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EVALUATION OF SOME LACTIC ACID BACTERIA IN THE LIGHT OF ITS ROLE IN CHEESE RIPENING

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ABSTRACT

The present investigation is concerned with evaluating the proteolytic activity of some lactic acid bacteria. Nine lactic acid bacterial 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, *Lactobacillus delbrueckii ssp. bulgaricus* CNRZ397 *and Streptococcus thermophilus* ACA - DC0022. 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 probiotic culture for improving soft cheese ripening made from buffaloe's milk concentrated by ultrafiltration (UF).

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 flavor compounds, such as various alcohols, aldehydes, acids, esters, and sulfur compounds (Smit *et al.*

2005). The specificities of cell envelop protease play an essential role in the production of bitter peptides (Broadbent *et al.* 2002). 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 (Christensen and Steele 2003; Sridhar *et al.* 2005). 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 (Sridhar *et al.* 2005). 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 (Martínez- Cuesta *et al.* 1998; Meijer *et al.* 1998; Madkor *et al.* 2000). Enriching the *L. lactis* proteolytic 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. (Wegmann *et al.* 1999; Guldtfeldt *et al.* 2001; Luoma *et al.* 2001; Courtin *et al.* 2002; Henrich *et al.* 2002; Joutsjoki *et al.* 2002; Sridhar *et al.* 2005). However, while it can be concluded that balanced proteolysis is important for flavor 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 flavor formation from proteins (Smit *et al.* 2005). Thus, engineering the proteolytic system alone is hardly the key for accelerating flavor 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 flavor 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 (Buist *et al.* 1997; De Ruyter *et al.* 1997; Guldtfeldt *et al.* 2001; Tuler *et al.* 2002; Hickey *et al.* 2004).

In addition to good viability in the intestine, technological 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 (Dinakar and Mistry, 1994; McBrearty *et al.*, 2001) ; Gouda (Gomes *et al.*, 1998) and soft cheeses (Shehata *et al.*,2001 ; Sultan;1987; Gobbetti *et al.*, 1997; Menendez *et al.*,2000; Vinderola *et al.*, 2000; El- Abd *et al* ., 2003 ; El-Zayat and Osman, 2001). However, several studies in which commercial or noncommercial *Lactobacillus* adjuncts were used have been published (Fox *et al.*1996; Fox *et al.* 1998) 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, the present investigation is concerned with evaluating the proteolytic activity of some lactic acid bacteria in order to select some of them to be used in a further study as adjunct probiotic culture for improving soft cheese ripening made from buffaloes milk concentrated by ultrafiltration (UF).

MATERIALS AND METHODS

Strains:

Nine strains of lactic acid bacteria 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, *Lactobacillus delbrueckii* ssp. *bulgaricus* CNRZ397 *and Streptococcus thermophilus* ACA-DC0022.

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 for 20 min. The resultant clear supernatant was used for testing protease activity. .

Lactic acid bacteria count:

Lactic acid bacteria count was enumerated on MRS Agar medium and the plates were incubated at 37°C for 48 hrs, according to Dave and Shah (1996).

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 LAB 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 (1976).

Proteolytic activity determination:

Protease activity of culture supernatant was determined by the method of Chopra and Mathur (1983). 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 lactic acid bacterial 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 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.*, 1985; Brandsaeter, and Nelson, 1956). *Lactobacillus rhamnosus*, gave best results at pH 3.88. Marked variations among all the strains were at pH 3.87 and 4.04.

Table (1): pH value of some lactic acid bacterial strains grown in MRS broth at 37 °C. during incubation period.

	Bacterial strains	Incubation Time (hours)				
		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
9	*Streptococcus thermophilus* ACA-DC0022	5.98	4.59	3.60	3.98	3.91

Table 2 shows the changes in total count of some lactic acid bacterial 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 (2): Total count (log cfu / ml) of some lactic acid bacterial strains grown in MRS broth at 37 °C. during incubation period.

	Bacterial strains	Incubation Time (hours)				
		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
9	*Streptococcus thermophilus*ACA-DC0022	7.897	8.591	8.851	8.623	8.612

Table 3 shows changes in protease activity (unit/ml) of some lactic acid bacterial 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 indicate that most of the strains were actively producing exocellular protease in the early stationary phase of cell growth.

Kawai *et al.* (1999) and Wang *et al.* (2007) mentioned that, the maximum protease activity (0.14 U/ ml) appeared at the beginning of stationary phase. The increase in protease activity seemed to be consistent with the decrease of pH value of the culture supernatant.

Table (3): Protease activity (unit/ml) of some lactic acid bacterial strains grown in MRS broth at 37 °C. during incubation period.

	Bacterial strains	Incubation Time (hours)				
		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
9	*Streptococcus thermophilus*ACA-DC0022	0.705	0.752	0.983	1.687	1.650

Tables (4) indicate 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 Hegest *et al* (2005) 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. Therefore, 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.

Table (4): Protease specific activity of some lactic acid bacterial strains grown in MRS broth at 37 °C. during incubation period.

Bacterial strains	Incubation Time (hours)				
	0	12	24	36	48
1 *Lactobacillus casei* NRRL B-1922	0.00210	0.00620	0.009	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
9 *Streptococcus thermophilus* ACA-DC0022	0.00376	0.00501	0.00630	0.0091	0.0116

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تقييم بَعْض بكتريا حمض اللاكتيك فى ضوء دورها فى تسوية الجبن

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تهدف هذه الدراسة إلى التعرف على بعض سلالات بكتريا حمض اللاكتيك من حيث إنتاجها لإنزيم البروتييز والذى يلعب دورا هاما فى تحسين جودة الجبن الناتج عند إضافته. وقد تضمنت هذه الدراسة تسع سلالات من بكتريا حمض اللاكتيك وهى:

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, *Lactobacillus delbrueckii* ssp. *bulgaricus* CNRZ397 and *Streptococcus thermophilus* ACA - DC0022.

حيث تم تنميتها على بيئة MRS السائلة والتحضين على 37 م° لمدة 48 ساعة وقد تم أخذ عينات من المزارع المنماه وذلك خلال الفترات الزمنية الآتية: 0 , 12 , 24 , 36 , 48 ساعة من التحضين. وتم إختبارها لتقدير قيم الـ pH وأعداد البكتريا وأعطت هذه السلالات عموما إنخفاض فى قيم الـpH value مع مرور الوقت وزيادة فى أعداد بكتريا حمض اللاكتيك وكانت *Lactobacillus casei* NRRL B441 أعلى السلالات عددا حيث أعطت (9.344 log cfu/ml) عند التحضين على 37 م° لمدة 24 ساعة.

ثم تم تقدير نشاط إنزيم البروتييز فى الراشح الرائق المتحصل عليه بعد الطرد المركزى للبيئة المنماة عليها السلالات على نفس درجة الحرارة وأوقات التحضين السابقة. ولقد لوحظ أن أعلى السلالات إنتاجا لإنزيم البروتييز بعد التحضين على37 م°. ولمدة 36 ساعة هى:

Lactobacillus rhamnosus NRRL B-445, *Lactobacillus plantarum* NRRL B4004, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus helveticus* CNRZ32.

وكانت قيم نشاط الإنزيم الناتجة تنازليا كالتالى: 3.110 ثم2.740 ثم 2.500 ثم 2.370 (Unit /ml) على الترتيب. كما أظهرت الأربع سلالات أعلى نشاط نوعى لإنزيم البروتييز مقارنة بباقى السلالات. وعلى العكس أعطت السلالاتين التاليتين أقل قيم لنشاط إنزيم البروتييز:

Lactobacillus casei NRRL B-1922, *Lactobacillus reutri* NRRL B-14171

وعلى ذلك تقرر إختيار السلالات الأربعة الأعلى انتاجا لإنزيم البروتييز لإستخدامها فى تحسين جودة الجبن الطرى الناتج من اللبن الجاموسى المركز بالترشيح الفوقى فى دراسة لاحقه.