decrease in pH as compared to *L. acidophilus*, *L. brevis* and *L. casei* when used as starter organisms for dairy products. Kholif et al. (2011) reported increase in log count up to 48 h with simultaneous decrease in pH by strains that *L. casei*, *L. acidophilus* and *L. delbrueckii*. Cell concentration has great effect on proteolysis as well as antimicrobial activity as depicted in our study and also reported by many previous studies (Ramesh, 2011; Franca et al., 2009).

For high bio-product synthesis inoculum size must be controlled to ensure optimum nutrient uptake in a culture medium. The inoculum size resulted in significant effect on antimicrobial activity which varied among strains also as higher inoculum level reduced the antimicrobial activity. Tadesse et al. (2006) reported higher growth of LAB in MRS broth (a complete nutrient media) led to higher acid production and metabolites with antimicrobial property while LAPTg broth with inferior nutrition caused lower cell concentration with higher antimicrobial activity. Thus higher number of bacteria produced may have adverse effect on bioactivity. In contrast Misra and Kuila (1992) reported same antibacterial activity at 5, 10 and 15% inoculum levels against four organisms that is *E. coli*, *Shigella dysenteriae*, *Staphylococcus aureus* and *Bacillus cerus*.

The inoculum size resulted in significant effect on proteolytic activity which varied among strains also and with increasing inoculum level (1-3%) proteolytic activity increased. Small inoculum size as 1% level for *L. acidophilus* NCDC 14 means insufficient number of bacteria secreted reduced amount of protease leading to lower hydrolysis. An increase in inoculum density led to higher biomass productivity with higher hydrolysis but lower bioactivity (Danquah and Forde, 2007) which agrees with present study also. However in another study Agyei et al. (2012) reported at higher cell densities (>5%) growth rate of *L. delbrueckii* sub sp. *Lactis* ATCC 7830 decreased with lower CEP production due to increasing limitation of key nutrients. An increase in inoculum density usually leads to higher biomass productivity but lower average specific growth rate (Koutsoumanis and Sofos, 2005).

Temperature is an important environmental factor affecting all the physiological activities in a living cell and controls the growth, microbial activities, and normal functioning of the cellular enzymes. Antimicrobial activity of *Lactobacillus* strains was higher at 37°C than 42°C might be due to higher growth rate. In a similar study for

development of Bifidus milk, Misra and Kuila (1992) studied effect of incubation temperature on antibacterial activity. At 32°C and 40°C no zone of inhibition was observed against *Shigella dysenteriae*, *E. coli* and *Staphylococcus aureus* while at 37°C good inhibition zone was obtained. The optimum temperature for growth of *B. bifidum* was 37°C and maximum production of antimicrobial substance was expected at this temperature as in the present study also optimum peptide production for both strains was obtained at 37°C (data not shown).

With decreasing proteolysis at 42°C (Figure 9) antimicrobial activity has also decreased as suggested by positive correlation coefficient ( $r^2=1$ ). The sharp decrease in proteolytic and antimicrobial activity at 42°C may be due to thermal inactivation of biosystems at temperature higher than the optimum (Agyei et al., 2012). Increasing limitation of key nutrients and accumulation of growth inhibitory metabolites or maximum cell lysis when fermentation leads to maximum growth rate could be another cause of decrease in cell concentration at 42°C reported by Otte et al. (2011) for *L. helveticus*. Although *L. delbrueckii* NCDC 09 was highly proteolytic but the peptides released by *L. acidophilus* NCDC 14 fermentation had higher antimicrobial activity. In a similar study higher proteolytic bacteria *L. casei* did not show higher ace-inhibitory activity which means higher proteolysis may not lead to higher bioactivity (Donkor et al., 2007). Furthermore bioactivity is also strain dependent as Fugalsang et al. (2003) reported varying amounts of ace-inhibitors produced by most LAB during milk fermentation varied with strains.

### Corelation between proteolysis and antimicrobial activity

As shown in Figure 3a and b proteolysis was highest at 48 h for *L. acidophilus* NCDC 14 and at 24 h for *L. delbrueckii* NCDC 09. At different incubation periods positive correlation ( $r^2=0.99, 0.64$ ) was observed between proteolysis and antimicrobial activity for both strains. With increasing proteolysis antimicrobial activity increased but after optimum incubation period antimicrobial activity decreased with increasing proteolysis. As shown in Figure 6a and b increasing inoculum level increased the proteolysis but not antimicrobial activity. Higher proteolysis lysed antimicrobial peptides leading to loss of activity and other new peptides to appear (Donkor et al., 2007). For *L. delbrueckii* NCDC 09 higher antimicrobial zone diameter observed at lower inoculum level (1%) can explain for negative correlation ( $r^2= -0.94$ ) among proteolysis and antimicrobial activity. Similarly for *L. acidophilus* NCDC 14 antimicrobial activity increased only up to 2% level in contrast to 3% for proteolysis. The results are in agreement with the study of Pan and Guo (2010) that is ace-inhibitory activity released during the first hydrolysis reaction time and further digestion did not

**Table 1.** Optimum conditions of fermentation for generation of antimicrobial peptides by *L. acidophilus* NCDC 14 & *L. delbrueckii* NCDC 09.

Strain	Parameter	Antimicrobial activity zone dia. (mm)	Peptide content (mg/ml)
<i>L. acidophilus</i> NCDC 14	48 h	20 ± 1	2.25±0.003
	2%	19.5 ± 0.5	1.79±0.10
	37°C	16.5 ± 0.5	2.03±0.1
<i>L. delbrueckii</i> NCDC 09	24-36 h	15±2, 16.5 ± 1.5	4.2±0.06-5.19±0.04
	1%	16 ± 1	3.50±0.28
	37°C	16 ± 0	5.20±0.15

increased activity. Thus proteolytic activity that affects the release of various peptides observed in this study might have important consequences on the extent of antimicrobial activity in fermented milk.

### Optimized conditions for generation of antimicrobial peptides by selected *Lactobacillus*

Fermented milks showed good antimicrobial activity with significant differences among them. Incubation period, inoculation volume and incubation temperature showed significant effect on antimicrobial activity. Both strains showed higher antimicrobial activity at different incubation periods that is *L. acidophilus* in 48 h, *L. delbrueckii* NCDC 09 in 12-24 h which could be correlated to proteolysis and peptide content as activity increased and then decreased at a particular incubation periods. With proteolysis in range of 7.34-8.5 mM Leucine and 20-27.1 mM Leucine for *L. acidophilus* NCDC 14 and *L. delbrueckii* NCDC 09 respectively antimicrobial activity increased (17 mm< and 15 mm<) and above this stated range activity decreased. *L. acidophilus* and *L. delbrueckii* NCDC 09 showed higher activity at different inoculum level that is 2 and 1% respectively. Both strains showed good antimicrobial activity at 37°C which seemed to be optimal temperature for growth. Optimized conditions for antimicrobial peptides generation with peptide content by selected *Lactobacillus* are presented in Table 1.

### Conclusion

This work has shown, for the first time, that incubation period (48 and 12-24 h), inoculum size (2 and 1%) incubation temperature (37°C) is important variables determining the production of antimicrobial peptides from skim milk by *L. acidophilus* NCDC 14 and *L. delbrueckii* NCDC 09. The optimal condition yielded 2.43-2.90 mg/ml and 20-27 mg/ml peptides from skim milk by *L. acidophilus* NCDC 14 and *L. delbrueckii* NCDC 09 fermentation respectively. The usefulness and application of antimicrobial peptides in coming years, is likely to

expand beyond their medicinal properties as well as their use in food. Detailed optimization of fermentation parameters is essential for the generation of peptides at optimum process and economic conditions. The results of this study give useful information towards the achievement of such ends, in addition to providing and extending utility of antimicrobial peptides from skim milk by *L. acidophilus* NCDC 14 and *L. delbrueckii* NCDC 09 fermentation.

### Conflict of interests

The authors did not declare any conflict of interest.

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