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Effect of enzymatic hydrolysis of proteins on growth of *Bifidobacterium bifidus* in milk

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Abstract: The effect of enzymatic hydrolysis of proteins in milk using neutrase on the growth of the probiotic strain *Bifidobacterium bifidus* was evaluated by estimation of microbial growth, acidity, viscosity and flavour production. A significant increase in the growth of *B bifidus* was observed in neutrase-hydrolysed milk. The setting time of bifidus-cultured milk was advanced by about 12 h at 5% degree of hydrolysis. Enzymatic hydrolysis of proteins prior to cultivation also significantly increased the viscosity of the product. An approximately 60% increase in viscosity of the product was observed in neutrase-hydrolysed milk. Production of steam-volatile monocarbonyls as an indication of development of flavour was also higher in neutrase-hydrolysed milk. The concentration of steam-volatile monocarbonyls was 2.47 μmol per 100 ml in neutrase-hydrolysed milk but only 1.84 μmol per 100 ml in control milk at the setting point of the curd.

Keywords: milk protein; neutrase hydrolysis; *Bifidobacterium* growth; viscosity

INTRODUCTION

Caseins, being major milk proteins, are well known for their nutritive and functional properties but have limited effect when used as conventional components. To enable complete and effective utilisation of these proteins in functional foods, changes in their conformation are desirable, which can be achieved through controlled hydrolysis by selected proteinases.[1]

The use of *Bifidobacterium* spp as probiotic strains has been widely accepted owing to their beneficial effects on human beings of all age groups. They impart excellent therapeutic properties to foods.[2–4] The most useful media for administering bifidobacteria to patients are milk and milk products. There is considerable interest in determining optimal growth conditions for bifidobacteria in dairy products. One possible method for improving the survival and viability of these micro-organisms is the use of growth promoters. Hydrolysed milk proteins are among the various suitable growth promoters.[5,6]

The enzyme neutrase has its optimum activity at the pH of milk and hence can be used for the effective hydrolysis of milk. The performance of *Bifidobacterium bifidus* in neutrase-hydrolysed milk and the influence of enzymatic hydrolysis on the physicochemical and functional characteristics of the product were investigated in this study.

MATERIALS AND METHODS

Enzyme and culture

Neutrase with activity 0.5 AU g^{-1} (Novo Nordisk Co Ltd, Denmark) was used for modification of proteins. *B bifidus* culture was obtained from the Dairy Microbiology Department, Dairy Science College, Bangalore, India.

Enzymatic hydrolysis of milk

Neutrase was dissolved in phosphate buffer (0.02 M, pH 6.5) and added to milk standardised to 3.0% protein and 4.8% lactose, maintained at 45 °C, at an enzyme/substrate ratio of 1:20 000 (calculated on basis of protein content of milk). Samples were drawn at intervals of 5, 10 and 15 min and immediately immersed in boiling water for 10 min to inactivate the enzyme. Finally, samples were sterilised at 121 °C for 15 min.

The degree of hydrolysis was determined spectrophotometrically by the tyrosine value method.[7]

Preparation of bifidus-cultured milk

B bifidus was inoculated aseptically into both unhydrolysed (control) and neutrase-hydrolysed sterilised milks at a rate of 5%. Samples were incubated at 37 °C until the curd was set, as determined by visual observation of the formation of coagulum after every 2 h.

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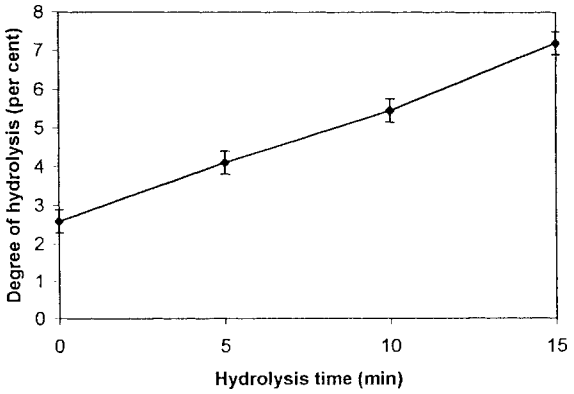

Figure 1. Degree of hydrolysis of proteins in neutrase-hydrolysed milk.

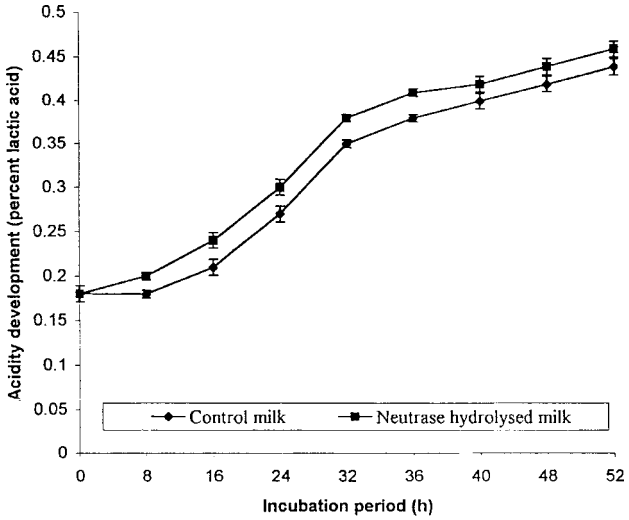

Figure 2. Acidity development in neutrase-hydrolysed milk.

Analysis of bifidus-cultured milk

The acidity of samples was measured by titration against 0.1 N NaOH.[8]

The bifidobacterial numbers in control and neutrase-hydrolysed milks were estimated by the direct microscopic count method.[9]

The viscosity of samples was determined using a falling ball viscometer.[10]

The steam-volatile monocarbonyl content was estimated by conversion into 2,4-dinitrophenylhydrazones according to the procedure described by Bhat and Ramamurthy.[11]

ANOVA was carried out to determine significant differences between treatments.[12]

RESULTS AND DISCUSSION

Proteins in milk were modified by enzymatic hydrolysis using neutrase. The proteolytic action of neutrase on milk proteins as a function of time is shown in Fig 1. There was a proportionate increase in degree of hydrolysis (DH) with increasing time. It was also found that bitter peptides with flavour defects were present beyond 5% DH, as observed by Venkatesh.[13] In the present study, hydrolysis for 15 min at an enzyme/substrate ratio of 1:20 000 gave about 5% DH; hence hydrolysis was restricted to a maximum of 15 min throughout the experiment.

The effects of protein hydrolysis on the growth of *B bifidus* in milk, represented by direct microscopic count (DMC; expressed as $\log_{10}$ ml^{-1}) and corresponding titratable acidity (expressed as % lactic acid), are shown in Table 1 and Fig 2 respectively. A significant increase in bifidobacterial counts was observed in protein-hydrolysed milk with increasing extent of hydrolysis. The rate of development of acidity was also faster in protein-modified milk. Control samples required 48 h while neutrase-hydrolysed samples took 36 h for curd setting (Table 2). The acidity development was almost the same, 0.40% lactic acid, at the respective times of setting.

Proteinase and peptidase systems play a vital role in supplying peptides and essential amino acids, thus allowing the growth of starters to high cell densities so that rapid fermentation can occur. Growth promotion of lactic acid bacteria by peptides is well known.[14] Thus the reduction in setting time of bifidus-cultured milk and the higher rate of developed acidity may be due to the availability of smaller peptides or amino

Incubation period (h)	*Modification time (min)*			
	Control	*5*	*10*	*15*
0	6.000	6.060	6.217	6.079
8	7.491	7.491	7.663	7.653
16	7.740	7.690	7.806	7.863
24	7.792	7.778	7.959	7.973
32	8.004	7.991	8.053	8.096
36	8.021	8.025	8.071	8.113
40	8.045	8.079	8.113	8.136
48	8.096	8.139	8.096	8.079
52	8.079	8.113	8.079	8.045
Mean	$7.696^{a} \pm 0.66$	$7.707^{ab} \pm 0.65$	$7.780^{b} \pm 0.61$	$7.781^{b} \pm 0.66$

Growth (direct microscopic count) expressed as $\log_{10}$ ml^{-1}.
Proteins in milk were hydrolysed to 5% degree of hydrolysis.

Table 1. Effect of protein modification by neutrase hydrolysis on growth of *Bifidobacterium bifidus* in milk

Table 2. Curd setting time (h) of neutrase-hydrolysed milk cultured with *Bifidobacterium bifidus*

	Modification time (min)			
Trial	*Control*	*5*	*10*	*15*
1	48	46	36	36
2	46	46	40	34
3	48	50	40	36
4	50	44	36	38
5	48	44	48	36
Mean	$48^{c} \pm 1.4$	$46^{c} \pm 2.4$	$40^{b} \pm 4.8$	$36^{a} \pm 1.4$
Reduction in setting time (%)	—	4.16	16.66	25.00

Critical difference (CD) between treatments = 3.90.
The curd setting time was noted by visual observation of the formation of coagulum after every 2 h.

Table 3. Effect of protein modification by neutrase hydrolysis on viscosity (cP) of bifidus-cultured milk

	Modification time (min)			
Trial	*Control*	*5*	*10*	*15*
1	72.50	76.00	103.50	116.10
2	72.60	76.50	103.60	116.50
3	72.80	75.70	103.85	116.30
4	72.90	76.30	103.80	116.80
5	73.70	76.20	103.65	117.50
Mean	$72.90^{a} \pm 0.47$	$76.14^{b} \pm 0.30$	$103.68^{c} \pm 0.14$	$116.64^{d} \pm 0.54$
Increase in viscosity (%)	—	4.44	42.22	60

CD between treatments = 0.532.

acids in protein-hydrolysed milk at the initial stage, leading to faster growth of these organisms.

The performance of *B bifidus* was also investigated in terms of viscosity and flavour production in bifidus-cultured milk. Uniform viscous products add to the palatability of the fermented milks, especially those served in stirred form. Table 3 shows the effect of protein hydrolysis on the viscosity of bifidus-cultured milk.

The viscosity of milk hydrolysed for 15 min (116.64 cP) was significantly (about 60%) higher than that of the control (72.9 cP). This significant increase in viscosity may be attributed to the exposure of more hydrophilic and hydrophobic groups by the action of neutrase during hydrolysis, which may lead to quicker aggregation of protein molecules and add to the effect of production of lactic acid by *B bifidus* for setting of the curd.

Table 4. Production of steam-volatile carbonyls (μmol per 100 ml) in bifidus-cultured milk

	Samples		
Trial	*Control milk*	*Control milk curd*	*Modified milk curd*
1	1.83	2.00	2.40
2	1.82	2.10	2.20
3	1.80	2.07	2.45
4	1.87	2.10	3.00
5	1.88	1.98	2.30
Mean	$1.84^{a} \pm 0.33$	$2.05^{b} \pm 0.56$	$2.47^{c} \pm 0.31$

CD between treatments = 0.22.
Steam-volatile monocarbonyls were determined immediately after the curd was set.

Flavour production in protein-modified bifidus-cultured milk from neutrase-hydrolysed milk was studied by estimating the steam-volatile monocarbonyl (SVMC) content of the product. The results are depicted in Table 4. The SVMC concentrations were estimated at the respective setting times of bifidus-cultured milk in order to avoid any contribution due to differences in acid production or growth of organisms. From Table 4 it can be seen that the SVMC content of modified milk at its setting time was significantly higher (2.47 μmol per 100 ml) than that of control milk (1.84 μmol per 100 ml). This may have significance for the flavour of fermented milk prepared from neutrase-hydrolysed milk. Neutrase treatment is shown to increase the production of amino acids such as valine, leucine and phenylalanine,[15] which may undergo catalytic degradation by the enzymes produced by yoghurt culture to produce aroma compounds.

CONCLUSION

The study revealed that the slow-growing probiotic culture *B bifidus* finds a suitable environment to grow luxuriously in enzyme-hydrolysed milk. The use of enzyme-hydrolysed milk for bifidus-cultured milk preparation results in a reduced setting time and is thus of benefit to the industry. Further, improvements in viscosity and flavour characteristics are reflected in the final quality of the product, which may enhance consumer acceptability.

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