



Detection of enzymes produced by lactic acid bacteria isolated from traditionally made Serbian cheese and their role in the formation of its specific flavor

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ABSTRACT

Nine species (sixteen isolates) of lactic acid bacteria (LAB) isolated from traditionally made Serbian cheese were evaluated for their enzymatic activities in order to select indigenous strains of technical interest for the manufacture of cheese. These strains were selected based on their previously determined biochemical and physiological characteristics, as well as their antimicrobial activity, and were identified as *Lactococcus lactis* subsp. *lactis* (one isolate), *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* (five isolates), *Lactobacillus fermentum* (two isolates), *Lb. plantarum* (one isolate), *Lb. brevis* (one isolate), *Enterococcus faecalis* (three isolates), *E. faecium* (one isolate), *E. durans* (one isolate) and *E. hirae* (one isolate). The enzymatic activities (acid and alkaline invertases, alkaline phosphatase, alkaline protease, α -amylase) were measured by using the spectrophotometric method. The results indicated that all *Lactobacillus* isolates showed protease, amylase, and alkaline phosphatase activities, while the activities of acid and alkaline invertases were not observed. The *Lactococcus* isolates showed protease, acid invertase and alkaline phosphatase activities, except the KGPMF50 isolate, which showed no alkaline phosphatase activity. The tested *Enterococcus* isolates showed weakly and strain-specific enzymatic activity. The results indicated that the enzymes produced by the investigated strains have a role in the formation of the specific flavor of cheese and that these isolates, especially *Lactobacillus* isolates, showed the potential for use in the dairy industry or applied biotechnology.

Keywords: enzymatic activity, lactic acid bacteria, traditionally made cheese, biotechnology, dairy industry.

ИЗВОД

Циљ овог рада био је испитивање способности продукције ензима девет одабраних врста (16 изолата) бактерија млечне киселине (БМК) изолованих из српског сира произведеног на традиционалан начин, како би се утврдило који аутохтони сојеви имају технолошки утицај у процесу производње сира. Изолати су одабрани на основу претходно утврђених биохемијских и физиолошких карактеристика, као и антимикробне активности и идентификовани су као: *Lactococcus lactis* subsp. *lactis* (један изолат), *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* (пет изолата), *Lactobacillus fermentum* (два изолата), *Lb. plantarum* (један изолат), *Lb. brevis* (један изолат), *Enterococcus faecalis* (три изолата), *E. faecium* (један изолат), *E. durans* (један изолат) и *E. hirae* (један изолат). Ензимска активност, која је обухватала детекцију киселе и алкалне инвертазе, алкалне фосфатазе, алкалне протеазе и α -амилазе, је одређена коришћењем спектрофотометра. Резултати указују да су сви изолати *Lactobacillus* показали активност протеазе, амилазе и алкалне фосфатазе, док активност киселе и алкалне инвертазе није примећена. Изолати лактокока су показали активност протеазе, киселе инвертазе и алкалне фосфатазе, осим изолата KGPMF50, који није показао активност алкалне фосфатазе. Испитани изолати ентерокока показали су слабу и изолат специфичну ензиматску активност. Резултати указују да продуктовани ензими испитиваних изолата имају улогу у формирању специфичног укуса сира и да сви изолати, посебно изолати из рода *Lactobacillus*, показују потенцијал за примену у млечној индустрији или у примењеној биотехнологији.

Кључне речи: ензимска активност, бактерије млечне киселине, традиционална производња сира, биотехнологија, млечна индустрија.

1. Introduction

Traditional cheeses, made from raw, unpasteurized milk, without adding any bacterial starter cultures or rennet of animal origin, are the source of autochthonous lactic acid bacteria (non-starter LAB). In these cheeses, bacterial enzymes can affect maturation, color, texture, flavor, and nutritional value. They are mainly used in the food industry to optimize the process, improve efficiency, quality, shelf life, and, most importantly, to achieve the desired organoleptic

characteristics of the final product (Avenidaño et al., 2016).

The LAB isolated from Sokobanja cheese showed antimicrobial potential and great acidification ability in previous studies (Muruzović et al. 2018a; 2018b). Liu et al. (2013) indicated that LAB has the ability to produce some useful enzymes. Wijesundera et al. (1997) found that lactobacilli can enhance proteolysis and improve flavor formation in cheese. The metabolic and enzymatic potentials of non-starter LAB lie in their dominance in total viable microorganisms in cheeses

(Peterson et al., 1990) and their metabolic versatility (Fox et al., 1998). So far, enzymatic activity has been studied mainly for LAB isolated from wine (Mtshali 2007). But, Novik et al. (2007) and Patel et al. (2012) indicated that some species of *Lactobacillus*, *Lactococcus*, *Pediococcus*, and *Bifidobacterium* isolated from fermented foods have the ability to produce carbohydrate degrading enzymes. It is known that fermented kinds of milk can be supplemented with some probiotic strains. Fermented milk manufactured in this way can be made more functional in human nutrition (Patel et al., 2013). Furthermore, if LABs are capable of synthesizing essential biomolecules (vitamins, enzymes, or bacteriocins), they can affect the functional and technological properties of the food products.

This study aimed to evaluate the enzymatic activity (acid and alkaline invertases, alkaline phosphatase, alkaline protease, invertase) of sixteen LAB isolates from traditionally made cheese from Southeastern Serbia (Sokobanja area) to find their role in the specific flavor of the cheese.

2. Materials and methods

Isolates used in the study

The tested strains of LAB were isolated from three-day-old Sokobanja cheese (Muruzović et al., 2018a; 2018b; Grujović et al., 2019). The strains were isolated during different sampling seasons (spring, summer, and autumn) from different culture media and were identified to the genus level by physiological and biochemical tests. Then, sixteen strains were chosen, based on their antimicrobial activity relative to five indicator strains (*Escherichia coli* ATCC 25922, *Proteus mirabilis* ATCC 12453, *Klebsiella oxytoca* KGPMF1, *Klebsiella ornithinolytica* KGPMF8, and *Aeromonas hydrophila*). The chosen strains of LAB were identified as *Lactococcus lactis* subsp. *lactis* (one strain), *Lc lactis* subsp. *lactis* biovar. *diacetylactis* (five strains), *Lactobacillus fermentum* (two strains), *Lb. plantarum* (one strain), *Lb. brevis* (one strain), *Enterococcus faecalis* (three strains), *E. faecium* (one strain), *E. durans* (one strain), and *E. hirae* (one strain), by using MALDI-TOF mass spectrometry. The strains were frozen at -80°C in MRS broth with 20% glycerol. They were subcultured in the same medium at 37°C for 16–24 h before the various assays were performed.

Screening of LAB for their proteolytic and lipolytic activity

The proteolytic activity of LAB was examined according to Harrigan and McCance (1976), with some modifications. The substrate was formed by mixing MRS agar medium and milk (1.6% fat) in a ratio of 1:1. The inoculated media were incubated at 37°C/24 h. The appearance of a clear zone around the colonies of bacteria confirmed their proteolytic activity. *Bacillus subtilis* ATCC 6633 and *Escherichia coli* ATCC 25922 were used as positive and negative control, respectively.

The lipolytic activity of bacteria was examined according to Harrigan and McCance (1976), with some modifications. The substrate was formed by adding an egg yolk (4%) into the MRS agar. The inoculated media were incubated at 37°C/24 h. The appearance of an

opalescent zone around the colonies of bacteria confirmed their lipolytic activity. *B. subtilis* ATCC 6633 and *E. coli* ATCC 25922 were used as a positive and negative control, respectively.

Preparation of fermentation liquids of LAB

Strains of isolates were grown in 10 ml of MRS broth (1% inoculum) at 37°C for 24 h until they had reached the stationary phase (Kenny et al. 2003). The cultures were then centrifuged at 10000 rpm, for 30 min, at a temperature of 4°C. The fermentation liquid supernatants were separated and frozen at -20°C until use. The enzymatic activities were tested from these samples.

Evaluation of enzymatic activities

Determination of acid and alkaline invertase activities

Invertase activity was determined by the method described in Sumner and Howell (1935) with some modifications. The determination of alkaline invertase activity in the fermentation liquid of the isolates involved the preparation of a reaction mixture containing 0.5 ml of crude enzyme extract, 0.5 ml of 0.02 M phosphate buffer (pH 8.0), and 1 ml of 1% (w/v) of sucrose. The contents of the tube (sample) were mixed and incubated in a water bath at 37°C for 15 min. In the second (control) tube, the same ingredients were added as in the test tube, and that tube was placed on ice until the incubation of the test tube was completed. One ml aliquots of the contents of both tubes were taken and 2 ml of the dinitrosalicyl reagent was added. The test tubes were incubated at 100°C/5 min. The tubes were cooled at room temperature and the amount of liberated reducing sugars was spectrophotometrically determined at 540 nm (Miller, 1959). The enzymatic activity of acid invertase was determined in the same way in a reaction mixture containing 0.5 ml of crude enzyme extract, 0.5 ml 0.01 M sodium acetate buffer (pH 4.5), and 1 ml of 1% (w/v) sucrose. The invertase activity unit (IU) is defined as the amount of enzyme which catalyzes the production of 1 µM of glucose per minute at 37°C.

Determination of alkaline phosphatase activity

The enzymatic activity of alkaline phosphatase was determined based on the amount of inorganic phosphorus, using Allen's method (Allen 1940) with some modifications. The enzymatic activity was measured in a reaction mixture containing 1 ml of glycol buffer (pH 9.0) with Mg²⁺, 1 ml of enzyme extract, and 1 ml of β-glycerophosphate (substrate). The tubes containing the reaction mixture were incubated at 37°C/30 min. After the incubation, the enzyme reaction was interrupted with the addition of 3 ml of 10% TCA. The tubes were placed on ice for 15 minutes, after which the contents of the tubes were filtered, and the filtrate was collected. Control tubes were prepared in the same way, but without incubation, TCA was immediately added, and the tubes were held on ice for 15 min and filtered. To determine the concentration of phosphorus in the sample, Allen's reaction was performed. One ml aliquots of both the test and control filtrates were poured into two tubes, and 0.4 ml of amide, 0.4 ml of 60% PCA, 0.2 ml of NH₄

molybdate, and 3 ml of distilled water were added to each tube. The tubes were incubated at room temperature, for 11 min. The absorbance of prepared solutions was measured at 720 nm.

Determination of alkaline protease activity

The proteolytic activity of the fermentation liquid was determined based on the presence of enzymes in 1 ml of the liquid medium by Anson's method (Anson 1938) with some modifications, based on the amount of tyrosine liberated by the hydrolysis of casein under the action of proteolytic enzymes. The proteolytic activity of the fermentation liquid was determined as follows: 1 ml of the fermentation fluid was added to one tube, and 5 ml of 2% (w/v) solution of casein was added to another tube. Both tubes were heated in a water bath at 37°C/15 min. Thereafter, 2 ml of heated casein in the test tube was mixed with the fermentation liquid (test) and returned to incubate for another 10 min. The enzyme reaction was stopped by the addition of 5 ml of cold 5% (w/v) trichloroacetic acid (TCA). The mixture was blended and then passed through filter paper. In another control tube, 1 ml of the fermentation fluid was pipetted and 2 ml of cold casein and 5 ml of 5% (w/v) TCA were immediately added. The mixture was blended and then passed through filter paper. Two ml of the filtrate was taken from the test and control tubes, and separated, following which 5 ml of 6% (w/v) Na₂CO₃ and 1 ml of Folin-Ciocalteu reagent were added to each filtrate. The mixture from the tubes was blended and left at room temperature /30 min until the blue color had developed. In a separate tube (blank), 1 ml of distilled water was placed instead of tyrosine and all other ingredients were added. The absorbance value was read on the spectrophotometer at 660 nm.

Determination of α-amylase activity

The Bernfeld method (Bernfeld 1955), with some modifications, was used to determine α-amylase activity. The activity of α-amylase in the fermentation liquid was determined by the preparation of a reaction mixture containing 0.5 ml of crude enzyme extract, 1.5 ml of buffer (pH 6.9) (20 mM sodium phosphate, and 6.7 mM NaCl) and 1 ml of 1% (w/v) starch. The contents of the tube (sample) were mixed and incubated in a water bath at 37°C/15 min. In the control tube, the same ingredients were added as in the test tube, and the tube was placed on ice until the incubation of the test tube was completed. One ml aliquots of the contents of both tubes were taken and 1 ml of dinitrosalicyl reagent (5.3 M K-Na-tartrate and 96 mM of 3,5-dinitrosalicylic acid) was added. The test tubes were incubated at 100°C/15 min. The tubes were cooled to room temperature and then the absorbance was spectrophotometrically read at 540 nm.

Statistical analysis

All data for measuring the amount of enzymes were presented as means ± standard deviations using Microsoft Excel (Redmond, Washington, DC, USA). Statistics 20. ANOVA analysis (SPSS Inc., Chicago, IL, USA) was carried out to determine statistical differences ($p < 0.05$) between the different bacterial strains of the same genus with respect to the values of acidification and enzyme activity. The strains were tested three times for each enzymatic activity.

3. Results and discussion

Screening for proteolytic and lipolytic activity

As revealed by the results of screening for proteolytic and lipolytic activity, isolates from the genera *Lactobacillus*, *Lactococcus* and *Enterococcus* did not exhibit lipolytic activity, but they showed proteolytic activity. Exceptions were isolates from the genus *Enterococcus*, because they did not show sufficiently clear results related to proteolytic activity when compared with the positive (*B. subtilis* ATCC6633) and negative (*E. coli* ATCC 25922) controls. Therefore, in the next part of the experiments, lipolytic activity was not investigated.

The enzymatic activities of isolated lactic acid bacteria

The enzymatic activities of four isolates of the genus *Lactobacillus* were investigated and the results are presented in Table 1. All isolates showed protease, amylase, and alkaline phosphatase activities, while the activities of acid and alkaline invertases were not observed. The activity of protease was significant, compared with the other tested enzymes ($p < 0.05$). Isolate *Lb. fermentum* KGPMF28 showed the best enzymatic activity among all lactobacilli.

The enzymatic activities of six isolates of the genus *Lactococcus* were investigated and the results are presented in Table 1. All isolates showed protease, acid invertase, and alkaline phosphatase activities, except KGPMF50, which showed no alkaline phosphatase activity. The activities of alkaline invertase and amylase were not observed. The activity of protease was significant, compared with the other tested enzymes ($p < 0.05$). The enzymatic activity among all lactococci was strain specific.

The enzymatic activities of six isolates of the genus *Enterococcus* were investigated. The results are presented in Table 1. The activities of protease, acid invertase, and alkaline invertase were not observed, while the activity of amylase was not observed for *E. hirae* KGPMF9. The activity of alkaline phosphatase was not observed for KGPMF47 and KGPMF48 isolates. This result suggests that the tested isolates of enterococci showed no proteolytic activity. The results indicated that the isolates showed weak and strain-specific enzymatic activities.

In this paper, the enzymatic activities of LAB isolated from Sokobanja cheese (Southeastern Serbia) were evaluated. Also, the role of produced enzymes in the specific cheese flavor was assessed. As classified by Muruzović et al. (2018a), this cheese belongs to a group of full-fat, acid-curd soft cheeses. The detection of the enzymatic activities of the isolates was crucial since the bacteria in a different natural environment demonstrated the potential for becoming good producers of extracellular enzymes. So far, it has been demonstrated that enzymes play a very important role in food quality (Avendaño et al., 2016). Microbial enzymes have been used in various fields, such as chemical, agricultural, and biopharmaceutical industries.

Table 1.
Enzymatic activities of LAB isolates

Isolates	Protease (IU/ml)	Acid invertase (IU/ml)	Alkaline invertase (IU/ml)	Amylase (IU/ml)	Alkaline phosphatase (IU/ml)
<i>Lb. fermentum</i> KGPMF28	5.8 ± 0.00	0 ± 0.00	0 ± 0.00	0.564 ± 0.00	0.006 ± 0.00
<i>Lb. fermentum</i> KGPMF29	2.1 ± 0.00	0 ± 0.00	0 ± 0.00	0.257 ± 0.00	0.005 ± 0.00
<i>Lb. brevis</i> KGPMF35	1.4 ± 0.00	0 ± 0.00	0 ± 0.00	0.135 ± 0.00	0.003 ± 0.00
<i>Lb. plantarum</i> KGPMF62	2.3 ± 0.00	0 ± 0.00	0 ± 0.00	0.301 ± 0.00	0.002 ± 0.00
<i>Lc. lactis</i> subsp. <i>lactis</i> KGPMG23	0.7 ± 0.00	0.03 ± 0.00	0 ± 0.00	0 ± 0.00	0.004 ± 0.00
<i>Lc. lactis</i> subsp. <i>lactis</i> biovar. <i>diacetylactis</i> KGPMG50	3.9 ± 0.00	0.035 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
<i>Lc. lactis</i> subsp. <i>lactis</i> biovar. <i>diacetylactis</i> KGPMG54	5.3 ± 0.00	0.03 ± 0.00	0 ± 0.00	0 ± 0.00	0.002 ± 0.00
<i>Lc. lactis</i> subsp. <i>lactis</i> biovar. <i>diacetylactis</i> KGPMG55	4.6 ± 0.00	0.017 ± 0.00	0 ± 0.00	0 ± 0.00	0.006 ± 0.00
<i>Lc. lactis</i> subsp. <i>lactis</i> biovar. <i>diacetylactis</i> KGPMG57	5.2 ± 0.00	0.04 ± 0.00	0 ± 0.00	0 ± 0.00	0.010 ± 0.01
<i>Lc. lactis</i> subsp. <i>lactis</i> biovar. <i>diacetylactis</i> KGPMG59	4.2 ± 0.00	0.09 ± 0.00	0 ± 0.00	0 ± 0.00	0.002 ± 0.00
<i>E. hirae</i> KGPMF9	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0.014 ± 0.01
<i>E. durans</i> KGPMF10	0 ± 0.00	0 ± 0.00	0 ± 0.00	0.216 ± 0.00	0.006 ± 0.00
<i>E. faecium</i> KGPMF14	0 ± 0.00	0 ± 0.00	0 ± 0.00	0.186 ± 0.00	0.007 ± 0.01
<i>E. faecalis</i> KGPMF47	0 ± 0.00	0 ± 0.00	0 ± 0.00	0.208 ± 0.00	0 ± 0.00
<i>E. faecalis</i> KGPMF48	0 ± 0.00	0 ± 0.00	0 ± 0.00	0.230 ± 0.00	0 ± 0.00
<i>E. faecalis</i> KGPMF49	0 ± 0.00	0 ± 0.00	0 ± 0.00	0.243 ± 0.00	0.005 ± 0.00

The values are presented as mean ± standard deviation

In the case of cheese ripening, the formation of flavors is a slow process that involves various chemical and biochemical conversions of milk components. There are three main pathways in the conversion of milk components: the conversions of lactose (glycolysis), fat (lipolysis), and caseins (proteolysis). The starter cultures used in these fermentations (*Lc. lactis*, *Lactobacillus* sp., *Streptococcus thermophilus*) are the main source of the enzymes involved in these pathways (Bockelmann and Hoppe Seyler 2001). Lactobacilli, originating from the milk environment, might grow in the dairy products and participate in enzyme activities which lead to the formation of flavors and can play a crucial role in the ripening of cheese (Smit et al. 2005). Muruzović et al. (2018a; 2018b) investigated acidification ability in pure milk and milk enriched with 2% glucose and 1% yeast extract and showed that LAB isolates from Sokobanja cheese had good acidification ability. The best activity was shown by lactobacilli. In this paper, lactobacilli had significantly better enzymatic activity than the other two tested genera.

LAB contributes relatively little to lipolysis (Molimard and Spinnler, 1996; Medina et al., 2001), which was confirmed in this study because the LAB demonstrated no lipolytic activity.

Lb. plantarum isolated from Genestoso cheese showed strong caseinolytic activity (González et al., 2007). In this paper, proteolytic activity significantly differed between the genera of LAB and between the strains of the same genus. Lactobacilli and lactococci showed proteolytic activity, while enterococci showed no proteolytic activity. These findings confirmed previous screening results. Several strains of the genus *Lactococcus* showed greater proteolytic activity than some lactobacilli. These results are consistent with studies reported by Herreros et al. (2003) and González et al. (2010). The results in our study indicated no

significant difference in protease activity between lactobacilli and lactococci isolates.

Amylolytic LAB influenced food processing industries, especially in the production of food additives such as organic acids (lactic acid) and enzymes (α -amylase) (Panda and Ray 2018). LABs with amylase activity are much sought after because they can reduce the cost of substrate pretreatment (Shibata et al. 2007). Shin et al. (2008) indicated that LAB from the genus *Enterococcus* showed high amylase activity, which was confirmed in our study.

Todorov et al. (2017) investigated the activity of alkaline phosphatase among lactobacilli isolated from Bulgarian *lukanka* (the meat product) and showed that activities were not exhibited by most of the tested strains. The best activity was shown for *Lb. brevis* strains. Also, Medina et al. (2001) showed that lactococci isolated from milk and cheeses from Argentina had a good activity of alkaline phosphatase. Our study indicated that lactobacilli had a good activity of alkaline phosphatase, while the activity among lactococci and enterococci was strain specific.

It is well-known that LABs have the ability to produce some extra-cellular proteins, which include bacteriocins, enzymes, lipoproteins, and surface layer proteins (Kleerebezem et al., 2010), which is essential for their survival in different ecological niches (Zhou et al., 2010). Research by Muruzović et al. (2018c) indicated that LAB isolates from Sokobanja cheese possess the ability to survive environmental stress conditions. Based on the research results on their enzymatic activities, LAB isolates have the potential for use in biotechnology.

4. Conclusion

Since the tested cheese was made without adding bacterial starter cultures, it could be concluded that enzymes of the LAB had a role in the degradation of

caseins, which led to flavor formation in cheese. It was established that the tested isolates showed no lipolytic activity. The activity of the other tested enzymes was strain specific. This study demonstrated that Sokobanja cheese is a potential resource for the isolation of LAB enzyme producers, which can be used as starter cultures. The results of this study contribute to a better knowledge of possibilities for further investigation of LAB, which is important for the detection of potential new starter cultures in the dairy industry or their application in biotechnology.

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