

## Proteolytic Activity of Strain *Enterococcus Faecalis* A71 during Growth in Milk

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The aim of this research was to study the proteolytic activity of strain *Enterococcus faecalis* A71 isolated from traditional homemade cheese of Azerbaijan. The ability of isolated strain to hydrolyze milk proteins was tested after induction of the proteinase production in milk. Investigated strain was able to hydrolyze  $\alpha_{s1}$ -,  $\alpha_{s2}$ -,  $\beta$ -caseins, and BLG fractions of milk. Proteolysis was observed after 3 h cultivation in milk and increased with the time of incubation. After 24 h incubation in milk strain *Enterococcus faecalis* A71 hydrolyzed 96% of  $\beta$ -, 71% of  $\alpha_{s1}$ -, 74% of  $\alpha_{s2}$ -caseins and 64% of BLG fraction of milk. Growth and acidifying activity of strain was also determined. Growth determination was determined by calculation of CFU/ml. *E. faecalis* A71 was able to coagulate milk after 6 h cultivation and showed good ability of growth in milk. Due to the high proteolytic activity *E. faecalis* A71 could represent new adjunct cultures for the dairy industry.

**Key words:** lactic acid bacteria, proteolytic system, milk proteins, proteolysis

### INTRODUCTION

One of the most important features of lactic acid bacteria, considering their ability to grow in milk and other protein containing media, is their proteolytic system. It has been well established that many lactic acid bacteria, isolated from milk products, are multiple amino acid auxotrophs. The requirement for amino acids is strain dependent and can vary from 4 up to 14 different amino acids. In milk, the amounts of free amino acids and peptides are very low. Lactic acid bacteria, therefore, depend for growth in milk on a proteolytic system that allows degradation of milk proteins (Bjurlin et al., 2002). Caseins constitute about 80% of all proteins present in bovine milk. The four different types of caseins found in milk are  $\alpha_{s1}$ -,  $\alpha_{s2}$ -,  $\beta$ - and  $\kappa$ -caseins. Caseins contain all amino acids necessary for growth of lactic acid bacteria in milk to high cell density. The degradation of caseins plays a crucial role in the development of texture and flavour. Certain peptides contribute to the formation of flavour, whereas others, undesirable bitter-tasting peptides, can lead to off-flavour (Fira et al., 2001; Kunji et al., 1996). Detailed understanding of these processes may lead to engineered lactic acid bacteria with improved proteolytic properties.

The structural components of the proteolytic systems of lactic acid bacteria can be divided into three groups on the basis of their function: proteinases that breakdown casein to peptides, peptidases that degrade peptides, and the transport systems that translocate the breakdown products across the cytoplasmic membrane (Bjurlin et al., 2002; Kunji et al., 1996). The proteinase is clearly involved in the initial degradation of caseins, yielding a large number

of different oligopeptides. The initial analyses of the casein breakdown products liberated by the proteinases have indicated that, with a few exceptions, only large peptides are formed (Exterkate et al., 1993; Kunji et al., 1996). Consequently, further breakdown by extra cellular peptidases was considered to be critical to fulfil the needs for essential and growth-stimulating amino acids.

Biochemical and genetic aspects of the lactococcal proteolytic system have been extensively studied. Two types of proteinase (PI and PIII type) have been identified among lactococci on the basis of their specificity towards caseins. PI-type proteinases hydrolyse  $\beta$ -casein but not  $\alpha_{s1}$ -casein. In contrast, PIII type proteinases cleave both  $\beta$ - and  $\alpha_{s1}$ -caseins (Fira et al., 2001). The majority of lactococci do not synthesize strictly extra cellular secreted proteinases. Instead, they produce cell envelope-associated serine proteinases, which initiate casein degradation.

Very little is known about the proteinases of lactic acid bacteria from the natural environment, since the main objects of research are the strains routinely used in industrial processes. Therefore, study of proteolytic systems of lactic acid bacteria from the traditionally produced homemade fermented products would be very interesting because such LAB could be potentially a source of proteinases with different caseinolytic properties of commercial value. Genes encoding these proteinases could be used for construction of new starter cultures for the dairy industry by means of genetic engineering.

In this work presented a study of proteolytic activity of *Enterococcus faecalis* A71 strain isolated from traditional homemade cheese of Azerbaijan.

## MATERIALS AND METHODS

### Bacterial strains and cultivation conditions.

*Enterococcus faecalis* strain used in this study was isolated from traditional cheese obtained from individual household in Qazah region of Azerbaijan. Strain was reconstituted in sterile skim milk (12.5%, w/v) supplemented with 30% (w/v) glycerol and stored at  $-80^{\circ}\text{C}$ . Before using strain was propagated twice in M17 media.

**Proteolytic activity during growth in UHT skimmed milk.** To analyze the proteolytic activity in milk, overnight culture of studied strain was inoculated (5%, v/v) in UHT Skim milk (Délisse, France) and incubated at  $37^{\circ}\text{C}$  (El-Ghaish et al., 2010). Control was prepared by inoculation of equivalent volume of media (M17 or MRS) in UHT skim milk. At different time intervals, samples were taken and mixed with solubilization buffer for electrophoresis (50 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 3% 2-mercaptoethanol, 0.07% bromophenol blue) at a 1:10 volume ratio. Samples were heated at  $100^{\circ}\text{C}$  for 3 min and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Acidification ability was followed by measuring pH decrease after 3, 6, 9, 12 and 24 h incubation.

**Determination of growth and pH decrease in UHT skimmed milk.** Overnight culture of strain was inoculated (5%, v/v) in UHT Skim milk (Délisse, France) and incubated at  $37^{\circ}\text{C}$ . After shaking with vortex, the mixture of milk with inoculated culture was divided into appropriate aliquots to be analyzed each hour during 24h of incubation. pH decrease was measured by pH-meter. Growth determination was determined by calculation of CFU/ml. For this, decimal serial dilutions of the samples taken each hour were prepared in a sterile 0.85% (w/v) sodium chloride. One milliliter from  $10^{-6}$  and  $10^{-8}$  dilution was plated on M 17 agar (1.5%, w/v) and inoculated plates were incubated at  $37^{\circ}\text{C}$  for 48 h. At the end of incubation period the number of colonies was counted by colony counter and the results were expressed as colony-forming units (CFU) per milliliter. All analyses were performed in duplicate.

**SDS-PAGE.** Gels were run on vertical slab electrophoresis cells (BIORAD Mini PROTEAN 3 System, Hercules, CA, USA). Analysis of caseins hydrolysis was carried out on SDS-PAGE by loading 12% polyacrylamide gel with prepared samples (Laemmli, 1970). The migration buffer contained 50 mM Tris, 0.384 M glycine and 0.1% SDS. After running at 10mA on the stacking gel and 20 mA on the running gel, proteins and peptides were observed by staining gels with Coomassie Brilliant Blue R-250 (Sigma-Aldrich)

followed by a convenient destaining in a solution made of ethanol (30% v/v) and acetic acid (5% v/v) in distilled water.

The gels were scanned with Image scanner III (GE Healthcare, USA). The degradation of proteins and the percentages of hydrolysis were quantified by densitometry analysis of gels with Fuji Film Image Gauge V3.0 software (Fuji Photo Film Co. Ltd. Japan). Data were expressed as the ratio of the area and intensity of the band. The reduction in the intensity of band during incubation with respect to the original intensity was expressed as percentage of hydrolysis (Ong et al., 2006).

## RESULTS

*Enterococci* constitute a large proportion of the autochthonous micro flora associated with artisanal food. They have been recognized as an essential part of the natural microbial population of many dairy products, where they can sometimes even dominate over *Lactobacilli* and *Lactococci* (Foulquié-Moreno et al., 2006; Suzzi et al., 2000). The predominance of *Enterococci* in fermented dairy products might be attributed to their capability to grow over a wide range of temperatures, to tolerate salt and acid pH (Giraffa, 2003) and to produce proteolytic enzymes involved in casein degradation (Jensen et al., 1975; Wessels et al., 1990).

Proteolytic system is very important feature of lactic acid bacteria (LAB), to which *Enterococci* belong, that enables them to grow in milk and other protein-containing media, releasing amino acids, which are essential for their growth. Proteolytic activity of *Enterococci* was studied by different authors, revealing *E. faecalis* as the most active species (Centeno et al., 1999; Psoni et al., 2006; Sarantinopoulos et al., 2001; Suzzi et al., 2000; Veljovic et al., 2009). In the present work we investigated the proteolytic activity of strain *Enterococcus faecalis* A71 isolated from traditional Azerbaijani cheese. The ability of isolated strain to hydrolyze milk proteins was tested after induction of the proteinase production in milk. In this case, hydrolysis of milk proteins was tested in conditions of non-regulated pH and proliferation. Kinetics of milk proteins hydrolysis is shown in Fig.1a. Investigated strain was able to hydrolyze  $\alpha_{S1-}$ ,  $\alpha_{S2-}$ ,  $\beta$ -caseins, and BLG fractions of milk. It starts hydrolyze caseins after 3h cultivation, and whey proteins after 6h cultivation. Similar results were observed in the study of El-Ghaish et al. (El-Ghaish et al., 2010), where they observed efficient hydrolysis of milk proteins by dairy *Enterococci* isolates. Hydrolysis of ALA was not observed. Proteolysis was observed after 3 h cultivation in

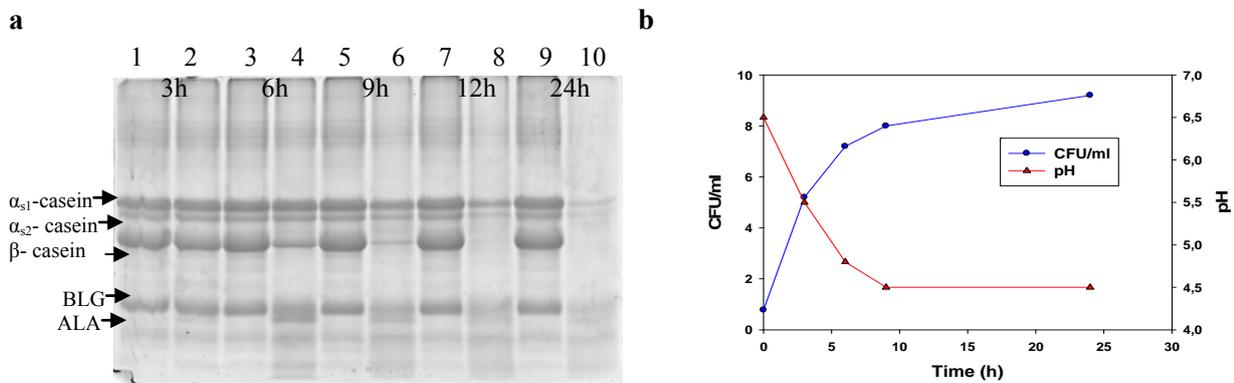
milk and increased with the time of incubation (Fig.1a). Highest degree of hydrolysis was observed for  $\beta$ -casein (more than 90% after 24 h incubation). Strain started hydrolyzes this protein after 3 h incubation. In another study (Psoni et al., 2006) authors also found that *Enterococci* isolates hydrolyzed faster  $\beta$ -casein than other caseins, but at the end of incubation time  $\alpha_S$ -caseins were more hydrolyzed.

Hydrolysis of  $\alpha_{S1}$ -casein was also observed after 3 h incubation, but was very low. However, hydrolysis  $\alpha_{S2}$ -casein was not observed at this time point, when the pH of the medium was still near neutral value (pH 6.0–6.5). After 24 h incubation, when the pH of milk decreased below 4.8, hydrolysis ratio of  $\alpha_{S1}$ -,  $\alpha_{S2}$ -, and  $\beta$ -caseins increased. We can see, that at the beginning of the exponential growth phase (after 3 h incubation)

when the pH of milk was near neutral value proteolytic activity was very low. It seems that investigated strain in studied conditions produce proteases with pH optimum near acidic conditions.

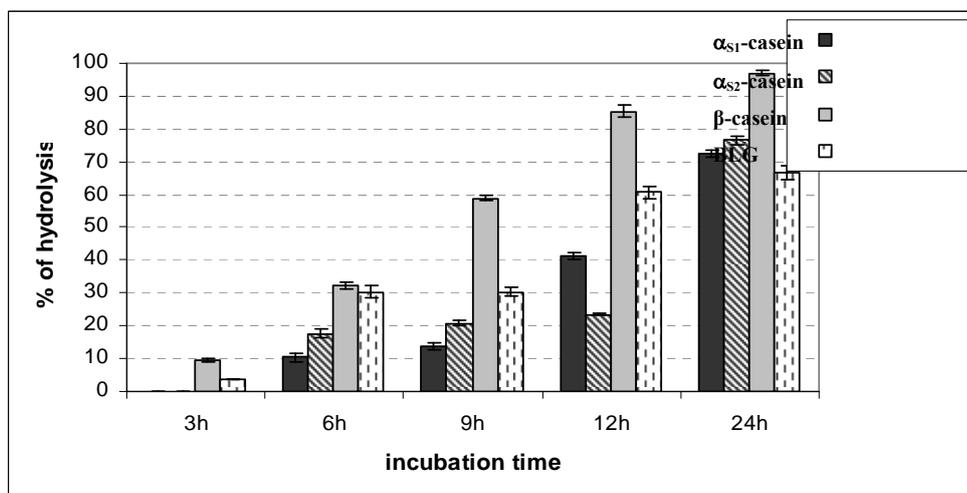
The highest degree of hydrolysis (96% of  $\beta$ -, 71% of  $\alpha_{S1}$ -, 74% of  $\alpha_{S2}$ -caseins and 64% of BLG) was observed at the end of incubation time when the pH decreased below 4.8 (Fig.2).

With regards to acidifying activity strain *E. faecalis* A71 was able to coagulate milk after 6 h cultivation. Kinetics of growth and pH decrease during cultivation in milk is presented in Fig 2b. After 24 h cultivation in milk the pH decreased till 4.5. High acidifying activity of *Enterococcus faecalis* isolates was also observed in another study (Suzzi et al., 2000). Investigated strain showed good ability of growth in milk.



**Figure 1.** Kinetics of growth and proteolytic activity in milk.

**a.** Kinetics of proteolytic activity - SDS-PAGE of samples after different time intervals cultivation in UHT skimmed milk. Lines 1, 3, 5, 7, 9 - control (substrate without cells analyzed at the same time intervals), lines 2, 4, 6, 8, 10 - samples (substrate incubated in the presence of cells) taken after 3, 6, 9, 12 and 24 h incubation with substrate. **b.** Kinetics of growth and pH decrease.



**Figure 2.** Percentage of caseins and BLG fractions hydrolysis at different time intervals of milk fermentation with *Enterococcus faecalis* A71 strain.

Isolated strain *E. faecalis* A71 probably contribute to the differences in flavor, texture and taste of Azerbaijani traditional cheeses due to the high proteolytic activity and could represent new adjunct cultures for the dairy industry. However, we are aware that further studies regarding safety aspects of this strain, such as resistance to antibiotics and presence of virulence factors are necessary before the statement can be made that it has no effect on food safety.

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### ***Enterococcus Faecalis* A71 Ştamının Süddə Inkubasiya Zamanı Proteolitik Aktivliyinin Öyrənilməsi**

Tədqiqatın əsas məqsədi proteolitik fermentlər ifraz edən *Enterococcus faecalis* A71 ştamının süddə inkubasiya zamanı proteolitik aktivliyinin öyrənilməsi olmuşdur. Tədqiq olunan ştamın proteolitik aktivliyi elektroforetik üsulla yoxlanılmışdır. *Enterococcus faecalis* A71 ştamı bütün kazein fraksiyalarını hidroliz etmişdir. Hidroliz 3 saat süddə inkubasiyadan sonra başlamışdır. 24 saat süddə inkubasiyadan sonra *Enterococcus faecalis* A71 ştamı  $\beta$ - kazeinin 96%-ini,  $\alpha_{s1}$ - kazeinin 71%-ini,  $\alpha_{s2}$ -kazeinin 74%-ni və BLG fraksiyanın 64%-ni hidrolizə etmişdir. Ayrılmış fəal ştam proteolitik fermentlərin produsentidir və qida sənayesində istifadə üçün potensiala malikdir.

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### **Изучение Протеолитической Активности Штамма *Enterococcus Faecalis* A71 в Молоке**

Целью данной работы было изучение протеолитической активности штамма *Enterococcus faecalis* A71, изолированного из традиционного сыра, произведенного в Азербайджане. Наличие гидролиза белков молока проверяли с помощью электрофореза в додецил-сульфат-натрий-полиакриламидном геле. Протеолитические ферменты выделяемые исследуемым штаммом расщепляли  $\alpha_{s1}$ -,  $\alpha_{s2}$ -, и  $\beta$ -казеины, а также  $\beta$ -лактоглобулин (БЛГ) молока. После 24 часов инкубации в молоке, протеолитические ферменты исследуемого штамма гидролизировали 96%  $\beta$ -, 71%  $\alpha_{s1}$ -, 74%  $\alpha_{s2}$ -казеинов и 64% of БЛГ фракции молока. Также была изучена кинетика роста и подкисления среды при росте штамма в молоке. Кинетику роста определяли с помощью подсчета КФЕ/мл. Штамм *Enterococcus faecalis* A71 проявил хорошую способность роста и коагуляции в молоке. Полученный штамм является потенциальными кандидатами в качестве стартерной культуры для использования в молочной промышленности.