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CAPABILITY OF LACTIC ACID BACTERIA TO INHIBIT PATHOGENS IN SEWAGE SLUDGE SUBJECTED TO BIOTECHNOLOGICAL PROCESSES

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Abstract

The investigation included 30 isolates of lactic bacteria originating from different sources (composted green materials, EM "Greenland" preparation as well as *Lactobacillus brevis* and *L. plantarum* cultures), and the following pathogens: *Salmonella* sp., *Listeria monocytogenes, L. innocua, Escherichia coli, Streptococcus faecium, Staphylococcus aureus, Pseudomonas aeruginosa, Proteus mirabilis* and *Bacillus cereus*. Lactic bacteria were inoculated pointwise and incubated under anaerobic conditions, then covered with a layer of fluxed medium with a pathogen tested. After incubation pathogen growth inhibition zones were read (the test for bacteriocins). The influence of other metabolites (e.g. lactic acid) on the survival of pathogens was tested under aerobic conditions (agar disc method). Bacteria isolated from composted green material showed the weakest inhibitory activity. The other lactic bacteria affected pathogens much more effectively, and were more active under aerobic conditions. Bacteriocins of selected strains of lactic bacteria were produced only on solid media and were separated with the method of pH-related adsorption/desorption. Wide range of metabolites produced by lactic bacteria allows to introduce them into the systems of aerobic and anaerobic technologies of sewage sludge hygienization.

Key words: lactic acid bacteria, bacteriocin, antagonism, pathogen, composting.

Lactic fermentation bacteria are widely spread in the natural environment, mainly on plants. That is why they are present in composted material enriched with green additives. Due to their enzymatic and antagonistic properties they contributed to the efficient degradation of organic matter and, at the same time, inhibit the growth of other microorganisms, pathogenic for plants, animals and people. Associated vaccines containing, among others, the *Lactobacillus* strains introduced into composts have been used in the world for some time. The main factors responsible for antagonistic properties of these bacteria are bacteriocins. We know their efficiency in the inhibition of the growth of such pathogens as *Salmonella* sp., *Listeria* sp., *Escherichia coli, Bacillus cereus, etc*. present in food (1, 3, 5, 9). Besides, bacteriocins of lactic bacteria produce other substances which act as antagonists, e.g. H_2O_2, organic acids, lactoperoxidase, diacetyl, and CO_2 (4, 7, 11, 12). They enter into the composition of a group called EM (effective microorganisms) used in the shape of vaccines for the production of biofertilizer in the composting process. These properties can be of a great importance for composting of sewage sludge which almost always contain vast amount of pathogenic microflora. An attempt has been made in the study to answer the question whether lactic bacteria can contribute to the elimination of numerous pathogens in sewage sludge subjected to biotechnological processes, such as composting and anaerobic fermentation.

Material and Methods

Lactic acid bacteria. The investigation comprised 29 isolates of lactic bacteria joined in 3 separate groups according to their origin:

- *Lactobacillus brevis* 0845 and *L. plantarum* 0858 strains coming from the Industrial Microorganisms Collection of the Institute of Fermentation Technology and Microbiology of Engineering College of Łódź (group I);
- 20 isolates numbered from 1 to 20 obtained from composted green material (group II);
- 7 isolates marked with letters A-G obtained from EM "Greenland" preparation (Greenland Technologia EM, Puławy) (group III).

Isolation of bacteria from groups II and III and culture of all lactic bacteria were conducted on MRS medium. They were identificated as *Lactobacillus* genus according to the criteria suggested by Schillinger and Luck (10). All the chosen isolates of groups II and III were Gram-positive, catalase-negative and homofermentating bacilli.

Pathogenic bacteria. The following strains of 9 pathogens coming from the collection of pure cultures of the Hohenheim University in Stuttgart and from the Sanitary and Epidemiological Station in Bydgoszcz were used: *Salmonella* sp., *Listeria monocytogenes* 2728/18, *L. innocua* 2403/18, *Escherichia coli, Streptococcus faecium, Staphylococcus aureus* S-28/M/78, *Proteus mirabilis* DSM 788, *Pseudomonas aeruginosa* and *Bacillus cereus*. The culture was grown on peptone water (Peptone Water, Merck, 7228), and on solid medium TSA (Tryptone-Soja-Agar, P-0090, BTL, Łódź Enzymes and Peptones Institute). The estimation of the antagonistic effect of lactic acid bacteria in relation to pathogens was carried out under aerobic and anaerobic conditions.

Influence of lactic fermentation bacteria on pathogens under aerobic conditions. An agar disc test was used to define the possible impact of predominant metabolites of lactic bacteria such as lactic acid and H_2O_2, produced under aerobic conditions, on the pathogens. The first step of the experiment was the cultivation of the bacteria on enriching media – the lactic bacteria grew on MRS medium at 30°C for 24 h, while the pathogenic bacteria – on peptone water at 37°C for 24 h. After incubation the suspension of lactic bacteria was brought to the equal value of T_{550} (Spectrofotometer Beckman) and the deep inoculation of 1ml of each lactic bacteria culture on MRS medium (15 ml) was made. After 48 h incubation at 30°C, discs 9 mm in diameter were cut out from the agar and put on Petri dishes with TSA solid medium covered with suspensions of 48-h cultures of the pathogenic bacteria. After an incubation at 30°C for 48 h the growth inhibition zones of pathogenic bacteria growth, caused by lactic acid bacteria, were measured.

Influence of bacteriocins on pathogen growth. 18-h cultures of lactic acid bacteria on MRS broth were inoculated pointwise on a MRS-agar. Five ml of the tenfold dilution of each bacterial culture was placed on the medium. The cultivation was conducted under anaerobic conditions (Anaerocult, Merck) for 18-24 h at 30°C. Anaerobic conditions were created in order to inhibit the synthesis of lactic acid and hydrogen peroxide. Pathogenic bacteria in turn were inoculated on a liquid breeding-ground (pepton water) and incubated at 37°C for 24 h. Lactic bacteria cultures were treated with chloroform for 20 min and after its evaporation the medium was covered with suspensions of particular pathogens (0.25 ml of tenfold solution of each culture) in 10 ml of fluxed TSA medium. The cultivation was carried out at 37°C for 48 h under aerobic conditions. Antagonistic activity of lactic bacteria was estimated on the basis of measurements of the size of a pathogen growth inhibition zone.

Isolation and titration of bacteriocins. The investigations comprised two strains – *Lactobacillus plantarum* and *L. brevis*. The isolation of bacteriocins from cultures on a solid medium was made according to the method of precipitation with chloroform from a liquid medium (8), precipitation with ammonium sulfate (2) and pH-related adsorption/desorption (13).

Statistical analysis. The results were analysed statistically in the Statistica program with the rest of the Least Significant Difference (LSD). The presence of significant differences between particular groups of lactic acid bacteria in relation to the pathogens, and differences within the groups according to experimental conditions (aerobic, anaerobic), were calculated.

Results

The influence of all tested isolates of lactic acid bacteria on particular pathogens has been presented in Table 1. The sizes of growth inhibition zones of pathogenic bacteria under aerobic and anaerobic conditions have been compared. *L. plantarum* and *L. brevis* from group I did not inhibit the growth of *L. innocua* strain under aerobic conditions. Apart from that they affected all pathogens tested in both experimental systems in a very wide range, and *L. monocytogenes, Staph. aureus* and *E. coli* strains were the most susceptible to their antagonistic effect. Lactic acid bacteria isolated from compost (group II) produced very small growth inhibition zones of pathogenic bacteria. Bacteriocins activity caused the appearance of inhibition zones of 1-2 mm, while under aerobic conditions the zones ranged from 3 – 4 mm. The most active isolates were 1, 3, 12 and 16. The bacteria numbered as 4-8, 13-15, 19 and 20 showed a minimal antagonistic activity in relation to pathogens, or the total lack of the activity. Group III of lactic microorganisms isolated from the EM "Greenland" preparation showed the stronger inhibition caused by the effect of bacteriocins in relation to three pathogens: *E. coli, P. aeruginosa* and *P. mirabilis*. The strains A and F did not show any inhibitory activity under anaerobic conditions. The biggest growth inhibition zones were observed in isolates C and G (20 mm). The mean zone width under aerobic conditions ranged from 1 to 7 mm. Of 3 tested methods of isolation of bacteriocins only the method of adsorption/desorption gave satisfactory results. So we can draw a conclusion that the bacteria tested produce bacteriocins growing on solid media. Their titer was 8.

It turned out that there were significant differences between the groups I and II as well as II and III independently of the experimental conditions. The arithmetic means for inhibition zones under aerobic and anaerobic conditions of particular groups show that group III inhibited the pathogen growth most powerfully under aerobic conditions, and group I–under anaerobic conditions. The weakest antagonistic properties were noticed in group II independently of the incubation conditions.

Table 1

Growth inhibition zones (mm) of pathogens affected by bacteriocins (anaerobical conditions) and metabolites of aerobic processes

Lactic acid bacteria		Pathogens																	
		*L.m.**		*L.i.*		*B.c.*		*Staph.*		*Strep.*		*Salm.*		*E.coli*		*P.a.*		*P.m.*	
Group	strain/isolate	an.	a.**	an.	a.	an.	a.	an.	a.	an.	a.	an.	a.	an.	a.	an.	a.	an.	a.
I	*L.plantarum*	5.0	6.0	3.0	-	2.0	1.0	5.0	6.0	2.5	2.0	3.5	4.0	6.0	3.0	3.0	3.0	4.0	3.0
	L.brevis	4.0	5.0	4.0	-	4.0	1.0	4.0	6.0	3.0	1.0	6.0	3.0	4.6	4.0	2.0	2.0	2.5	3.0
II	1	-	-	3.0	1.0	-	-	-	2.0	1.0	2.0	-	-	-	-	2.0	2.0	2.0	-
	2	1.0	-	-	-	-	-	-	2.0	2.0	2.0	3.5	-	-	-	1.0	-	-	-
	3	1.0	-	-	1.0	1.0	-	-	2.0	-	2.0	3.0	-	3.0	-	2.0	2.0	-	-
	4	-	-	-	-	-	-	-	2.0	-	-	-	-	1.0	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	2.5	-	4.0	-	-	1.0	-	-	-	-
	6	-	-	-	-	-	-	-	-	3.0	-	3,0	-	2.5	-	-	-	-	-
	7	-	-	-	-	1.0	-	-	-	-	-	-	2.0	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-	-	2.0	2.0	-	-	-	-	-	-
	9	-	2.0	-	-	-	-	-	-	-	-	-	2.0	-	-	-	-	-	-
	10	2.0	2.0	-	-	-	-	-	-	1.0	1.0	-	2.0	-	-	-	-	-	-
	11	2.0	2.0	-	-	-	-	-	-	1.0	1.0	-	2.0	-	-	-	-	-	-
	12	2,0	2.0	3.0	1.0	-	-	-	-	3.0	1.0	1.0	2.0	-	-	-	-	-	-
	13	3.0	5.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	14	4.0	7.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	15	-	-	-	-	-	-	-	1.0	-	-	-	-	-	-	-	-	-	-
	16	1.0	4.0	3.0	2.0	-	-	3.0	-	-	-	-	-	3.0	3.0	2,0	2.0	2,0	4.0
	17	-	-	-	-	-	-	1.0	-	1.0	-	1.0	2.0	-	-	1.0	1.0	-	-
	18	-	-	3.0	1.0	-	-	1.0	-	1.0	-	2.0	2.0	-	-	1.0	1.0	-	-
	19	-	-	-	-	-	-	-	-	-	-	-	2.0	-	-	-	-	-	-
	20	-	-	-	3.0	-	-	-	-	-	-	-	2.0	-	-	-	-	-	-
III	A	-	5.0	-	3.0	-	3.0	-	5.0	-	3.0	-	5.0	-	-	-	3.0	-	-
	B	-	3.0	-	2.0	5.0	4.0	-	3.0	-	4.0	-	5.0	11.0	3.0	14.0	3.0	7.0	3.0
	C	-	3.0	-	6.0	7.0	4.0	-	4.0	-	6.0	-	6.0	11.0	2.0	21.0	3.0	5.0	-
	D	-	6.0	-	1.0	-	2.0	-	2.0	-	3.0	-	7.0	7.0	-	6.0	3.0	-	-
	E	-	3.0	-	2.0	-	4.0	-	4,0	-	2.0	-	5.0	3.0	4.0	4.0	4.0	3.0	4.0
	F	-	3.0	-	1.0	-	3.0	-	5.0	-	-	-	5.0	-	5.0	-	4.0	-	4.0
	G	-	3.0	-	3.0	-	3.0	-	2.0	-	3.0	-	2.0	12.0	5.0	21.0	5.0	6.0	4.0

* *L.m. – Listeria monocytogenes, L.i. – L. innocua, B.c. – Bacillus cereus, Staph. – Staphylococcus aureus, Str. – Streptococcus faecium, S. – Salmonella sp., E. coli – Escherichia coli, P.a. – Pseudomonas aeruginosa, P.m. – Proteus mirabilis*

** an. – anaerobical conditions, a. – aerobical conditions, "-" – none inhibition

Table 2
Significance of the differences among 3 groups of lactic acid bacteria in the impact on pathogens under aerobic and anaerobic conditions

Aerobical condition				Anaerobical condition			
Mean width of inhibition zones (mm)		LSD test between groups		Mean width of inhibition zones (mm)		LSD test between groups	
Group I	2.888	I:II	0.0062*	Group I	3.668	I:II	0.0008*
Group II	0.460	II:III	0.0000*	Group II	0.494	II:III	0.0004*
Group III	4.123	I:III	0.1730	Group III	2.492	I:III	0.2052

*statistically significant differences

Discussion

Specific biochemical properties of lactic fermentation bacteria make it possible to use them not only in food industry, but in contaminated human environment. Sewage sludge not subjected to hygienization processes or mistreated becomes a carrier of pathogenic microorganisms, such as *Clostridium, Salmonella, Escherichia, Streptococcus, Staphylococcus, Listeria, Proteus, Vibrio, Bacillus etc.* That is why it is so important to eliminate this state as soon as possible. One of the methods is composting of sludge together with green material, which is a reservoir of lactic acid bacteria. So selected cultures of these bacteria are frequently used and composed into sets, called also vaccines.

In this study an attempt has been made to evaluate the biochemical properties of lactic acid bacteria isolated from different sources in respect of their antagonistic effect on selected pathogenic bacteria. The investigations were carried out under aerobic and anaerobic conditions, since there are different types of antibacterial metabolites produced in each of the mentioned environments. Under aerobic condition lactic acid and hydrogen peroxide are of the greatest importance, while under anaerobic conditions the most important are bacteriocins. The width of growth inhibition zones resulting from the action of definite substances were estimated in both cases. The results obtained indicate that the most versatile antagonists belong to group I (*L. plantarum, L. brevis)*, because they inhibit pathogen growth both under aerobic and anaerobic conditions (excepting the effect on *L. innocua* under aerobic conditions). Strong inhibition zones were also noticed in isolates of group III (from "Greenland" preparation) under aerobic conditions, while under anaerobic conditions these stains produced remarkable zones only against *E. coli, P. aeruginosa and P. mirabilis*. Isolates of group II did not show such strong properties as the other isolates of groups I and III. They are visibly least active against pathogens. *Escherichia coli* and *Salmonella* sp. were influenced by the highest number of the tested isolates.

Ogunbanwo *et al.* (6) reported that *L. brevis* OG1 was able to produce a bacteriocin which had a wide inhibitory spectrum in relation to both Gram-positive and Gram-negative bacteria causing food decay, as well as to pathogens. The strain of that bacteria inhibited the growth of 21 of 32 indicatory pathogenic strains, where the biggest inhibition zone was produced against *Enterococcus faecalis* EF1. The ability to hold the correct balance among microorganisms in the alimentary system by *Lactobacillus* sp. was also noticed by Sandine (9). Their increasing number in the environment and metabolites produced by bacteriocins resulted in the reduction in the number of pathogenic bacteria constituting the hazard forhuman health. Soerjadi (11) has also indicated out *Lactobacillus* as one of the most important antagonists of *Salmonella* sp. In presented studies group I which contained *L. brevis* and *L. plantarum* showed strong antagonistic activity against all the tested pathogens independently of experimental conditions (aerobic or anaerobic), which creates a possibility of their wide use.

The investigations on the influence of lactic acid bacteria on selected pathogens indicated that *L. plantarum* and *L. brevis* as well as the isolates from the EM "Greenland" preparation can reduce the number of pathogenic microorganisms very effectively. From the results it is evident that they can be successfully introduced into composted green materials as antagonistic vaccines.

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