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Research / Araştırma

ANTIMICROBIAL ACTIVITY SPECTRUMS OF SOME *BACILLUS* STRAINS FROM VARIOUS SOURCES

Ayşe Avcı*, Seda Üzmez, Firdevs Büşra Alkan, İpek Bagana, Esra Nurçeli, Enes Çiftçi

Sakarya University, Faculty of Engineering,
Department of Food Engineering, Serdivan, Sakarya, Turkey

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Abstract

During the recent years, production of antimicrobial substances by *Bacillus* strains have been attracted due to their broad antimicrobial spectrum. In the current study, some *Bacillus* strains which were previously isolated from some fermented foods as well as soil have been screened for antimicrobials production. Six of the isolates have been detected as antimicrobial substance producers. Antimicrobial activity was determined using disc diffusion assay against eight important food pathogens (*Bacillus cereus, Escherichia coli* O157:H7, *E. coli, Listeria monocytogenes, Salmonella Enteritidis, Salmonella Typhimurium, Staphylococcus aureus*. and *Pseudomonas aeruginosa*). The cell-free supernatants of the isolates used in current study did not inhibit the growth of *S. aureus*, however all inhibited *E. coli* O157:H7. The results showed that their antimicrobial spectrums were broad including both Gram negative and Gram positive bacteria. All the isolates produced the maximum amount of antimicrobials at 24 h and increased incubation periods caused the decrease in the production of antimicrobials.

Keywords: Antimicrobial substances, *Bacillus*, bacteriocin.

ÇEŞİTLİ KAYNAKLARDAN İZOLE EDİLMİŞ OLAN BAZI *BACİLLUS* SUŞLARININ ANTİMİKROBİYEL AKTİVİTE SPEKTRUMLARI

Özet

Son yıllarda, *Bacillus* suşları ile antimikrobiyel madde üretimine olan ilgi bunların geniş antimikrobiyel aktivite spektrumlarından dolayı artmıştır. Bu çalışmada, çeşitli fermente gıdalar ve topraktan izole edilmiş olan bazı *Bacillus* suşlarının antimikrobiyel madde üretimleri araştırılmıştır. Çalışılan mikroorganizmalardan 6'sının antimikrobiyel madde üreticisi olduğu belirlenmiştir. Antimikrobiyel aktivite 8 önemli gıda patojeni (*Bacillus cereus, Escherichia coli* O157:H7, *E. coli, Listeria monocytogenes, Salmonella Enteritidis, Salmonella Typhimurium, Staphylococcus aureus* ve *Pseudomonas aeruginosa*) kullanılarak disk difüzyon yöntemi ile belirlenmiştir. Bu çalışmada kullanılan izolatların süpernantları *S. aureus*'a etki etmemiş, ancak hepsi *E. coli* O157:H7'yi inhibe etmiştir. Elde edilen sonuçlar, mikroorganizmaların hem Gram negatif hem de Gram pozitif patojenlere etki eden geniş spektrumlu antimikrobiyel maddeler ürettiğini göstermiştir. En iyi antimikrobiyel madde 24 saatte üretilmiş ve artan inkübasyon sürelerinde antimikrobiyel aktivitenin düştüğü gözlenmiştir.

Anahtar kelimeler: Antimikrobiyel maddeler, *Bacillus*, bakteriyosin.

* Corresponding author /Yazışmalardan sorumlu yazar;
aysea@sakarya.edu.tr, (+90) 264 295 5464, (+90) 264 295 5608

INTRODUCTION

Bacteriocins are hydrophobic peptides which are ribosomally synthesized by generally Gram positive bacteria (1-3). They have antimicrobial effects on various microorganisms, including foodborne pathogens, by killing or inhibiting the growth (4, 5). Bacteriocins are considered as natural antimicrobials alternative to antibiotics, as they have been formed in many fermented foods and consumed by human for more than thousand years (6). During the last decades, great attention has been given for the screening of bacteriocin producing bacteria owing to their beneficial effects on food preservation (7, 8). Several reports are available representing the improved shelf-life and safety of foods by the help of bacteriocins as biopreservatives (9, 10).

Due to their natural occurrence in foods, production of bacteriocin has been extensively studied by lactic acid bacteria (LAB) including the species of *Lactobacillus, Pediococcus, Leuconostoc, Weisella*, and *Enterococcus* (1, 7, 8, 11, 12). In addition, most LAB are regarded as GRAS (generally recognized as safe) (10). LAB originated from fermented foods such as Turkish sucuk, boza, and cheese have been determined to produce bacteriocins which also contribute to the shelf-life of these products (13-15). However it has been reported that antimicrobial spectrum of LAB is narrow. Especially Gram negative pathogens in foods are more robust to bacteriocins from LAB (16).

Various species from *Bacillus* genus have been reported as bacteriocin producers such as *B. subtilis, B. coagulans, B. cereus, B. thuringiensis, B. megaterium*. Amongst them *B. subtilis, B. coagulans* and *B. licheniformis* are mostly investigated ones that produce subtilin, coagulin and bacitracin, respectively (3, 17). Antimicrobial spectrum of bacteriocins vary and some strains of *Bacillus* were reported as having broad antimicrobial spectrum (18). Therefore, bacteriocins by *Bacillus* spp. are of great interest for their potential application as food preservatives and therapeutic agents (19). In this study, we aimed to screen bacteriocin producing *Bacillus* strains which were isolated from different sources and determining their antibacterial spectrum over some foodborne pathogens.

MATERIALS AND METHODS

Microorganisms

Twelve *Bacillus* isolates that were previously isolated from various sources such as pickles, olives, and soil were screened for their antimicrobial effects on some food pathogens. They were defined as *Bacillus* spp. based on the morphological and some biochemical tests. Test organisms used for antimicrobial activity detection were *Bacillus cereus, Escherichia coli, E. coli* O157:H7, *Listeria monocytogenes, Salmonella Enteritidis, Salmonella Typhimurium, Staphylococcus aureus*. They were obtained from culture collection of Sakarya University, Food Engineering Department, except *Pseudomonas aeruginosa* which was supplied from Ankara University, Food Engineering Department.

Cultural conditions

Bacillus isolates were maintained in nutrient broth (Merck, Darmstadt, Germany) containing 50% glycerol at -18°C. They were activated by propagating on nutrient agar (Merck, Darmstadt, Germany) plates followed by incubation at 33°C for 24 h. Thereafter, single colonies were picked up and transferred into nutrient broth which was prepared in 100 mL Erlenmeyer flasks as 30 mL portions. Incubations were carried out on a shaking incubator (120 rpm) at 33°C for 24 h. This liquid culture was used as inoculum for the production of antimicrobials. In order to determine production of antimicrobials, Erlenmeyer flasks (100 mL) containing 30 mL of nutrient broth were inoculated with 5% (v/v) fresh *Bacillus* cultures and incubated aerobically at 33°C for 72 h on a shaking incubator (120 rpm). Separate samples were collected every 24 h for the determination of antimicrobial activity and growth. Analysis were performed at least in duplicate. Test organisms were maintained in tryptic soy broth (TSB; Merck, Darmstadt, Germany) containing 50% glycerol at -18°C. They were activated in TSB at 37°C for 24 h, except *L. monocytogenes* which was incubated at 30°C.

Antimicrobial activity assay

Samples were centrifuged at 10000 rpm for 10 min and supernatants were filtered through 0.45 µm Millex membrane filter (Merck Millipore,

Darmstadt, Germany). The resulting filtrates were used for antimicrobial activity measurements. Antimicrobial activity was determined using disc diffusion method as described by Cadirci and Cıtak (20) with some modifications. Briefly, 50 µL of 24 h actively grown test microorganisms (~10^8 cfu/mL) were spread on TSA plates which were then allowed around 30 min to diffuse cultures. Sterilized filter paper discs (Whatman No.1; 6 mm in diameter) were placed on the agar plates containing pathogens. Subsequently, 10 µL of cell-free supernatants were carefully applied in the center of the filter papers. Plates were incubated at 37°C, except *L. monocytogenes* which was incubated at 30°C for 24 h. At the end of the incubations, clear zones were measured by using a ruler.

Monitoring growth of Bacillus strains

Growth of the *Bacillus* strains were measured using UV-VIS spectrophotometer (UVmini 1240; Shimadzu, Japan). For this, samples containing bacterial cells were diluted 5-fold with deionized water and homogenized by vortexing. Optical densities of cell suspensions were measured at 600 nm.

Growth curve of *Bacillus* sp. GIT2 and production of antimicrobials during the growth

Bacillus sp. GIT2 which was one of the best isolates producing antimicrobials was chosen for the detection of antimicrobial production during the growth. Throughout the 48 h incubation time, periodical samples were taken and optical densities were measured to construct growth curve. Separate samples were taken for the determination of the antimicrobial activity by disc diffusion method. *L. monocytogenes* was used as indicator organism in this experiment.

RESULTS AND DISCUSSION

Antimicrobial activities of 12 *Bacillus* isolates have been determined. Six of them (*Bacillus* sp. BAST2, *Bacillus* sp. BMZE4, *Bacillus* sp. GIT2, *Bacillus* sp. ZBP10, *Bacillus* sp. ZGT3, *Bacillus* sp. ZGT9) have shown antimicrobial activity on various test organisms. All were Gram positive, rod shaped, catalase positive and spore forming strains isolated from some fermented foods and soil samples. Sources of the isolates having antimicrobial activity are given in Table 1.

Table 1. Origins of the Bacillus isolates possessing antimicrobial activity.

Isolate	Origin
Bacillus sp. BAST2	Homemade cucumber pickles
Bacillus sp. BMZE4	Homemade fermented olives
Bacillus sp. GIT2	Soil (Istanbul)
Bacillus sp. ZBP10	Soil (Sakarya)
Bacillus sp. ZGT3	Soil (Sakarya)
Bacillus sp. ZGT9	Soil (Sakarya)

The microorganisms were grown in nutrient broth and daily samples were taken and assayed for antimicrobial activities using disc diffusion method. The results obtained after 24 h are depicted in Table 2. Inhibitory spectrum of the bacteria varied and inhibition zone ranged from 6.5 to 10.3 mm. Among them *Bacillus* sp. ZGT9 was the strain having the narrowest inhibition spectrum which did not inhibit four of the test organisms. The other strains inhibited the growth of at least five bacteria over the eight test organisms.

As a general consideration, bacteriocins from Gram positive bacteria inhibit Gram positive bacteria and they are less effective against Gram negative bacteria [19]. For instance, Khalil *et al* (18) reported a *B. megaterium* strain inhibiting

Table 2. Antimicrobial spectrums of *Bacillus* isolates that were grown in nutrient broth at 33°C for 24 h on shaking incubator (120 rpm).

Indicator Bacteria		Zone of inhibition (mm)					
	BAST2	BMZE4	GIT2	ZBP10	ZGT3	ZGT9	
Gram Positive	*B. cereus*	10.3±0.4	7.8±0.4	10.0±0.0	7.3±0.4	8.8±0.4	9.3±0.4
	L. monocytogenes	N.A*	8.5±0.7	9.5±0.7	8.0±0.0	8.5±0.7	N.A
	S. aureus	N.A	N.A	N.A	N.A	N.A	N.A
Gram Negative	*E. coli* O157:H7	8.8±0.4	8.3±0.4	9.5±0.7	8.0±0.0	9.5±0.7	9.0±0.0
	E. coli	10.0±0.0	10.0±0.0	N.A	8.0±0.0	10.0±0.0	N.A
	P aeruginosa	9.0±1.4	N.A	9.5±0.7	8.0±0.0	8.5±0.0	8.8±0.4
	S. Enteritidis	8.5±0.7	8.5±0.7	9.5±0.7	9.0±0.0	N.A	N.A
	S. Typhimurium	6.5±0.0	N.A	8.0±0.0	N.A	9.0±0.0	7.0±0.0

*N.A: No Activity

Gram positive bacteria such as *S.* Typhimurium, *B. licheniformis* ZJU12, but not the Gram negatives. In another study, Cherif et al. (17) searched the antimicrobial activity of *B. thuringiensis* subsp. *entomocidus* HD10 against Gram negative bacteria and found no activity. However in our study, it was not the case that the action of the antimicrobials from the *Bacillus* isolates had different mode of action which was not attributed to Gram characteristics of the test organisms. None of the isolates inhibited the growth of Gram positive *S. aureus*. On the other hand, all of the strains interestingly inhibited the growth of *E. coli* O157:H7 which is Gram negative. On contrary to general approach, similar findings to our results were also reported that proves the antimicrobial activity of bacteriocins from *Bacillus* against the bacteria having different Gram reactions (18, 19, 21). Some lactic acid bacteria were also reported with the same property (10). In the literature, it was cited that narrow spectrum of bacteriocins limits the application in foods (21). Thus, discovery of novel strains with large antibacterial spectrum would help to expand food applications.

Bacteriocins are mostly effective against closely related strains (17). It has been argued that bacteriocins are generally originated from plasmids which are incidentally produced as a by-product of protein mechanism. Due to the incidental mutation on the plasmids, the bacteria can inhibit the competitive flora and dominate in the medium (22). Our findings are in accord with this phenomenon that all of the isolates have shown antimicrobial activity against *B. cereus* (Table 2).

An interesting outcome of this study is that all the strains had inhibitory effect over *E. coli* O157:H7 which is one of the most important foodborne pathogens causing serious health problems almost in all regions of the world. It is mainly found in milk and meat products and have a low infectious dose (~50 CFU) (23). To our knowledge, there are few studies in the literature about the effects of bacteriocins on *E. coli* O157:H7. Bacteriocins from *Enterococcus feacium, Pediococcus pentosaceus* (16), *L. mesenteroides* (24), *P. acidilactici* (25) have been shown as ineffective on *E. coli* O157:H7. There are some reports representing the inhibitory action of bacteriocins on this pathogen. For instance, Rodriguez *et al.* (26) tested the survival of *E. coli* O157:H7 in the presence of bacteriocin producing LAB in cheese during ripening. They found an inhibition after 30 days of storage when they used *Lc. lactis* CL2 which produces nisin and pediocin.

Antimicrobial activities of the isolates were also measured after 48 and 72 hours of the production against the same pathogens. It has been observed that the most of the strains did not show antibacterial effects after prolonged incubations, except *Bacillus* sp. GIT2 and *Bacillus* sp. ZBP10. Antimicrobials from *Bacillus* sp. GIT2 inhibited *B. cereus, E. coli* O157:H7, *L. monocytogenes, S.* Enteritidis, and *S.* Typhimurium after 48 h and 72 h. Those from *Bacillus* sp. ZBP10 inhibited *B. cereus*, *E. coli* O157:H7, *E. coli*, *L. monocytogenes, P. aeruginosa* and *S.* Enteritidis (Results are not shown).

Growths of the isolates were given in Table 3. As can be seen from the table, cell concentrations of the strains are distant from each other even though they were grown in the same conditions. *Bacillus* sp. ZGT9 had the highest cell concentration and *Bacillus* sp. GIT2 had the lowest. These results indicate that there is no correlation between cell concentration and antimicrobial production. Because *Bacillus* sp. ZGT9 was the bacterium having narrowest antibacterial spectrum that had shown inhibitory effect on only four pathogens (*B. cereus, E. coli* O157:H7, *P. aeruginosa*, *S.* Typhimurium). In a similar manner, *Bacillus* sp. GIT2 which has the lowest growth amongst the strains had the better zone of inhibition, as well as inhibition spectrum. Growth decreased at 48 h, as microorganisms entered death phase and continuous decrease have been observed at 72 h of the incubation.

Table 3. The growth of *Bacillus* isolates in nutrient broth measured as optical density.

Microorganism	Microbial growth (OD_{600})		
	24 h	48h	72 h
Bacillus sp. BAST2	4.87±0.25	3.00±0.3	3.22±0.17
Bacillus sp. BMZE4	4.62±0.05	3.40±0.02	2.99±0.13
Bacillus sp. GIT2	3.57±0.03	1.86±0.11	2.02±0.19
Bacillus sp.ZBP10	5.56±0.28	3.16±0.33	2.38±0.35
Bacillus sp. ZGT3	5.79±0.05	4.38±0.01	3.30±0.09
Bacillus sp. ZGT9	7.40±0.29	3.57±0.37	2.92±0.03

As *Bacillus* sp. GIT2 had possessed better inhibition zone against *L. monocytogenes* and *E. coli* O157:H7, we have chosen this bacterium to make further investigations on the relation of antimicrobials production with the growth. For this, periodical samples were taken and subjected to antibacterial activity measurements against *L. monocytogenes* and growth curve of the bacterium was constructed from the optical density measurements (Figure 1).

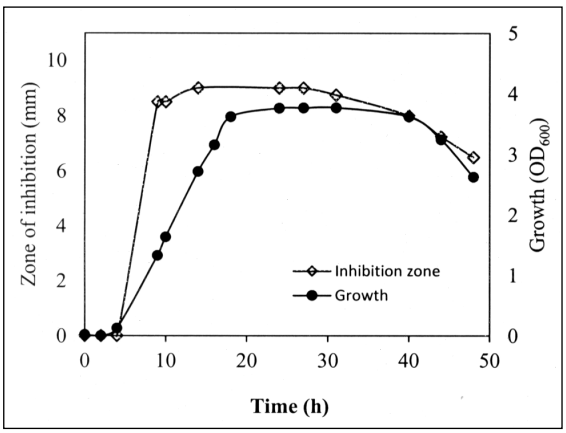

Figure 1. Relation of growth and antimicrobial activity of *Bacillus* sp. GIT2. *L. monocytogenes* was used as test organism for the determination of antimicrobial activity.

After 4 h of lag phase, the bacterium entered exponential phase at which production of antimicrobials have started. The first antimicrobial activity has been observed in the sample taken at the 9th hour of the growth. It almost reached to maximum during the exponential phase and retained until the end of the stationary phase (30th hour of the growth). Afterwards measured activities started to decrease. According to the results, it can be suggested that production of antimicrobials is associated with growth. Most of the bacteria, including LAB and *Bacillus* strains, generally produce bacteriocins as primary metabolites (21, 27-30). Decrease in the antimicrobial activity attributed to the proteolytic enzymes produced during the growth of the cells (3).

CONCLUSIONS

Antibacterial activities of some *Bacillus* strains which were isolated in our lab have been determined. It has been observed that they had relatively wide antimicrobial activity spectrum. Unlike most of the bacteriocin producing bacteria, they inhibited Gram negative pathogens including *E. coli* O157:H7 and *S.* Typhimurium. This feature makes these bacteria a potential for use as biopreservatives. However further investigations are necessary for the characterization of the antimicrobials from those strains.

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