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# Significance of Growth Rate, Acceptability of Fermented Milk and Release of Peptides by Lactic Cultures

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***Abstract***

*Ten lactic cultures (S. thermophilus MD2, S. thermophilus MD8, Lc. lactis 009, L. fermentum AI2, L. fermentum AI3, L. fermentum 138, L. plantarum AD29, L. rhamnosus NS4, L. rhamnosus NS6 and E. feacalis ND3) isolated from vegetables and fermented foods were evaluated for their potential to ferment milk, produce good quality curd and ability to release peptides in fermented milk. It was found that S. thermophilus MD8, S. thermophilus MD2 and L. rhamnosus NS6 were found to exhibit maximum growth rate (1.15, 0.98 and 0.49 respectively) and acid production rate (4.65, 5.14 and 5.59 respectively) in skimmed milk compared to other LAB. The curd/fermented milk prepared with S. thermophilus MD2, S. thermophilus MD8 and L. rhamnosus NS6 showed that the maximum acceptability as reflected by highest scores for overall acceptability i.e., 7.19, 7.07 and 7.04 respectively. Therefore, these three isolates were further studied for ACE (angiotensin-1 converting enzyme) inhibitory activity by spectrophotometric method and peptides released in milk medium after fermentation by RP-HPLC (reverse phase high performance liquid chromatography). L. rhamnosus NS6 exhibited relatively higher ACE inhibitory activity as well as released maximum peptides during RP-HPLC analysis than other two isolates. This reflects better acceptability and their application in preparation of acceptable quality fermented milk products.*

***Keywords:*** *Fermentation, growth rate, lactic acid bacteria (LAB), milk, sensory score*

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## INTRODUCTION

Lactic acid bacteria (LAB) are generally associated with habitats rich in nutrients, especially in food products like milk, meat, beverages and vegetables. Some are also members of the normal flora of the mouth, intestine and vagina of mammals. The term lactic acid bacteria were used to mean "milk-souring organisms". According to current taxonomy, based on 16s rRNA sequencing and other molecular techniques, the LAB are grouped in the phylum *Firmicutes* and order, *Lactobacillales*. LAB belong to the family *Lactobacteriaceae* that consists of gram-positive, catalase negative, facultative anaerobic, non-motile, non-spore forming rods and cocci shaped bacteria which produce lactic acid as the major end product during the fermentation of carbohydrates. Milk is particularly suitable as a fermentation substrate owing to its carbohydrate-rich, nutrient-dense composition. Fresh bovine milk contains 5 % lactose and 3.3 % protein and has a water activity near 1.0 and a pH of 6.6–6.7, perfect conditions for most microorganisms. LAB fermentative in nature, therefore they are ideally suited for growth in milk. In general, they will out-compete other microorganisms for lactose and by virtue of acidification, will produce an inhospitable environment for competitors. Milk fermentation process has been relied on the activity of LAB, which plays a crucial role in converting milk as raw material to fermented milk products. The most important properties of LAB are their ability to acidify milk and to generate flavour and texture, by converting milk protein due to their proteolytic activities [1]. Fermented milk products are reported to contribute to human health through several mechanisms [2]. Some LAB can synthesize folates, which are destroyed by the heat and have been shown to confer many health benefits [3]. Given the early recognition of the importance of milk in human nutrition and its widespread

  

consumption around the world, it is not surprising that cultured dairy products have evolved on every continent. Their manufacturing was already well established thousands of years ago, based on their mention in the old testament as well as other ancient religious texts and writings. Yogurt is also mentioned in Hindu sacred texts and mythology. Present study was planned with an objective to screen isolates of LAB based on their selected functional fermentation characteristics like better growth rate, faster acid production and acceptable sensory properties as well as the ability to release peptides in the fermented milk.

## MATERIALS AND METHODS

The LAB cultures used in the present study (Table 1) were obtained from Dairy Microbiology Department, SMC College of Dairy Science, Anand, Gujarat, India. The LAB cultures were propagated in sterilized reconstituted skim milk (10 % T.S.) and maintained by sub-culturing every week. The bacteriological media, chemicals and reagents were purchased either from Hi-media (Bangalore), Merck (Germany) or Sigma. Tonned milk (Amul) was purchased from local market of Anand (Gujarat, India) for making the curd.

***Table 1:*** *Gene Accession Number and Isolation Source of LAB Isolates.*

| Sr. No. | Name of Isolate | Isolate Number | Gene accession No | Source of Isolation |
|---|---|---|---|---|
| 1 | *S. thermophilus* | MD2 | GQ253961 | Market Dahi |
| 2 | *S. thermophilus* | MD8 | GQ253962 | Market Dahi |
| 3 | *Lc. lactis* | 091 | - | Procured from NDRI |
| 4 | *L. fermentum* | 138 | JN792459 | Fresh Turmeric |
| 5 | *L. plantarum* | AD29 | JN792465 | Dahi |
| 6 | *L. rhamnosus* | NS4 | KJ156963 | Shrikhand |
| 7 | *L. rhamnosus* | NS6 | KJ156964 | Shrikhand |
| 8 | *E. feacalis* | ND3 | KJ156959 | Dahi |
| 9 | *L. fermentum* | AI2 | JN792468 | Dhokla Batter |
| 10 | *L. fermentum* | AI3 | JN792457 | Dhokla Batter |

### Study of Growth Curve

The growth of all the isolates was studied by estimating viable lactic counts as well as development of titratable acidity (% lactic acidity). The activated cultures were inoculated in 100 ml RSM flasks at the rate of 2 %. After mixing them thoroughly, 10 ml was transferred to previously autoclaved test tubes. The culture tubes were incubated at 37 °C and samples were taken out at different intervals of 0, 4, 8, 12 and 24 h for viable lactic counts and titratable acidity measurement.

### Lactobacilli and Streptococci count

Lactobacilli and Streptococci counts of bacterial cultures were determined as per the method described by Indian Standards [4]. One ml of sample was taken out from the tubes and added to 9 ml phosphate buffer tubes. From this required numbers of serial dilutions were prepared. One ml diluted sample from appropriate tubes was transferred to labeled petri plates (in duplicates), poured with either MRS or M17 agar. The contents were spread thoroughly by tilting and rotating the plates and allowed it to solidify and then additional layer (5–7 ml) of the same agar was poured completely over the solidified medium. After solidification, the plates were incubated at 37 °C for 24–48 h. typical colonies counted and expressed as cfu/ml.

### Determination of Growth rate

Growth rate was calculated from the data of log CFU at different time intervals up to 12 h for individual LAB isolates using following formula:

$$\text{Growth Rate (G)} = \frac{(\log N - \log N0)}{2.41}$$

Where,

log N = log CFU/ml after 12 h of incubation

log N0 = log CFU/ml at 0 h of incubation and 2.41 is constant

**Determination of Titratable Acidity and Rate of Acid Production**

The titratable acidity of cultures was estimated by the procedure as described in Indian Standard [4]. Acidification rate was calculated with the % LA at 0 h and 12 h of incubation. Based on the titratable acidity (% Lactic acid) data obtained at an interval of 4 h up to 12 h for individual strains, acidifying rate was calculated. The formula for the calculation of acidifying rate is as follows:

Acidifying Rate = $\frac{X-X0}{12}$ $2-00$

Where,

X = Titratable Acidity at 12 h, X0 = Titratable Acidity at 0 h

**Method for the Preparation of Curd**

Tonned milk (Amul) was heated to 90 °C for 10 min in boiling water bath. It was cooled and transferred to sterile 100 ml beakers. LAB strains were inoculated individually at the rate of 2 % v/v to each beaker. The contents of each beaker were mixed thoroughly and covered with aluminium foils. The beakers with contents were incubated at 37 °C for 12 h followed by transfer to refrigerator (5 ± 1 °C) for overnight.

**Sensory Evaluation**

The product was subjected to the sensory evaluation by an expert panel of nine judges using nine point hedonic scales [5]. The product was served at 10 °C to the panel. The score for colour and appearance, flavour, body and texture and overall acceptability were recorded.

**ACE Inhibitory Activity by Spectrophotometric Method**

The method was suggested by Papadimitriou *et al.* [6]. For this experiment, active cultures were inoculated at the rate of 2 % in sterilized reconstituted skim milk (10 % TS), incubated at 37 °C for 24 h then centrifuged the fermented milk at 10,000 rpm for 10 min at 4 °C (Eppendorf centrifuge, US). The supernatant was collected and it was filtered through 0.2 µm membrane filter. Thereafter, 50 µl of 5 mM HHL (10.74 mg HHL in 5 ml sodium borate buffer, pH 8.3) solution was mixed with 500 µl deionized water and 100 µl of sample. The reaction is initiated by the addition of 20 µl (4 mU in 250 µl) of ACE enzyme and the mixture was incubated for 30 min at 37 °C. Then, the reaction was terminated by the addition of 1000 µl of 1 [N] HCl. The hippuric acid liberated by the ACE was extracted with 1.7 ml ethyl acetate and then heated at 100 °C for 20 min in waterbath.

The residue containing hippuric acid is dissolved in 2 ml of de-ionised water and the absorbance of the solution is measured spectrophotometrically using Systronic PC based double beam spectrophotometer 2202, India at 250 nm against blank. The extent of inhibition is calculated as follows:

% ACE Inhibitory Activity

$$= \left[\frac{(A-B)-(C-D)}{(A-B)}\right] \times 100$$

Where,

A = The absorbance of solution containing ACE but no sample

B = The absorbance of solution containing ACE but no sample and HCL

C = The absorbance of solution containing ACE, sample and HCL

D= The absorbance of solution containing ACE, sample but no HCl

**Release of Peptides by LAB through RP-HPLC**

Fermented milk and unfermented milk (control) were centrifuged at 14,000 rpm (Eppendorf centrifuge, US) for 10 min at 4 °C. Then, supernatants were collected and ultra-filtered through 3 kDa cut off membranes (Merck millipore) at 2000 rpm for 15 min at 10 °C. Permeates were collected, filtered through a 0.45 µm disposable hydrophilic filter and 20 µl water soluble extract (WSE) was injected in the HPLC (Shimadzu, Japan) through microinjector (Hamilton, Switzerland). In the present study, RP-HPLC was used for the separation purpose of different peaks. RP-HPLC (Shimadzu LC-20, Japan) was performed as described by Rodríguez-Figueroa *et al.* [7]; Papadimitriou *et al.* [6].

A binary gradient HPLC system was used fitted with C18 column (Phenomenex) white pore analytic column (5 µ, 158x4.6 mm). Sample was applied using microinjector with 20 µl loop. Eluent-A was 0.1 % v/v TFA in deionised water and Eluent-B was 0.08 % v/v TFA in mixture of 60: 40 acetonitrile and

  

de-ionised water. Separation was conducted at room temperature at flow rate 1 ml/min with eluent-A for 10 min and linear gradient, from 0–80% of eluent-B, for 10 min. The column finally eluted with 100 % eluent-B for 10 min. Absorbance of elute was monitored at 214 nm using variable wavelength spectrophotometric detector.

## STATISTICAL METHODS

All the tests were carried out in three replications independently in duplicates and data were subjected to statistical analysis using completely randomized design (CRD) as per the methods described in Steel and Torrie [8]. The significance was tested at 5 % level of significance using mean value. The values for microbial counts were log transformed before analysis.

## RESULTS AND DISCUSSION

### Determination of Growth Rate of LAB Isolates in Milk

The pattern of viable lactic counts of MD2 and MD8 clearly indicated that both the cultures were fast growing and entered into log phase within first 4 h and reached to the peak of log phase at 12 h (Figure 1). Subsequently, they showed declination phase till 24 h. None of the isolates except MD2 and MD8 entered into declination phase up to 24 h, indicating that they were slow growers compared to other isolates. The growth pattern of NS4 and NS6 (*L. rhamnosus* strains) were found similar which might be due to same genus and species. Similar results were obtained with the *L. fermentum* strains (138, AI2 and AI3). However, *L. plantarum* AD29 showed sudden increase in cell concentration after 8 h.

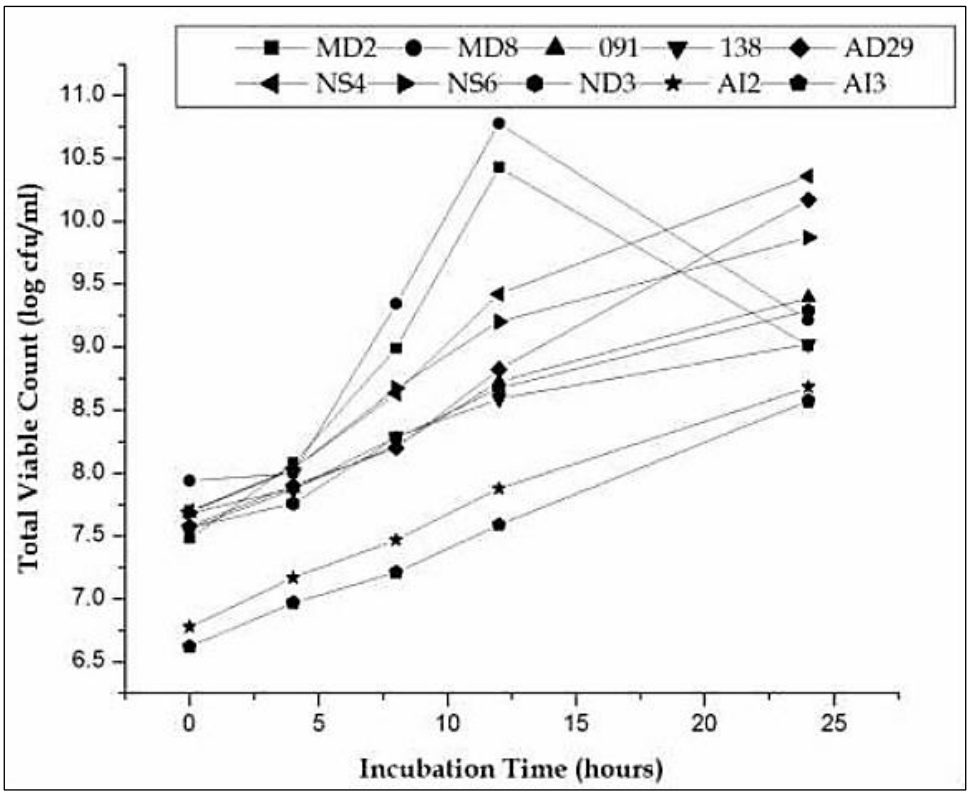


***Fig. 1:*** *Total Viable Count (CFU/ml) of LAB Isolates in RSM (10 % TS) at 37 °C.*

Based on the data obtained from growth curve, growth rate was calculated (Table 2). The growth rate of LAB isolates was found to be in the range of 0.26–1.15. Among all the strains MD8 was the fastest growing strain (1.15) while AI3 exhibited slower growth rate (0.26). It was observed that growth rate of MD2, MD8, NS4 and NS6 differed significantly (P <0.05 %) from each other.

While growth rate of 138, AI3 and AI2 were seen to be at par. Donkor *et al*. studied that *S. thermophilus* (St 1342) achieved a significantly (P <0.05) higher cell concentration ($10^9$ CFU/ml) in comparison to *L. delbrueckii* ssp. *bulgaricus* Lb 1466; the slow growth of the latter resulted in low viability ($10^7$ CFU/ml) at the end of the fermentation period of 24 h [9].

In our laboratory Dave studied, growth curve of *S. thermophilus* MTCC 5460, MTCC 5461 and D3 [10]. The average log CFU/ml was 6.69 (0 h), 7.20 (2 h), 8.80 (4 h), 8.25 (6 h) and 8.40 (12 h).

***Table 2:*** *Growth Rate of LABIsolates in Skim Milk at 37 °C.*

| LAB isolates | Growth rate | Acidifying Rate |
|---|---|---|
| *S. thermophilus* MD2 | 0.98±0.06 $^{e}$ | 4.65±0.07$^{c}$ |
| *S. thermophilus* MD8 | 1.15±0.03 $^{f}$ | 5.14±0.26$^{a,b}$ |
| *Lc. Lactis* 091 | 0.35±0.06 $^{b}$ | 3.86±0.11$^{e,f}$ |
| *L. fermentum* 138 | 0.30±0.03 $^{a, b}$ | 3.56±0.34$^{f}$ |
| *L. plantarum* AD29 | 0.38±0.08 $^{b}$ | 4.24±0.41$^{d}$ |
| *L. rhamnosus* NS4 | 0.58±0.03 $^{d}$ | 4.99±0.01$^{d,c}$ |
| *L. rhamnosus* NS6 | 0.49±0.01 $^{c}$ | 5.59±0.41$^{a,e}$ |
| *E. feacalis* ND3 | 0.38±0.07 $^{b}$ | 4.16±0.26$^{d,e}$ |
| *L. fermentum* AI2 | 0.30±0.04 $^{a, b}$ | 3.98±0.37$^{d,e,f}$ |
| *L. fermentum* AI3 | 0.26±0.03 $^{a}$ | 3.56±0.11$^{f}$ |
| **CD (0.05)** | 0.080 | 0.47 |

*Values with different superscripts differ significantly(P <0.05) in each column (mean ± SD).*

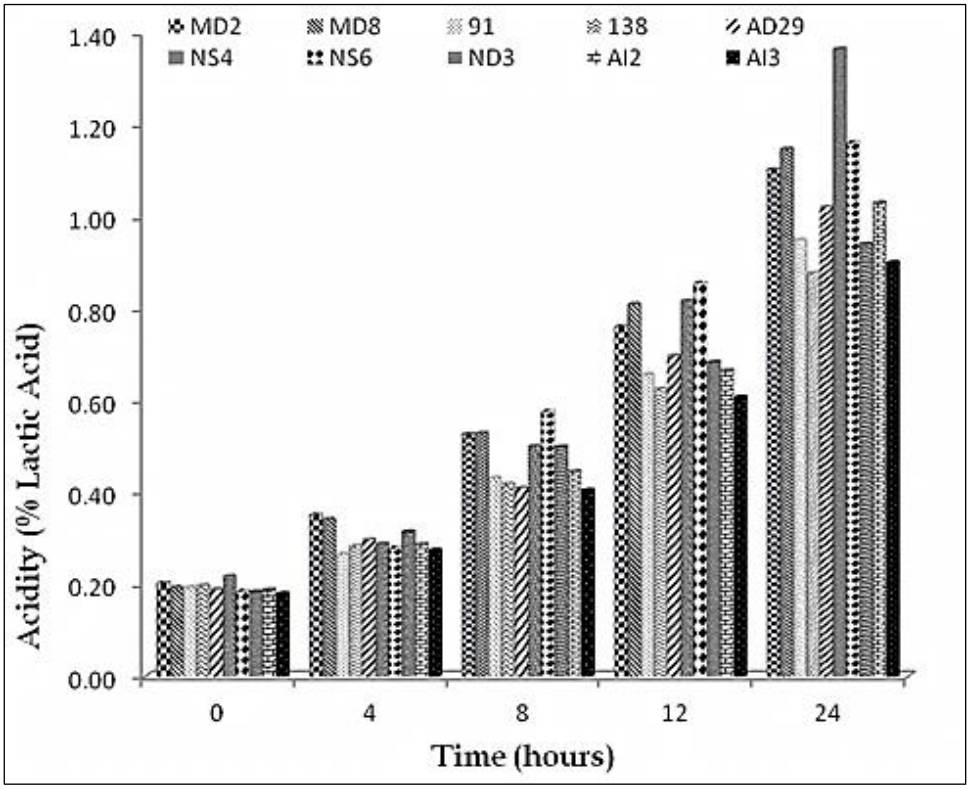


***Fig. 2:*** *Percentage Acidity Production in RSM (10 % TS) by LAB Isolates During 24 h of Incubation at 37 °C.*

**Determination of Acidifying Rate of Individual LAB Isolate in Milk**

The individual cultures were inoculated at 2 % rate in RSM (10 % TS) and titratable acidity (% lactic acid) was measured at different time interval up to 24 h of incubation. The growth curve for titratable acidity was prepared which is depicted in Figure 2. During the growth in skim milk MD2 and MD8 (*S. thermophilus* strains); NS4 and NS6 (*L. rhamnosus* strains); 138, AI2 and AI3 (*L. fermentum* strains) showed similarity in acidity development.

  

Acidifying rate was determined by calculating the amount of acidity developed during 12 h of incubation by the formulae mentioned in materials and methods. As seen from Table 3, rate of acid production by LAB isolates was in the range of 3.56–5.59. NS6 was found to be maximum acid producer (5.59) compared to rest of the cultures while 138 and AI3 produced minimum acidity (3.56). Donkor *et al.* found that even though *L. delbrueckii* ssp. *bulgaricus* Lb 1466 grew slowly in RSM, it producedsubstantial amount of lactic acid, second to *S. thermophilus* St 1342, and the highest ($P < 0.05$) decline in pH at the end of fermentation (~ 4.3) [9]. The study also showed that probiotic organisms produced some lactic acid even though not as high as in the case of yoghurt culture, *L. delbrueckii* ssp. *bulgaricus* Lb 1466.

Growth curve of *S. thermophilus* MD2, MD8 and D3, studied earlier by Dave showed average increase in % lactic acid as 0.25 (0 h), 0.39 (2 h), 0.82 (4 h), 0.99 (6 h) and 1.18 (12 h) [10]. Mehmood *et al*. studied the acid production by individual LAB strain in fermented milk at 2 h interval up to 8 h [11]. It was found that *S. thermophilus* S6, *S. thermophilus* S7 and *L. acidophilus* R20 produced significantly ($P < 0.05$) higher acidity i.e., 1.05, 1.04 and 1.02 respectively after 6 h, compared to the rest LAB isolates. This report supports our study.

**Sensory Profile of Curd Prepared Using Individual LAB Isolate**

Sensory profile of the curd depicted in Table 3, indicate the flavor score of the curd in the range of 5.15–7.11. The curd prepared with MD2 and NS6 gave distinctly higher flavour score i.e., 7.11 and 6.93 respectively compared to others in descending order NS6, MD8, AI2, AI3, 091, NS4, 138, AD29 and ND3. Isolates of ND3 and AD29 received flavour score less than 6.00 i.e., 5.15 and 5.67 respectively, hence these cultures were rejected. The body and texture score of curd was in the range of 5.44–7.30. The curd prepared with MD2 gave highest body and texture score i.e.,7.30 among all the test strains. As ND3 (5.44) and AD29 (5.70) scored less than 6.00, the strains were not accepted on the basis of body and texture parameter. MD2, MD8, NS6, AI2, AI3, 091, NS4 and 138 were at par with respect to body and texture score.

***Table 3:*** *Sensory Profile of Curd Prepared with LAB Isolates.*

| LAB Isolates | Flavour | Body & Texture | Colour & Appearance | Overall Acceptability |
|---|---|---|---|---|
| *S. thermophilus* MD2 | $7.11\pm0.48^{a}$ | $7.30\pm0.42^{a}$ | $7.26\pm0.64^{a}$ | $7.19\pm0.61^{a}$ |
| *S. thermophilus* MD8 | $6.81\pm0.39^{a,b}$ | $6.89\pm0.48^{a}$ | $7.19\pm0.39^{a,b}$ | $7.07\pm0.34^{a}$ |
| *Lc. lactis* 091 | $6.48\pm0.71^{a,b,c}$ | $6.41\pm0.83^{a,b,c}$ | $6.67\pm0.44^{a,b,c,d,e}$ | $6.67\pm0.62^{a,b,c}$ |
| *L. fermentum* 138 | $6.07\pm0.06^{d}$ | $6.30\pm0.32^{a,b,c}$ | $6.48\pm0.17^{c,d,e,f}$ | $6.15\pm0.06^{c,d,e}$ |
| *L. plantarum*AD29 | $5.67\pm0.59^{d,e}$ | $5.70\pm0.68^{b,c}$ | $6.19\pm0.42^{e,f}$ | $5.67\pm0.58^{d,e}$ |
| *L. rhamnosus* NS4 | $6.30\pm0.06^{b,c,d}$ | $6.33\pm0.51^{a,b,c}$ | $6.44\pm0.22^{d,e,f}$ | $6.33\pm0.29^{b,c,d}$ |
| *L. rhamnosus* NS6 | $6.93\pm0.26^{a,b}$ | $6.89\pm0.51^{a}$ | $6.93\pm0.17^{a,b,c,d}$ | $7.04\pm0.17^{a,b}$ |
| *E. feacalis* ND3 | $5.15\pm0.46^{e}$ | $5.44\pm0.78^{c}$ | $5.81\pm0.63^{f}$ | $5.48\pm0.56^{e}$ |
| *L. fermentum* AI2 | $6.70\pm0.42^{a,b,c}$ | $6.59\pm0.82^{a,b}$ | $6.52\pm0.39^{b,c,d,e}$ | $6.81\pm0.34^{a,b,c}$ |
| *L. fermentum* AI3 | $6.52\pm0.07^{a,b,c}$ | $6.48\pm0.23^{a,b}$ | $6.63\pm0.13^{a,b,c,d,e}$ | $6.59\pm0.13^{a,b,c}$ |
| **CD (0.05)** | 0.70 | 1.01 | 0.68 | 0.72 |

*Values with different superscripts differ significantly ($P < 0.05$) in each column (mean ± SD).*

The colour and appearance score of curd was in the range of 5.81–7.26. The curd prepared with MD2 and MD8 exhibited highest colour and appearance score i.e.,7.26 and 7.19, respectively compared to other LAB isolates whereas, lowest score was obtained with ND3 (5.48). As ND3 gave less than 6.00 score and it was rejected based on colour and appearance parameter. MD2, MD8, NS6, 091 and AI3 were found to be non-significant ($P > 0.05$ %) with respect to colour and appearance score.

The overall acceptability score of curd was in the range of 5.48–7.19. Maximum overall acceptability score was obtained by the curd prepared with MD2 (7.19).

Overall acceptability score of ND3 (5.48), AD29 (5.67) and 138 (6.13) was observed to be similar. Isolates ND3 and AD29 received less than 6.00 score, and hence were regarded as non-acceptable. MD2, MD8, NS6, AI2, 091 and AI3 were at par with respect to overall acceptability score. It is clear from the above discussion that the curd prepared by using MD2, MD8 and NS6 was better and most acceptable with respect to sensory scores as compared to all other isolates. Whereas curd prepared using ND3 received the lowest score.

Mehmood *et al.* studied the organoleptic quality of yoghurt prepared using single strains of LAB[11]. The overall acceptability score was highest for *L. bulgaricus* L2 (7.28), followed by *L. lactis* S17 (7.25), *S. thermophilus* S7 (7.18), *L. acidophilus* T1 (6.95), *L. bulgaricus* L1 (6.48), *L. casei* F13 (6.35), *L. acidophilus* R20 (6.23), *S. thermophilus* S6 (6.23), *L. lactis* S3 (6.20) and *L. casei* J10 (6.12). Based on the growth rate and acidifying rate in skimmed milk, the cultures MD2, MD8 and NS6 were found to be better than others. Similarly, all the parameters of sensory profile also showed that these (MD2, MD8 and NS6) cultures were most acceptable and can be used in preparation of good quality fermented milk products. Hence, these three cultures were further studied for their peptides production in milk.

**ACE Inhibitory Activity by Spectrophotometric Method**

ACE catalyses the degradation of bradykinin, a vasodilating peptide and the production of angiotensin II, a potent vasoconstrictor [12]. Angiotensin II induces also the release of aldosterone, which causes the retention of sodium ions by the kidney, elevated blood volume and thus, an increase in blood pressure. ACE-inhibitory substances were thus proposed to lower blood pressure. Many studies have been reported that ACE-inhibitory peptides can be produced by the enzymatic hydrolysis of milk proteins by the fermentation of milk with different strains of LAB. Such strains were then used to prepare functional fermented milks having antihypertensive activity.

It can be seen from the Table 4 that culture NS6 was exhibiting significantly (P <0.05) higher (28.39 %) ACE-I inhibitory activity than MD2 (15.27 %) and MD8 (11.95 %). All the 3 LAB isolates were found to be differing significantly (P <0.05). Solanki studied the ACE-I inhibitory activity of LAB isolates by spectrophotometrically in our laboratory [13]. The results indicated that the production of ACE-I inhibitors was not confined to single species or strain of bacteria but all the strains tested, produced peptide, which shows *in-vitro* ACE-I inhibitory activity. Strains *L. rhamnosus* NS4 and *L. bulgaricus* 009 gave maximum ACE-I inhibitory activity 79.66 % and 67.09 % respectively compared to other isolates.

Yamamoto *et al.* studied the ACE-I inhibitory activity of *L. helveticus* strain in three medium by spectrophotometric method [14]. The lowest ACE-I inhibitory activity was obtained for WPI-enriched milk, with IC50 values varying from 2.24–3.51 mg/ml, indicating that a large amount of medium would be required to inhibit 50 % of the enzyme activity. Higher ACE-I inhibition was measured for skim milk medium (1.15–1.68 mg/ml), whereas the caseinate enriched milk provided the highest ACE-I inhibitory activity (0.6–1.1 mg/ml). These results indicated that bacterial proteolysis appears to be essential to produce ACE-I inhibitors in fermented milk. Also, the nature of protein substrate used in the medium was found to be more important in the production of ACE-I inhibitors than the degree of protein hydrolysis.

***Table 4:*** *ACE Inhibitory Activity (%) of LAB Isolates in Skimmed Milk.*

| LAB isolates | % ACE Inhibitory Activity* |
|---|---|
| *S. thermophilus* MD2 | 15.27±0.66[a] |
| *S. thermophilus* MD8 | 11.95±0.40[b] |
| *L. rhamnosus* NS6 | 28.39±1.51[c] |
| **CD (0.05)** | 1.35 |

*Values with different superscripts differ significantly (P <0.05)(mean ± SD).*

**Release of peptides by LAB through RP-HPLC**

All the 3 LAB isolates were inoculated at 2 % rate in skim milk separately and incubated at 37 °C for 24 h. The supernatant was collected

and separated through 3 KDa membrane at 2000 rpm for 10 min at 10 °C for each culture. The filtrate was passed through 0.22 µm syringe filter and 20 µl filtrate of all the LAB isolates was injected into HPLC-RP loop individually. The chromatogram produced for each culture (for <3KDa peptides) is presented in Figure 3. A comparison of LAB isolates ability for hydrolyzing milk protein was shown by recording area counts from the peptides chromatographic profiles. Peptides produced by all the LAB isolates were maximum compared to raw milk peptides (control). Only three fractions of peptides were exhibited in raw milk due to some native proteolytic enzymes. However, culture NS6 released maximum peptide during HPLC-RP analysis as compared to MD2 and MD8 under specified conditions. It was also observed that maximum peptides were produced by NS6 from the protein content.

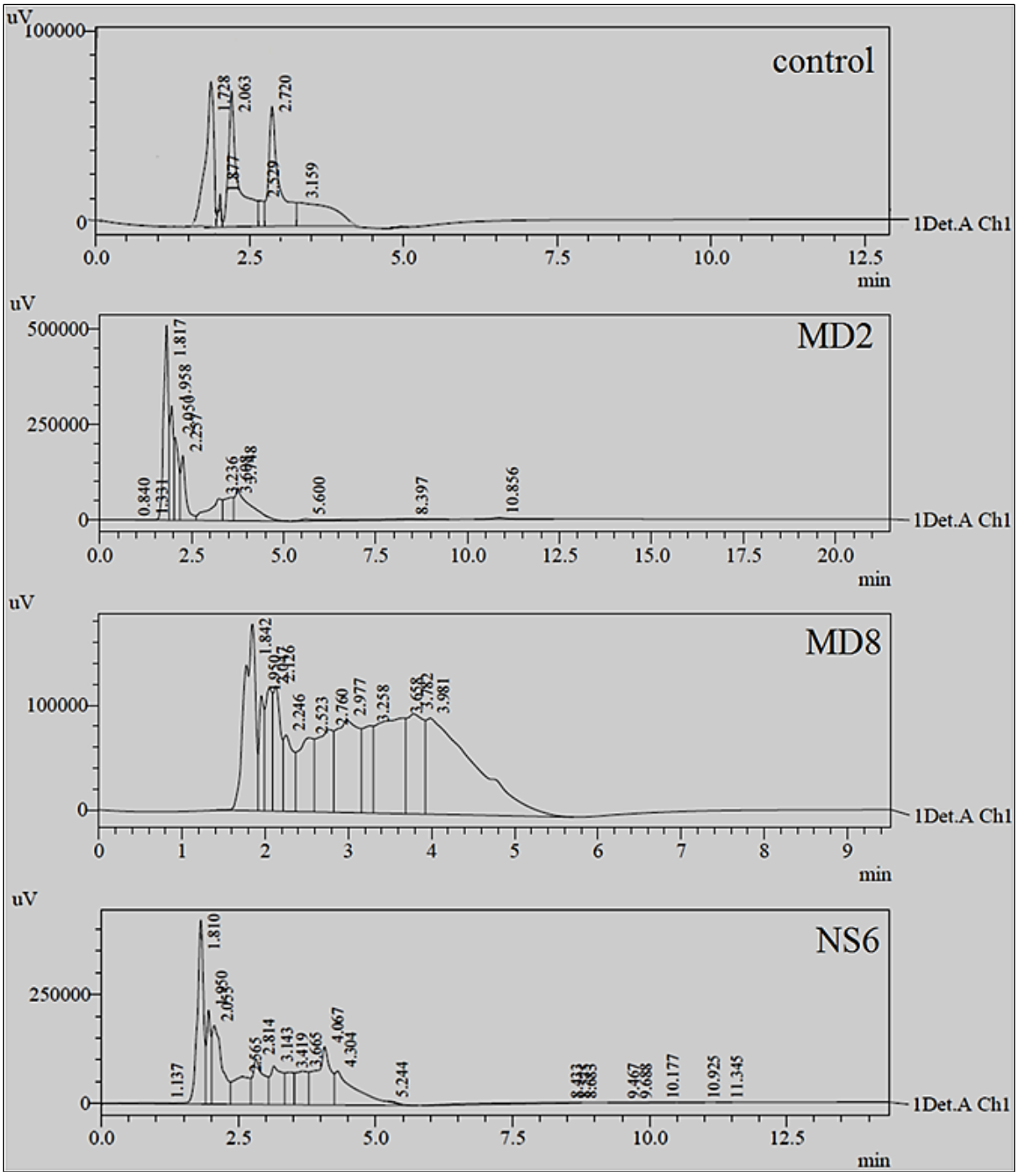


***Fig. 3:*** *Peptides Released in Milk Medium (Milk without Culture Innoculation was Taken as Control).*

Peptides content were highest after 1.8–2.2 min of elution by NS6 isolate compared to MD2 and MD8 during the flow of solvent B (90 %) (acetonitrile : water =60: 40) for 5–10 min when the concentration of acetonitrile was between 9 and 35 % which may be related to relatively hydrophobic nature of eluted peptide. It has been suggested that a close relation sheep exist between hydrophobicity and positively charged amino acids in the C-terminal position and bioactive peptides derived from milk protein [15]. Hati *et al.* studied the ACE-inhibitory activity and release of peptides in fermented skim milk under specified growth conditions of nine LAB isolates [16]. It was found that *L. rhamnosus* NS4 and *L. bulgaricus* 009 exhibited maximum ACE inhibitory activity compared to other cultures. Again, both these isolates showed maximum peptides separation during RP-HPLC analysis.

## CONCLUSION

Based on the growth rate and acidifying rate in skim milk medium, the selected cultures MD2, MD8 and NS6 were found to be better than the other isolates. Similarly, all the parameters of sensory profile exhibited that these (MD2, MD8 and NS6) cultures were most acceptable. Hence, these cultures are recommended for preparation of fermented milk products. Culture NS6 was found to release highest number of peptides and gave relatively higher ACE inhibitory activity in milk after 24 h of incubation than rest two isolates. So, there is a need to analyze the definite function of those peptides.

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