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Evaluation of Proteolytic Activity of Some Dairy Lactobacilli

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Abstract: The present investigation concerned with evaluating the proteolytic activity of some lactobacilli, 8 lactobacilli strains were grown on MRS broth medium to determine their proteolytic activity after incubation at 37°C for 0, 12, 24, 36 and 48 hrs. The tested strains were *Lactobacillus casei* NRRL B-1922, *Lactobacillus casei* NRRL B441, *Lactobacillus rhamnosus* NRRL B-445., *Lactobacillus helveticus* CNRZ32, *Lactobacillus plantarum* NRRL B4004, *Lactobacillus reutri* NRRL B-14171, *Lactobacillus acidophilus* CNRZ 593N and *Lactobacillus delbrueckii ssp. bulgaricus* CNRZ397. Among the tested strains *Lactobacillus rhamnosus, Lactobacillus helveticus, Lactobacillus plantarum* and *Lactobacillus delbrueckii* ssp. *bulgaricus* were the highest enzyme producers. The enzyme production was at the stationary phase, thus these organisms were chosen to be used in a further study as adjunct culture for improving soft cheese ripening made from buffaloe's milk concentrate.

Key words: Lactic acid bacteria · Protease activity · Protease specific activity

INTRODUCTION

In cheese manufacture, the proteolysis of casein is thought to play a pivotal role because amino acids resulting from proteolysis are the major precursors of specific flavour compounds, such as various alcohols, aldehydes, acids, esters and sulfur compounds [1]. The specificities of cell envelop protease play an essential role in the production of bitter peptides [2] and so on the use of Lactic acid bacteria (LAB) strains deficient in peptidase activity have also indicated that peptidases (Pep), are involved in the degradation of bitter peptides; these peptidases therefore impact the development of the organoleptic quality of the milk product [3, 4]. It was recently proposed that foodgrade strains of *L. lactis* expressing *L. helveticus* CNRZ32 PepO2 and PepO3, in combination with PepN, can be used to reduce bitterness in cheese [4]. For economic reasons, several approaches were exploited to accelerate the cheese ripening process. These have included methods such as elevation of storage temperature, addition of proteinases, the use of bacteriophage-encoded lysin or lytic bacteriophages and the addition of selected nonstarter LAB or lactobacilli adjuncts to cheese [5-7]. Enriching the proteolytic of LAB potential by constructing a recombinant starter strain expressing peptidases derived from *L. helveticus* or *L. delbrueckii* subsp. *lactis* under a constitutive or inducible promoter can also, be used to accelerate cheese proteolysis and, hence, the ripening process [4, 8-13]. However, while it can be concluded that balanced proteolysis is important for flavour formation and especially in prevention of bitterness in cheese, it is the conversion of the free amino acids, rather than proteolysis / peptidolysis that controls the rate of flavour formation from proteins [1]. Thus, engineering the proteolytic system alone is hardly the key for accelerating flavour formation in cheese.

The autolysis of LAB starters is considered to be another important element of cheese manufacture because this activity permits the release of cytoplasmic peptidases into the curd, which normally considered as a prerequisite for flavour formation to proceed. In this regard, a number of studies were sought to control the rate and level of lysis of lactococcus starter strains; these include phage - and autolysin - based mechanisms and leaky lactococcal starter cultures over expressing certain *L. lactis* or *L. helveticus* peptidases [9, 14-17]. In addition to good viability in the intestine, technological

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properties are a prerequisite for potential use of the strains as probiotic culture in cheese. The addition of probiotic cultures was tested in several cheeses. These included Cheddar [18, 19]; Gouda [20] and soft cheeses [21-27]. However, several studies in which commercial or noncommercial *Lactobacillus* adjuncts were used have been published Fox, [28, 29] in which, low numbers of selected mesophilic lactobacilli were added to the cheese milk. There is general agreement that the *lactobacilli* modify proteolysis; in particular, they result in a higher concentration of free amino acids and improve the cheese sensoric quality.

In view of the foregoing data, the present investigation concerned with evaluating the proteolytic activity of some lactobacilli in order to select some of them to be used in a further study as adjunct culture for improving soft cheese ripening.

MATERIALS AND METHODS

Strains: Eight strains of lactobacilli were obtained from the Food Sci. Dept., Faculty of Agriculture, Ain Shams University.

These Strains Were: *Lactobacillus casei* NRRL B-1922, *Lactobacillus casei* NRRL B441, *Lactobacillus rhamnosus* NRRL B-445., *Lactobacillus helveticus* CNRZ32, *Lactobacillus plantarum* NRRL B4004, *Lactobacillus reutri* NRRL B-14171, *Lactobacillus acidophilus* CNRZ 593N and *Lactobacillus delbrueckii* ssp. *bulgaricus* CNRZ397.

Propagation of Starter Cultures: Cultures were grown and maintained in autoclaved 115°C/10min reconstituted skim milk (12% total solids)fortified with 0.5% yeast extract and 0.1% calcium carbonate and incubated at 37°C for 16 hrs. Between transfers the culture was stored at 5°C.Before use, each culture was regularly examined for purity.

Screening Growth of Starter Cultures: Hundred ml of the MRS broth medium were placed in 250 ml Erlenmeyer flasks and autoclaved at 121°C /15 min. The sterile media were inoculated with 2% (v/v) of the activated cultures and thus incubated at 37°C for 0, 12, 24, 36 and 48 hrs. LAB count, cultures pH, protein content and proteolytic activity were determined at intervals for 12 hrs. At the end of incubation period, the media were centrifuged at (8000 × g at 4°C for 20 min). The resultant clear supernatant was assayed for protease activity.

Lactic Acid Bacterial Count: Lactic acid bacterial count was enumerated on MRS Agar medium and the plates were incubated at 37°C for 48 hrs, according to Dave *et al.* [30].

pH Value: The pH value of media was measured using Hanna Instruments pH meter type 170300, with combined glass electrode (Electric Instruments Limited). Values of pH were reported to nearest 0.01 units. Values of pH of lactobacilli grown in MRS broth at 37°C were measured at zero time and 12, 24, 36 and 48 hrs.

Determination of Protein Content: Protein content was determined colorimetrically at 595_{nm} using Coomassie brilliant blue G-250 (CBB) and bovine serum albumin (BSA), according to Bradford [31].

Proteolytic Activity Determination: Protease activity of culture supernatant was determined by the method of Chopra *et al.* [32]. One ml of the substrate (1% casein in 0.05 M phosphate buffer, pH 7.0) was incubated at 37°C for 15 min, then 1.0 ml of the culture supernatant which was obtained by centrifugation (8000 ×g at 4°C for 20 min) was added. After mixing, the reaction mixture was incubated at 37°C for 20 min. The reaction was terminated by adding 2.0 ml of 0.4 M / trichloroacetic acid (TCA) then filtrated and the mixture was further incubated at the same temperature for 20 min. For the blank, the substrate was precipitated with TCA before adding the enzyme solution and then treated as described above. To 1 ml of the filtrate obtained after TCA precipitation, 5.0 ml of 0.4 M sodium carbonate solution was added followed by 1.0 ml of folins reagent and incubated at 37°C for 20 min for color development and reading absorbance (A) at750_{nm}. A unit of protease activity is defined as the amount of enzyme required to release TCA - soluble fragment giving a blue color equivalent to one µg of tyrosine under the same condition of the assy.

Protease Specific Activity: Protease specific activity was calculated from dividing the determined protease activity values on the protein content results.

RESULTS AND DISCUSSION

Table 1 indicates the changes in pH of tested some lactobacilli strains grown in MRS broth at 37°C at zero time and 12, 24, 36 and 48 hrs incubation.Generally the pH decreased in all strains during incubation and this decrease was sharply during the first 24 hrs then

Table 1: Changes in pH value of some lactobacilli strains grown in MRS broth at 37°C during incubation period

	Incubation Time (hours)				
Bacterial strains	0	12	24	36	48
1 *Lactobacillus casei* NRRL B-1922	5.76	4.24	3.87	3.92	3.68
2 *Lactobacillus casei* NRRL B441	6.02	4.60	3.90	3.92	3.87
3 *Lactobacillus rhamnosus* NRRL B-445	5.74	4.30	3.88	3.97	3.86
4 *Lactobacillus helveticus* CNRZ 32	5.97	4.34	3.89	4.04	3.88
5 *Lactobacillus plantarum* NRRL B4004	5.81	4.28	3.83	3.90	3.80
6 *Lactobacillus reutri* NRRL B-14171	5.93	4.32	3.90	3.92	3.90
7 *Lactobacillus acidophilus* CNRZ 593N	5.85	4.42	3.90	3.96	3.88
8 *Lactobacillus delbrueckii* ssp. *bulgaricus* CNRZ397	5.97	4.31	3.91	3.87	3.94

Table 2: Changes in total count (log cfu / ml) of some lactobacilli strains grown in MRS broth at 37 °C during incubation period

	Incubation Time (hours)				
Bacterial strains	0	12	24	36	48
1 *Lactobacillus casei* NRRL B-1922	7.477	8.949	9.176	8.477	8.740
2 *Lactobacillus casei* NRRL B441	8.079	9.250	9.344	8.961	8.602
3 *Lactobacillus rhamnosus* NRRL B-445	7.511	8.989	9.177	8.977	8.012
4 *Lactobacillus helveticus* CNRZ 32	7.785	8.607	8.816	8.908	8.788
5 *Lactobacillus plantarum* NRRL B4004	6.755	8.710	9.258	9.250	8.785
6 *Lactobacillus reutri* NRRL B-14171	6.759	7.832	8.602	8.982	8.792
7 *Lactobacillus acidophilus* CNRZ 593N	6.414	8.528	8.788	8.550	8.188
8 *Lactobacillus delbrueckii* ssp. *bulgaricus* CNRZ 397	7.365	8.690	8.771	8.845	8.596

Table 3: changes in protease activity (unit/ml) of some lactobacilli strains grown in MRS broth at 37°C during incubation period

	Incubation Time (hours)				
Bacterial strains	0	12	24	36	48
1 *Lactobacillus casei* NRRL B-1922	0.300	0.850	1.130	1.540	1.480
2 *Lactobacillus casei* NRRL B441	0.514	0.778	1.127	1.910	1.490
3 *Lactobacillus rhamnosus* NRRL B-445	1.147	1.420	2.060	3.110	2.620
4 *Lactobacillus helveticus* CNRZ 32	0.725	0.830	1.190	2.370	2.099
5 *Lactobacillus plantarum* NRRL B4004	0.880	1.134	1.370	2.740	2.420
6 *Lactobacillus reutri* NRRL B-14171	0.725	0.801	1.160	1.670	1.486
7 *Lactobacillus acidophilus* CNRZ 593N	0.547	0.830	1.110	1.960	1.430
8 *Lactobacillus delbrueckii* ssp. *bulgaricus* CNRZ 397	0.678	0.816	1.469	2.500	2.400

Table 4: changes in protease specific activity of some lactobacilli strains grown in MRS broth at 37°C during incubation period

	Incubation Time (hours)				
Bacterial strains	0	12	24	36	48
1 *Lactobacillus casei* NRRL B-1922	0.00210	0.00620	0.0090	0.0110	0.0100
2 *Lactobacillus casei* NRRL B441	0.00251	0.00516	0.00808	0.0176	0.0111
3 *Lactobacillus rhamnosus* NRRL B-445	0.00830	0.01300	0.01800	0.0260	0.0190
4 *Lactobacillus helveticus* CNRZ 32	0.00365	0.00532	0.00965	0.0198	0.0131
5 *Lactobacillus plantarum* NRRL B4004	0.00430	0.00590	0.00800	0.0210	0.0176
6 *Lactobacillus reutri* NRRL B-14171	0.00615	0.00515	0.00806	0.0130	0.0111
7 *Lactobacillus acidophilus* CNRZ 593N	0.00290	0.00510	0.00710	0.0139	0.0102
8 *Lactobacillus delbrueckii* ssp. *bulgaricus* CNRZ 397	0.00459	0.00578	0.01070	0.0196	0.0177

slightly decreased till 48 hrs. As regards for lactobacilli, *L. plantarum* showed high proteolytic activity at pH 3.90. These results agree with those published by de Giori *et al.* [33] and Brandsaeter *et al.* [34]. *Lactobacillus rhamnosus*, gave best results at pH 3.88. Marked variations among all the strains were at pH 3.87 and 4.04.

Table 2 shows the changes in total count of some lactobacilli strains grown in MRS broth at 37°C for 0, 12, 24, 36 and 48 hrs. *Lactobacillus casei* gave the highest cell counts followed by *L. plantarum* and *L. rhamnosus* and the lowest was *Lactobacillus acidophilus* on 24 hrs.

Table 3 shows changes in protease activity (unit/ml) of some lactobacilli strains grown in MRS broth during incubation at 37 °C up to 48 hrs to determine the proteolytic activity. The culture supernatants as test solutions were obtained by centrifugation (8000 ×g at 4°C for 20 min). *Lactobacillus rhamnosus* NRRL B-445 gave the highest protease activity, followed by *L. bulgaricus, L. helveticus and L. plantarum* in desending order. These results indicated that most of the strains were actively producing exocellular protease in the early stationary phase of cell growth.

It was mentioned that, the maximum protease activity (0.14 U/ ml) appeared at the beginning of stationary phase [35, 36]. The increase in protease activity seemed to be consistent with the decrease of pH value of the culture supernatant.

Tables 4 indicats that protease specific activity was increased with the increase of incubation period till 36 hrs incubation in all strains then the specific activity decreased. The highest activities were found between 24 to 36 hrs of incubation (stationary phase), then the specific activity decreased. This can be due to the beginning of the cell autolysis.

Den Hengst *et al.* [37] found that the proteolytic system of *L. lactis* is repressed in nitrogen rich medium and is relieved when cells encounter limiting amount of branched chain amino acids.

In conclusion, from the aforementioned results, *Lactobacillus rhamnosus, Lactobacillus helveticus, Lactobacillus plantarum* and *Lactobacillus delbrueckii* ssp. *bulgaricus* were chosen for further studies to test their effect on the quality of UF buffalo's milk soft cheese.

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