

SCREENING OF ANTIMICROBIAL ACTIVITIES AMONG BULGARIAN LACTOBACILLI STRAINS

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Summary

Forty-six strains of genus Lactobacillus from the collection of the National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC) were screened by the use of the agar drop diffusion test, for antibacterial activity against nine test-microorganisms. Lactobacillus casei 300 exhibited activity against eight lactic acid bacteria and one yeast strains. The substance with antibacterial activity, produced by the NBIMCC 300 strain, was inactivated by proteolytic enzymes and possessed thermostability when heated to 80°C. This gave us a reason to conclude that this substance belonged to the bacteriocins' group.

Introduction

Among the antimicrobial substances produced by microorganisms, bacteriocins have gained an increasing interest in the recent years. They are small ribosomally synthesised proteins, which are able to kill bacteria, including a number of potential foodborn pathogens and food spoilage microorganisms [6, 9, 12]. The bacteriocins from the Generally Recognised As Safe (GRAS) lactic acid bacteria (LAB) have arisen a great deal of attention as a novel approach to control pathogens in food-stuffs [4, 8, 16, 17]. Data for bacteriocins, produced by LAB, and their application as preservatives in the food industry can be found in several reviews [12, 13].

Nisin, produced by some strains of *Lactococcus lactis*, is the best-characterised

bacteriocin [5, 10, 14]. It is an example of an approved GRAS status bacteriocin, which is applied as a food supplement. In a number of countries nisin is allowed to be used as a preservative in certain food-stuffs.

LAB bacteriocins usually are heat-stable, small peptides with a MW of 3 000 to 6 000 [1], and their genes are most frequently plasmid encoded. They typically inhibit the growth of closely related to the producer strains [18]. Some bacteriocins, produced by LAB, also can affect more distantly related species like *Bacillus subtilis*, *Lysteria monocytogenes* [1], etc.

This study was undertaken to determine the potential of genus *Lactobacillus* strains for the synthesis of antibacterial substances belonging to the bacteriocins' group.

Materials and Methods

Bacterial strains and media. The 46 studied *Lactobacillus* strains from the collection of the National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC) are presented in Table 1. Most of the indicator microorganisms used for the screening are included in Table 2. Besides them for the assay of activity of strain *Lb. casei* NBIMCC 300, several other strains from the NBIMCC collection are used - *Streptococcus salivarius* subsp. *thermophilus*

(NBIMCC 619 and 1272) and *Lb. lactis* subsp. *thermophilus* (NBIMCC 1375). A yeast strain - *Kluyveromyces marxianus* var. *lactis* (NBIMCC 1469), was also applied. *Lactobacillus* strains were cultivated in MRS broth [15] for the production of substances with antibacterial activity, and on MRS-agar, when the strains were used as test-microorganisms. The media used for the growth of the other strains are presented in Table 2.

Table. 1. Investigated strains.

Specieses	Nº in NBIMCC collection	t (°C)
<i>Lb. delbruecki</i> ssp. <i>bulgaricus</i>	268, 273, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 1273, 1381, 1382, 1411	45
<i>Lb. acidophilus</i>	11, 1379	38
<i>Lb. animalis</i>	1588	30
<i>Lb. helveticus</i>	507, 618, 1011	39
<i>Lb. salivarius</i>	1589	37
<i>Lb. casei</i>	300, 1010, 1013, 1014, 1373	33 - 37
<i>Lb. lactis</i>	1012	45
<i>Lb. plantarum</i>	289, 290, 297, 298	15
	1009	37
	1409, 1410	30
<i>Lb. brevis</i>	1585	30
<i>Lb. fermentus</i>	505	45
<i>Lb. reuterii</i>	1587	45
<i>Lb. viridescens</i>	1131	45
<i>Lb. leichmanii</i>	1130	37
<i>Lb. sp.</i>	506	37

Preparation of crude extract for antimicrobial activity screening. The investigated *Lactobacillus* strains were grown in 10 ml MRS for 48 h. Their cultivation temperatures are presented in Table 2. The biomass was separated by centrifugation (8000 x g, 30 min, 4°C) and the pH of the cultural supernatant was adjusted to 7.0 (by 5 M NaOH). Then the supernatant was lyophilized, dissolved in 1 ml distilled water and filtered through 0.22 µm pore-size filters. The obtained in this way crude extract was stored at -20°C and was used in the screening procedures.

Screening for the production of antibacterial substances and assay of antibacterial activity. The production of antibacterial substances was determined according to the method described by Muriana and Klaenhammer [11]. The quantitative estimation of the antibacterial substances activity was realised by the serial twofold dilution method of Barefoot and Klaenhammer [2]. It was expressed as the reciprocal value of the last serial dilution for which sterile zone was observed per millilitre of the initial media and was defined as conventional units - AU.

Ammonium sulphate precipitation of crude extract. The ammonium sulphate precipitation of the crude extract, obtained by the most active strain, was carried out according to Muriana and Klaenhammer [11]. The precipitate was collected by centrifugation at 15 000 x g for 2 h and resuspended in sodium phosphate buffer (50 mM pH 7.0). This material was a semipurified extract.

High temperatures and proteolytic enzymes sensitivity. The semipurified extract of strain NBIMCC 300 was heated at 80°C for 60 min in a water bath and then the antibacterial activity was quantitatively assayed against the indicator microorganism *Lb. casei* NBIMCC 1013. To determine the influence of the proteolytic enzymes, the following enzymes (1 mg/ml) were used:

trypsin, α -chymotrypsin, subtilisin and pronase. The semipurified extract/enzyme mixture was incubated for 60 min at 37°C. The control contained enzyme, preincubated for 10 min in a boiling water bath, and semipurified extract. As a test-microorganism, *Lb. casei* NBIMCC 1013 was used.

Assay of lactic acid. The quantitative lactic acid assay was enzymatically carried out. The reactive mixture contained 0.1 ml crude or semipurified extract, 0.2 ml 0.15 mM NAD solution, 2.7 ml 200 mM Tris HCl buffer pH 7.4. The reaction was started with the addition of 50 U lactate dehydrogenase and the initial and final absorption was measured at 340 nm [3]. An absorption coefficient of 6.22 was used in the calculations and the resulting lactate quantity was presented in mM/ml.

Table 2. Antimicrobial spectrum of activity of the studied strains.

Indicator microorganisms	Media ^a	t (°C) ^b	<i>Lactobacillus</i> strains with antibacterial activity against the indicator microorganism
507 NBIMCC <i>Lb. helveticus</i>	MRS agar	40	298, 299, 1586
552 NBIMCC <i>Hansenulla beckii</i>	YPD	24	275, 298, 1010, 1381, 1589, 2328
1013 NBIMCC <i>Lb. casei</i>	MRS agar	37	298, 300, 1130, 1588, 1589, 2117, 2188
1133 NBIMCC <i>Pseudomonas putida</i>	Nutrient agar	30	289, 506, 507, 1010, 1013, 1475, 1586, 1588, 1602, 2117, 2183, 2328
1410 NBIMCC <i>Lb. plantarum</i>	MRS agar	30	290, 299, 300, 1586
1586 NBIMCC <i>Lb. salivarius</i> ssp. <i>salivarius</i>	MRS agar	37	278, 300, 1373, 1457
2343 NBIMCC <i>Salmonella choleraesuis</i> ssp. <i>choleraesuis</i> ser. typhimurium	Nutrient agar	37	288, 289, 300, 1013, 1581, 1586
2344 NBIMCC <i>Salmonella choleraesuis</i> ssp. <i>choleraesuis</i> ser. typhimurium	Nutrient agar	37	288, 298, 300, 1013, 1586, 1588, 2188
6633 ATCC <i>Bacillus subtilis</i>	Nutrient agar	37	11, 273, 276, 280, 281, 286, 1273, 2188

^a Medium - the medium on which the indicator strains were cultivated

^b t°C - temperature of cultivation of indicator strains

Results and Discussion

Screening for compounds with antimicrobial activity. The results achieved by the investigation of 46 strains showed that 28 of them possessed antimicrobial activity. Some strains were found to produce antibacterial compounds with variable activity spectra against the indicator strains (Table 2).

The largest spectrum of inhibition was observed for: *Lb. casei* NBIMCC 300, which inhibited 5 test-microorganisms, and *Lb. plantarum* NBIMCC 298, *Lb. salivarius* NBIMCC 1586 and *Lb. casei* NBIMCC 1013, which were active against 4 strains. Crude extracts of these four strains were therefore

used to quantitatively assay their antibacterial activity. The results are shown in Table 3. Strain *Lb. casei* NBIMCC 300 showed the highest activity and it was used for the further investigations in this study.

Lb. casei NBIMCC 300 was active against eight LAB and one yeast strains. The highest activity was observed against *St. thermophilus* NBIMCC 1272 and *Lb. casei* NBIMCC 1013. The antimicrobial activity was gradually lost during 4°C storage, but was stable at -20°C for at least one year (data not shown).

Table 3. Antibacterial activity of some investigated strains.

A. Strain 298

Indicator microorganism	507	552	1013	2344
Activity (AU/ml)	1600	800	800	200

B. Strain 300

Indicator microorganism	505	619	1013	1272	1375	1410	1586	1588	2344
Activity (AU/ml)	800	800	6400	6400	1600	1600	200	800	3200

C. Strain 1013

Indicator microorganism	1133	1469	2343	2344
Activity (AU/ml)	1600	400	800	1600

Some strains were active against three Gram negative test microorganisms. The observed inhibition was not due to low pH (the active supernatant was adjusted to pH 7.0 before use) or hydrogen peroxide (the samples were lyophilized). The bacteriocins, produced by LAB, usually do not exhibit activity against Gram negative strains, although there is information by some authors [13] about the production of substances active against *Escherichia coli*. It is not clear, however, whether these compounds are bacteriocins or other agents of inhibition.

Ammonium sulphate precipitation of antibacterial active substance. As the precipitates formed flowing pellets, it was

impossible to remove the ammonium sulphate precipitates in MRS broth, containing Tween 80, by centrifugation. Therefore, the strain *Lb. casei* NBIMCC 300 was cultivated in MRS medium without Tween 80 in the ammonium sulphate precipitation experiments. The cells of the 48 h culture were separated by centrifugation and the supernatant obtained was solid ammonium sulphate treated to 80% saturation. After the removal of the pellet by centrifugation, the supernatant was treated again with solid ammonium sulphate to 100% saturation. The pellet of second saturation was dissolved in 0.5 ml distilled water. The solution was dialysed over night against 50 mM sodium phosphate buffer, pH 7.0 and the resulting sample, called

semipurified extract, contained the compound with antibacterial activity, detected against the NBIMCC 1013 and 1410 strains. The control, consisting of MRS broth (without Tween 80), treated in the same way, did not exhibit inhibition of the indicator strains.

No lactic acid was determined in the semipurified extract, while the crude extract contained 0.9 mM lactic acid per ml. Thus, the results achieved, brought us to the conclusion that the antimicrobial activity of *Lb. casei* NBIMCC 300 was not due to the lactic acid antagonistic action.

High temperatures and proteolytic enzymes sensitivity. A semipurified extract of *Lb. casei* NBIMCC 300 was tested for high temperatures and proteolytic enzymes sensitivity. It was found out that its activity

was preserved after 60 min treatment at a temperature of 80°C. Some bacteriocins of a low molecular weight, produced by LAB, exhibited high temperatures stability [1]. Thermostability is very convenient if the bacteriocins are to be used as food preservatives, because many food-processing procedures involve a heating step.

The antibacterial activity of *Lb. casei* NBIMCC 300 semipurified extract was completely lost after a treatment with four proteinases (Table 4). The control containing a semipurified extract incubated with previously heat-treated proteinase (10 min at 100°C) did not lose its activity. This gives a reason to conclude that the investigated component with antibacterial activity is of a protein nature.

Table 4. Proteolytic enzymes' influence on the antimicrobial activity of *Lb. casei* NBIMCC 300 semipurified extract.

Enzymes	Antimicrobial activity	
	After treatment with enzymes	Control
Trypsin	–	+
α-Chymotirpsin	–	+
Pronase	–	+
Subtilisin	–	+

(–) - lack of sterile zones; (+) - presense of sterile zones

The data obtained from the presented results show that *Lb. casei* NBIMCC 300 produces an antibacterial compound, which

can be a bacteriocin. This conclusion is due to the spectrum of activity and the protein nature of the semipurified extract.

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