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AN ADAPTATION STRATEGY TO IMPROVE THE PROTEOLYTIC ACTIVITIES OF LACTIC ACID BACTERIA ISOLATED FROM PICKLES

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Abstract

This study aimed to increase the proteolytic activity of 10 lactic acid bacteria (LAB) species isolated from pickles, which had low activity. For this purpose, a nine-step adaptation through the gradual addition of MRS Broth with peptone and a subsequent four-step adaptation through the gradual addition of MRS Broth with skim milk were applied. A slight improvement of the proteolytic activity following the gradual addition of MRS Broth with peptone was observed only in six LAB samples, of which *Pediococcus ethanoliduras* 513 showed the highest activity at the ninth step. At the 13th step corresponding to the last step of the skim milk adaptation, only *L. buchneri* 114, *L. brevis* 494, and *L. plantarum* 380 showed slightly higher activity compared to that at the ninth step. In conclusion, peptone and skim milk adaptations did not yield any industrially significant increase in the proteolytic activity of the 10 LAB strains isolated from pickles. An important increase was only obtained in the proteolytic activity of *L. plantarum* 380. Its initial activity of 0.095 mM pNA increased to 0.182 mM pNA and finally to 0.250 pNA at the ninth and 13th steps, respectively, but this increase is too small for industrial applications.

Keywords: LAB, proteolytic activity, adaptation, pickles

BİTKİ KAYNAKLI LAKTİK ASİT BAKTERİLERİNİN PROTEOLİTİK AKTİVİTELERİNİN GELİŞTİRİLMESİ İÇİN ADAPTASYON ÇALIŞMASI

Öz

Bu çalışmada turşulardan izole edilen bitki kaynaklı ve düşük proteaz aktivitesine sahip 10 adet laktik asit bakterisinin proteolitik aktivitesinin artırılması hedeflenmiştir. Bu amaçla, artan oranda pepton ilave edilmiş MRS Broth besiyerinde 9 aşamalı adaptasyon, ardından süt tozu ilaveli MRS Broth besiyerinde 4 aşamalı adaptasyon gerçekleştirilmiştir. Pepton adaptasyonunun 9. aşamasından sonra, yalnızca 6 adet LAB örneği başlangıca kıyasla bir miktar yüksek aktivite değeri göstermiş; bu aşamada en yüksek aktiviteyi *Pediococcus ethanoliduras* 513 göstermiştir. 13. aşamada (süt tozu adaptasyonu), yalnızca *Lactobacillus buchneri* 114, *L. brevis* 494 ve *L. plantarum* 380 örneklerinin aktivite değerleri 9. aşamaya göre bir miktar artmıştır. Sonuç olarak; pepton ve yağsız süt tozuna adaptasyon çalışmaları, deneme kapsamındaki bitki kaynaklı 10 adet LAB suşuna belirgin bir proteolitik akvite artışı kazandırmamış, 13 aşamalı adaptasyon çalışması boyunca yalnızca *L. plantarum* 380 örneğinin proteolitik aktivite değerinde, bir miktar artış olduğu saptanmıştır. Başlangıç 0.095 mM pNA olan aktivite, 9. aşamada 0.182 mM pNA ve 13. aşamada 0.250 mM pNA değerine erişmiştir. Proteolitik aktivitelerdeki artışlar, endüstriyel uygulamalar için çok yetersizdir.

Anahtar kelimeler: LAB, proteolitik aktivite, adaptasyon, turşu

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INTRODUCTION

Lactic acid bacteria (LAB) grow naturally in milk, dairy products, raw plants, and intestinal mucosa of animals and humans (Carr et al., 2002; Karasu, 2006). LAB, which have been used as a starter culture in different fermented food processes, gives aroma and increase acidity during the ripening of the plant, which is linked to the proteolytic enzyme system of LAB. Especially in the cheese process, starter cultures possessing high proteolytic activity have received significant attention since the end of the 20th century (Law and Haandrikman, 1997). Although there are significant differences in the proteolytic activities of LAB species, several have the proteolytic system that makes it possible for them to grow in protein rich substrates, such as milk and meat. These foods include protein and its derivatives; however, their free amino acid concentration is not sufficient for LAB growth. Due to the proteolytic enzyme system, high molecular weight peptides are degraded into short peptides and amino acids, which are essential for the growth of microorganisms. This is one of the basic metabolic activities of LAB (Hansen et al., 2001; Lahtinen et al., 2012).

Since studies on the LAB proteolytic system have mostly been conducted using *Lactococci* genus, the proteolytic system of the *Lactococci* has been detailed (Law, 1997; Liu et al., 2010). The proteolytic system basically consists of extracellular proteinases (PrtP), oligopeptide, di-tripeptide transport system, and some cytoplasmic peptidases (Moulay et al., 2006). The proteolytic pathway initializes with the casein cleavage during the growth of lactic acid bacteria in milk. In the first stage, the cell envelope proteinase (CEP or PrtP) converts casein to oligopeptides. In the second stage, the oligopeptides and di-peptides are carried into the cell by specific oligopeptide (OppT) and di-tripeptide transport systems (DtpT) (Doeven et al., 2005; Kunji et al., 1996). This transport process is completed by the further degradation of oligopeptides and di-peptides to amino acids by cytoplasmic peptidases. After the three aforementioned stages, amino acids are catabolized by the following reactions: deamination, decarboxylation, transamination, and outer chain modification (Kieronczyk et al., 2001).

It is generally known that when a microorganism has been exposed to stress, it can have a response allowing it to survive in different environments. This is defined as an adaptive response, induced tolerance or adaptation (Exterkate, 1985). This mechanism works through the synthesis of specific enzymes as a response to the stimulus of different environments (Murray et al., 2004; Tunail, 2009). In addition, previous studies have shown that a medium can regulate proteinase activity (Exterkate, 1979; Hugenholtz et al., 1984; Laan et al., 1993; Marugg et al., 1995; Meijer et al., 1996; Smid and Konings, 1990).

Daeschel et al. (1987) indicated that LAB of a vegetable origin have limited or no proteolytic activity and their amino acid degradation ability is lower compared to other microorganisms. This study aimed to investigate the positive effect of protein derivative addition on the proteolytic activity and growth of LAB isolated from pickles.

MATERIAL AND METHOD

Microorganism

Ten LAB species from Ankara University Food Engineering Department Culture Collection were used. These species were isolated from pickles selected from the Ankara Çubuk region, which have low proteolytic activity (Tokatlı, 2013). The name of the LAB species and their culture collection numbers are given in Table 1. MRS Broth (Merck) was used for the activation of cultures that were stored at -65 °C in Ankara University Food Engineering Department Culture Collection. The cultures were incubated at 30 °C for 24-48 h.

Table 1. Lactic acid bacteria species used in experiment

Species	Ank. Univ. Food Eng. Dept. Culture Collection No
Lactobacillus namurensis	192
Pediococcus parvulus	233
Lactobacillus buchneri	114
Pediococcus ethanolidurans	513
Lactobacillus brevis	494
Pediococcus ethanolidurans	229
Lactobacillus plantarum	178
Lactobacillus plantarum	4
Lactobacillus plantarum	239
Lactobacillus plantarum	380

Adaptation Plan and Medium Compositions

After the culture activation, two stages of adaptation comprising a total of 13 steps were conducted to adapt the microorganisms to the protein and its derivate. The peptone adaptation stage consisted of nine steps and there were four steps in the skim milk adaptation stage. The details are given in Table 2. Due to the formation of small peptides from peptone, the peptone addition was used as a transitional period prior to the skim milk adaptation and planned to take longer than skim milk adaptation stage. We conducted all the experiments in duplicate.

For the nine-step peptone adaptation stage, the peptone solution was prepared at a concentration of 50 g/L. For the four-step skim milk adaptation stage, a skim milk powder (Sigma) solution was prepared at 10% (w/v). These solutions were gradually added to the MRS Broth (Merck) according to the adaptation plan (10% addition rate). The compositions of the media are shown in Table 2. The inoculation rate was 5% at each step and the LAB were grown at 30 °C for 48-72 h.

Determination of Activation and Growth

The growth of the 10 LAB species were monitored using a UV-Vis spectrophotometer (Shimadzu, UV-1208) at 600 nm in both the activation and peptone adaptation stages. The pH values of the media were determined at the initial, ninth, and 13th steps with a benchtop pH meter (Mettler Toledo Seven Easy pH, China).

Proteolytic Activity Analysis

The chromogenic proteolytic method was used for the analysis of proteolytic activity. The principle of the chromogenic method is based on the degradation of chromogenic peptide to p-nitroanilide (p-NA) by LAB proteinase and the measurement of p-nitroanilide at 410 nm in a UV-Vis spectrophotometer (Savijoki et al., 2006). In this method, the LAB culture incubated in 10 mL MRS-Ca medium (10 mM CaCl2) at 30°C for 24 hours was centrifuged at 6000 rpm and 4°C for 10 min (Micro 22 Hettich, Germany). The bacterial precipitate was washed twice with a 10 mM $CaCl_2$-NaCl solution. Then, 5 mL Tris buffer (50 mM, pH 7.8) was added to the cells which were resuspended using a vortex to form an enzyme solution. The 200 µL enzyme solution was transferred to an eppendorf tube, to which 287.5 µL of phosphate buffer (0.2 M, pH 7.0), 225 µL of 5 M NaCl and 20 µL of S-Ala (N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide, 20 mM Sigma) were added. This solution was incubated at 30°C for 30 min in a water bath. To stop the reaction, 175 µL 80% (v/v) acetic acid was added, and then the reaction medium was centrifuged at 15000 rpm for 5 min. The optical density (OD) was measured at 410 nm and the concentration of the released pNA was calculated as mM pNA (Berdal et al., 1983; Savoy and Hebert, 2001).

Table 2. Medium compositions used in the adaptation plan (mL/10 mL)

Stage	Step	Medium Composition (mL/10 mL)		
		MRS broth	Peptone Solution (%5 w/v)	Skim Milk Solution (%10 w/v)
Initial	0	10.00	-	-
Peptone	1	9.00	1.00	-
Adaptation	2	8.00	2.00	-
Stage	3	7.00	3.00	-
	4	6.00	4.00	-
	5	5.00	5.00	-
	6	4.00	6.00	-
	7	3.00	7.00	-
	8	2.00	8.00	-
	9	1.00	9.00	-
Skim Milk	10	0.84	6.66	2.50
Adaptation	11	0.56	4.44	5.00
Stage	10	0.84	6.66	2.50
	12	0.28	2.22	7.50
	13	-	-	10.00

Statistical Analyses

The experimental data were analyzed using the Minitab 16 and the statistical differences among means were determined with Tukey's multiple range tests at the 5 % significance level.

RESULTS AND DISCUSSION

The Effect of Adaptation on LAB Growth

The ODs of LAB at the initial and ninth steps are shown in Tables 3 and 4. The OD values of LAB could not be measured at the 13th step because of skim milk concentration; therefore, the growth of microorganisms was observed based on the coagulation and pH of the media. While the OD of seven species increased from the 1st to 2nd step, the OD of three *Lactobacillus plantarum* species (4, 178 and 239) decreased. After the 2nd step, the OD values of all the samples gradually decreased.

To observe the effect of peptone addition to the medium, we compared the OD of LAB species at the initial step and the 9th step (Table 3). It was found that the OD values of all the species had decreased. *Lb. plantarum* 380 showed the highest decreasing rate with 44% and *Lb. buchneri* 114 showed the lowest rate of decrease with 18%. This decrease can be attributed to the decreasing nitrogen and carbon ratio in the medium.

The Effect of Adaptation on pH

Figure 1 shows the pH values of the growth medium at the initial, ninth and 13th steps after 48 h of incubation. When these three steps are compared, eight species had lower pH values at the initial step than they had in the next two steps. This shows that the growth at the initial step was better than those at the other steps. *Lb. buchneri* 114 had the highest reducing pH level by 2.39 units at the ninth step. Furthermore, at this step, all species except *Lb. brevis* 494 reduced the pH at the same level. Interestingly, *Lb. brevis* 494 reduced the pH at both the ninth and 13th steps more than at the initial step. The *Pediococcus ethanolidurans* species (229 and 513) and *Lb. plantarum* species (4, 178, 239 and 380) showed similar reductions of the pH levels.

Table 3. Optical density (600 nm) values after 48 h incubation during the peptone adaptation stage (nine-step)

Species	Steps								
	1	2	3	4	5	6	7	8	9
Lb. namurensis 192	1.327	2.078	1.984	1.902	1.783	1.654	1.576	1.473	1.184
P. parvulus 233	1.622	2.236	2.260	2.205	2.142	2.054	1.940	1.719	1.516
Lb. buchneri 114	1.671	1.716	1.882	1.931	1.967	1.902	1.986	2.097	1.589
P. ethanolidurans 513	1.709	2.280	2.301	2.270	2.157	2.060	1.927	1.719	1.416
Lb. brevis 494	1.886	2.157	2.051	2.027	1.936	1.869	1.836	1.701	1.492
P. ethanolidurans 229	1.499	2.075	1.995	1.945	1.848	1.722	1.715	1.577	1.430
Lb. plantarum 178	2.285	2.382	2.290	2.250	2.205	2.060	1.878	1.640	1.328
Lb. plantarum 4	2.295	2.275	2.250	2.197	2.201	2.069	1.900	1.668	1.494
Lb. plantarum 239	2.382	2.302	2.270	2.218	2.223	2.094	1.933	1.694	1.465
Lb. plantarum 380	2.142	2.223	2.205	2.146	2.131	1.995	1.821	1.561	1.561

Table 4. Optical density values at the initial and ninth step after 48 h incubation (600 nm)

Species	Step 0 (initial)	Step 9
Lb. namurensis 192	1.998	1.184
P. parvulus 233	2.193	1.516
Lb. buchneri 114	1.815	1.589
P. ethanolidurans 513	2.205	1.416
Lb. brevis 494	2.145	1.492
P. ethanolidurans 229	2.145	1.430
Lb. plantarum 178	2.456	1.328
Lb. plantarum 4	2.232	1.494
Lb. plantarum 239	2.456	1.465
Lb. plantarum 380	2.395	1.348

LAB are known to decrease the pH values of media in parallel with the growth of the bacteria. The OD values decreased gradually with the addition of peptone and skim milk through nine steps. Considering both the OD results and pH values at these three steps, the growth of bacteria was affected negatively by the addition of the protein derivative to media.

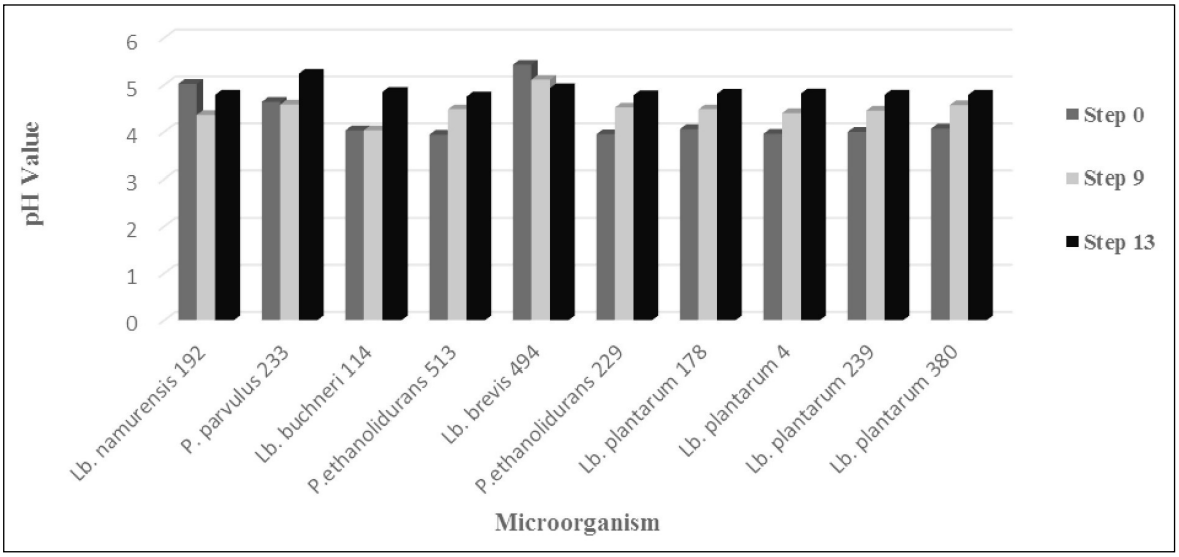

Figure 1. The pH values of species at the initial, ninth and 13th steps after 48 h incubation

The Effect of Adaptation on Proteolytic Activity of LAB

Table 5 shows the proteolytic activities (mM pNA) of LAB after 48-72 h incubation in the MRS-Ca medium. While six samples showed a higher proteolytic activity at the ninth step than at the initial step, four samples (*Pediococcus parvulus* 233, *Lb. buchneri* 114, *Lb. brevis* 494 and *Lb. plantarum* 380) showed lower activity. The highest proteolytic activity was measured as 0.413 mM pNA for *P. ethanolidurans* 513 and the lowest was 0.086 mM pNA for *Lb. buchneri* 114 at the ninth step. At the 13th step, only three species (*Lb. buchneri* 114, *Lb. brevis* 494 and *Lb. plantarum* 380) had higher proteolytic activities compared to their activities at the initial step. The highest proteolytic activity was 0.250 mM pNA for *Lb. plantarum* 380. No pNA was detected for *P. ethanolidurans* 229 at this step. During the adaptation stages, the enhancement in the proteolytic activity was only observed for *Lb. plantarum* 380 throughout the adaptation plan. Its activity was found as 0.095, 0.182 and 0.250 mM pNA at the initial, ninth and 13th steps, respectively.

Table 5. Proteolytic activities of LAB at the ninth and 13th steps of adaptation (mM pNA)

Species	Step 0 (initial)	Step 9*	Step 13*
Lb. namurensis 192	0.204	0.227	0.009
P. parvulus 233	0.286	0.240	0.204
Lb. buchneri 114	0.454	0.086	0.182
P. ethanolidurans 513	0.286	0.413	0.227
Lb. brevis 494	0.159	0.141	0.182
P. ethanolidurans 229	0.182	0.390	CD**
Lb. plantarum 178	0.209	0.286	0.182
Lb. plantarum 4	0.354	0.318	0.045
Lb. plantarum 239	0.032	0.182	0.060
Lb. plantarum 380	0.095	0.182	0.250

* A statistically significant difference was found between LAB species (P <0.05)
** CD: could not be detected

Daeschel et al. (1987) indicated that LAB of vegetable origin possess limited or no proteolytic activity and their amino acid degradation ability is lower compared to other microorganisms. Karasu (2006) found that *Lb. plantarum* species isolated from pickles and olives had low proteolytic activity (0.056-0.083 mg tyrosine/mL). Similarly, Kıvanç et al. (2011) showed similar results for *Lb. plantarum* (0.04-2.57 mg tyrosine/mL) isolated from boza (a traditional fermented Turkish beverage produced with grain). Furthermore, Turhan and Öner (2014) showed that *Lactobacillus* and *Lactococcus* genus species isolated from white cheese had high activity. They classified proteolytic activities as follows; 20 mg tyrosine/mL as high, 10-20 mg tyrosine/mL as medium and 10 mg tyrosine/mL as low. In this regard, these results imply that LAB isolated from dairy products have considerably higher proteolytic activity than those isolated from plants.

Hebert et al. (2000) conducted similar research on *Lactobacillus helveticus* and found that the biosynthesis of cell envelope proteinase (CEP) decreased in peptides and amino acids in an enriched medium (MRS Broth, Casitone, Casamino acid, etc.). Similarly, the CEP activity of *Lactobacillus rhamnosus* was affected by peptide and amino acid concentrations in the medium. In other respects, Sadat-Mekmene et al. (2011) found that *Lb. helveticus* achieved 9-12 times more CEP activity in milk than in the MRS Broth. They emphasized that the peptide-enriched medium repressed the CEP enzyme on the transcription level. Based on the information in the literature, it is clear that the proteolytic system of LAB is affected by the addition of peptides, amino acids, and other protein derivatives. Furthermore, the enzymes of proteolytic systems can be modulated by the medium content.

Tamang et al. (2009) investigated the functional properties of LAB isolated from local fermented vegetables in the Himalayas. The bacteria samples of plant origin were easily adapted to the milk ecology and produced acidity and coagulation. Although these species did not demonstrate any proteinase activity, they resulted in milk coagulation due to their peptidase activity.

In our experiments, we expected to obtain higher activity in both the peptone-added MRS Broth and the skim milk-added MRS Broth as observed by Sadat-Mekmene et al. (2011). Eight of the ten LAB samples following the 13th step adaptation plan showed lower proteolytic activity than at the initial step. For one sample, no activity could be detected and only *Lb. plantarum* 380 had increased proteolytic activity. As in the study by Tamang et al. (2009), coagulation was observed in the skim milk adaptation stage. A possible explanation for this result is the lack of the proteinase enzyme that caused the low proteolytic activity.

CONCLUSIONS

The OD of the 10 LAB species isolated from pickles decreased during growth in peptone-added MRS Broth medium. This decrease shows that peptone added medium is not suitable for growth and the reduced carbon source rate of the medium affected growth of LAB negatively.

A comparison of the proteolytic activity of LAB at the initial and ninth steps showed that the activities of six samples increased slightly. However, the activities of five samples decreased at the 13th step. This result was linked to the presence of small peptides in the peptone, which could be transported to the cell and used by it. Furthermore, casein, a complex molecule unlike peptone, could not be degraded by the cell.

In conclusion, only *Lb. plantarum* 380 was able to increase the proteolytic activity during the adaptation applications of both the peptone-added and skim milk-added broths. The proteolytic activity of *Lb. plantarum* 380 was 0.095, 0.182 and 0.250 mM pNA at the initial, ninth and 13th steps, respectively. This finding indicates that *Lb. plantarum* 380 has a higher activity capacity to adaptation protein-enriched medium than the other LAB species. Currently, this adaptation- increased proteolytic activity is not sufficient for industrial applications; however, to confirm our result, we suggest that further research should be conducted using *Lb. plantarum* 380 but with a longer adaptation period applying different additives.

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