ACTA MICROBIOLOGICA BULGARICA

Screening for Antimicrobial Activity of *Bacillus subtilis* and *Paenibacillus* Alvei Isolated From Rotten Apples Compost

Natalija Atanasova-Pancevska[1*], Ilina Popovska[1], Katarina Davalieva[2], Dzoko Kungulovski[1]

[1]*Department of Microbiology and Microbial Biotechnology, Institute of Biology, Faculty of Natural Sciences and Mathematics, "Ss. Cyril and Methodius" University, Skopje, Macedonia*
[2]*Research Centre for Genetic Engineering and Biotechnology "Georgi D Efremov", Macedonian Academy of Sciences and Arts, Skopje, Republic of Macedonia*

Abstract

New antimicrobial compounds are continuously required to combat antibiotic-resistant bacteria and pathogenic yeast as such resistance increasingly limits the effectiveness of current antimicrobial drugs. New screening approaches, including the search for novel targets and exploration of non-conventional places as sources of producer microorganisms, are needed.

Rotten apples compost samples from the composting plant in Resen, Macedonia, were screened for microbial colonies which form a clear zone of inhibition. The isolated bacterial strains were further screened for their antimicrobial activity against different bacteria and fungi by diffusion agar method. The isolates were later identified as *Paenibacillus alvei* and *Bacillus subtilis* by morphological studies and sequencing of the 16S rRNA bacterial gene. The maximum growth of *P. alvei* and *B. subtilis* for different cultivation media, pH, temperature, time of incubation and glucose concentration were determined. Maximum growth of *P. alvei* and *B. subtilis* was observed in MHB, at pH 6 after 24 hours of incubation at 30 °C for *P. alvei*, and at pH 7 after 48 hours at 40 °C for *B. subtilis.*

In conclusion, the identified strains with antimicrobial activity in this study are a useful addition to the worldwide effort for new antibiotic discovery.

Key words: *Paenibacillus alvei; Bacillus subtilis*; antimicrobial activity; optimization; 16S rRNA squencing.

Резюме

В борбата с резистентните бактерии и патогенни дрожди непрекъснато се изискват нови антимикробни съединения, тъй като тяхната резистентност ограничава ефективността на настоящите антимикробни лекарства. За това са необходими нови подходи за скрининг, включително и за търсенето на нови целеви места и неконвенционални източници на микроорганизми-продуценти.

Проби от гниещи ябълки, събрани от завода за компостиране в Ресен, Македония, са скринирани за колонии от микроорганизми, образуващи зона на инхибиране. Изолираните бактериалните щамове са изследвани допълнително за тяхната антимикробна активност срещу различни бактерии и гъби по метода за дифузия в агар. Изолатите са идентифицирани като *Paenibacillus alvei* и *Bacillus subtilis* чрез морфологични проучвания и секвениране на 16S рРНК. Установен е максималня растеж на *P. alvei* и *B. subtilis* върху различни среди за култивиране, pH, температура, време на инкубиране и концентрация на глюкоза. Максимален растеж на *P. alvei* се наблюдава в среда МБ и pH 6, след 24 часа инкубиране при 30°C и за *B. subtilis* ва същата среда с pH 7, след 48 часа инкубиране при 40°C.

В заключение, изолираните и идентифицирани щамове с антимикробиална активност в настоящето изследване са полезно допълнение към световните усилия за откриване на нови антибиотици.

Introduction

Due to the increasing numbers of resistant pathogenic bacteria and side effects caused by existing antibiotics, new antimicrobial compounds with effective properties are needed (Devasahayam *et al*., 2010).

*Corresponding author: natalijaap@gmail.com

Antimicrobial peptides are attracting increasing interest as a potential substitute and the best alternatives for biological control (Papagianni, 2003; Lee *et al*., 2008). Antimicrobial peptides are secondary metabolites produced by microorganisms via enzymatic catalysis involving a series of biosynthetic pathways (Demain *et al*., 1983). These peptides occur in living organisms and are produced as defense molecules against pathogens such as bacteria. Therefore, antimicrobial peptides are considered the first line of defense in invaded eukaryotic and prokaryotic cells (Papagianni, 2003).

It was found that the effect of different factors affecting growth such as incubation period, temperature, pH, aeration, addition of surfactants and the constituents of the production medium used, especially carbon sources, nitrogen sources and minerals, depends on the microbial strain and its growth rate (Vincent and Priestly, 1975; Kaur *et al*., 2001).

This study aimed to screen for antimicrobial activity of isolates from rotten apples compost against some microorganisms, to identify the strains using a molecular identification method based on 16S rRNA sequencing and to suggest the optimum conditions for maximum antimicrobial activity of the selected strains.

Materials and methods

Collection of compost samples

Rotten apples compost samples were collected from the composting plant in Resen, Macedonia. The collected samples were stored into sterile glass screw cap bottles. The collected compost sample was kept for screening and isolation of different microorganisms with antimicrobial potential.

Isolation of microorganisms with antimicrobial potential

Fifty grams of compost were taken and added to 250 ml sterile distilled water in a 500 ml Erlenmeyer flask. The flask was shaken on an orbital shaker for 30 min at 27 °C and serial dilutions from 10^{-1} to 10^{-6} were performed. From each dilution, about 0.5 ml of sample was taken and placed on Muller Hinton agar (MHA) medium along with antimycotic cycloheximide (5 g mL^{-1}) using pour plate technique and incubated at 27 °C for 1 week. After the incubation period, the plates were observed for microbial colonies which had formed a clear zone of inhibition. They were selected and picked up by a sterilized wire loop and sub-cultured on MHA to obtain pure bacterial colonies. The pure cultures were preserved on agar slants of Muller Hinton medium for further studies.

Screening for antimicrobial activity

The isolates were initially screened for their antimicrobial activity by a diffusion agar method (Hasegawa *et al*., 1990). They were inoculated into Muller Hinton broth medium and incubated for 48 hours at 27 °C. As test microorganisms were used freshly prepared (*i.e,* 24-hour-old) cultures of Gram-positive bacteria (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Micrococcus luteus*), Gram-negative bacteria (*Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 9027 inoculated in MHB medium), yeasts (*Candida albicans* ATCC 10231*, Saccharomyces cerevisiae* ATCC 9763) and molds (*Aspergillus niger* ATCC 16404*,* cucumber mold, tangerine mold and *Penicillium* sp. inoculated in Sabouraud dextrose broth medium). One hundred microliters of each test microorganism were transferred into 15 ml of appropriate molten medium at 45 °C and poured into a separate empty sterile petri plate. The agar was allowed to solidify for about 30 minutes and 10 µl of the isolates which showed antimicrobial activity were transferred on 5 mm sterile filter paper disks, previously placed on properly marked places on the surface of agar plates inoculated with test microorganisms. Sterile distilled water (10 µl) was used as a negative control and gentamicin (or cycloheximide) antimicrobial susceptibility test disks (10 µg) were used as a positive control. The petri plates were kept in a refrigerator for 1 hour before incubation to permit the diffusion of antimicrobial substances (Rizk *et al*., 2007). The plates were then incubated for 5 days at 25 °C for yeast and molds, and 72 hours at 37 °C for bacteria. After incubation, the diameters of the inhibition zones were measured.

Identification of the microorganisms by 16S rRNA sequencing technique

The phenotypic properties of the selected strains were determined using the methods described in Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). The selected antimicrobial strains were identified by sequencing of the 16S rRNA gene. First, DNA from each strain was isolated. Each pure colony was grown overnight in the appropriate medium, cells were harvested by centrifugation (14000 rpm, 10 min), washed twice with 1xPBS buffer (140 mM NaCl, 2.7 mM KCl, 100 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.3) and kept at -20°C until further processing. DNA extraction was done using PrepManUltra reagent (Applied Biosystems), following the protocol for culture broth samples. The concentration of DNA was

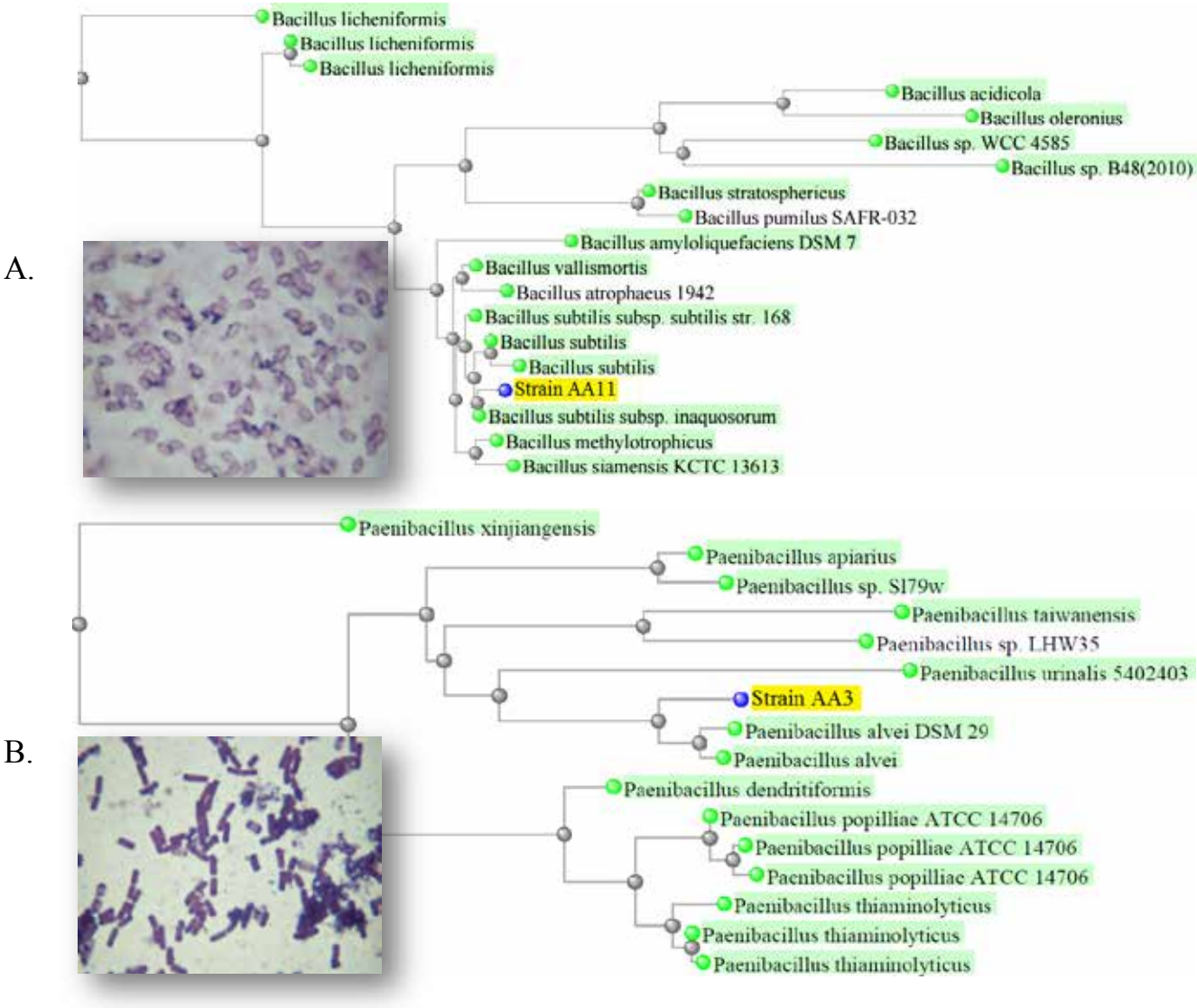

Fig. 1. Identification of bacterial strains with highest antimicrobial activity. Phylogenetics trees were obtained by BLAST pairwise alignments of NCBI bacterial 16S ribosomal RNA sequences against 16S rRNA sequences of AA11 and AA3 strains. The strains were identified as strains of *Bacillus subtilis* (**A**) and *Paenibacillus alvei* (**B**). The magnification of the microscopic images shown is 100×.

determined spectrophotometrically. DNA working solution of 2.7 – 3.1 ng/µl was prepared by diluting the stock DNA. The sequence of the 16S ribosomal RNA gene (rDNA) of bacterial strains was determined using MicroSeq Full Gene Kit (Applied Biosystems), composed of two parts: MicroSeq® Full Gene 16S rDNA Bacterial Identification PCR Kit and MicroSeq® Full Gene 16S rDNA Bacterial Identification Sequencing Kit. Amplification of the three fragments of the 16S ribosomal RNA gene was done using 7.5 µl DNA working solution in a reaction volume of 15 µl on 2720 Thermal Cycler (Applied Biosystems). Purification of the amplified products was done using ExoSAP-IT® reagent (USB) according to the manufacturer's instructions prior to sequencing. The cycle sequencing was performed with forward and reverse primers for each amplified product according to the instructions provided by the kit with one exception: the final volume of the sequencing reactions was 10 µl. After cycle sequencing, excess dye terminators and primers were removed from the cycle sequencing reactions by precipitation in separate tubes with 2 µl 5M Na-acetate and 50 µl ethanol. After incubation at room temperature for 30 min, the tubes were centrifuged at 14000 rpm for 30 min, the supernatant was discarded, the precipitate was dried for 5 min at room temperature and re-suspended in 20 µl of Hi-Di™ Formamide. Sequence analyses were performed on a 3500 Genetic Analyzer (Applied Biosystems).

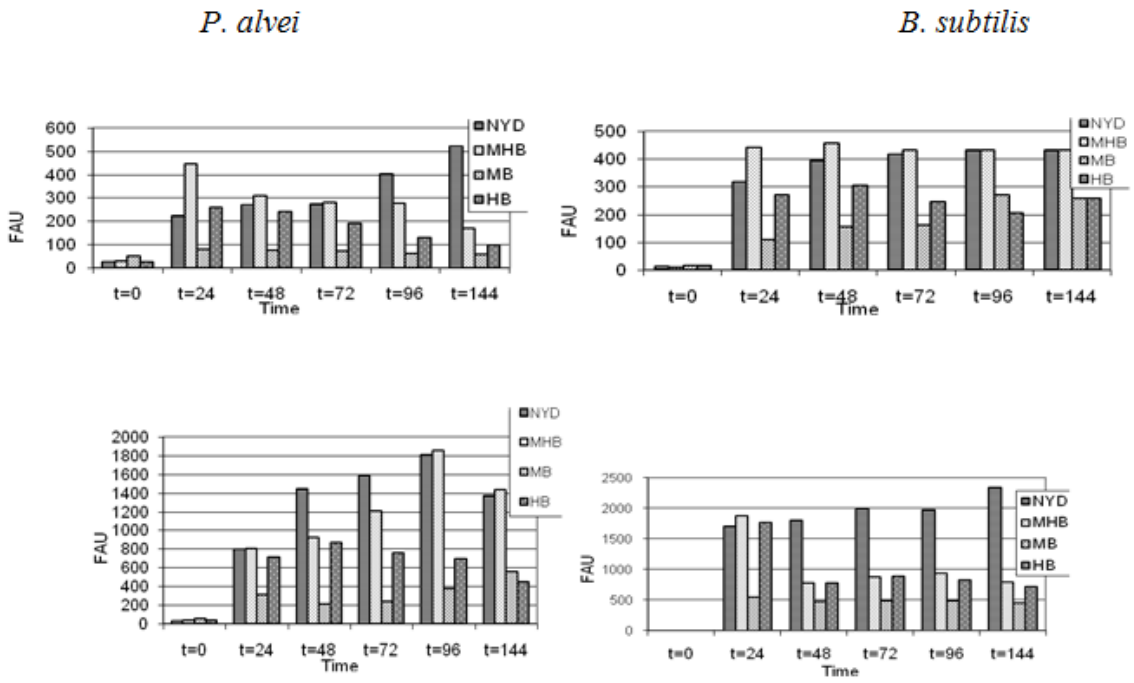

Fig. 2. Determination of optimal cultivation medium for growth of *P. alvei* and *B. subtilis* in a stationary series (first row) and series on a rotary shaker (second row).

Optimization of various parameters for maximum activity

Various parameters such as cultivation medium, pH, temperature, time of incubation and glucose concentration were tested to determine the optimal conditions for microbial growth.

In order to determine the most effective medium for mass cell production of isolates, four different media were tested: Muller Hinton broth, Nutrient broth, NYD medium (Li *et al*., 2009) and Mannitol broth. Fifty mililiters of each medium were aliquoted in four separate 100 ml Erlenmeyer flasks and autoclaved. Each flask was inoculated with 1.5 ml of 24-hour-old cultures from each isolate. Two Erlenmeyer flasks of each medium were incubated stationary at 25°C and the remaining two on a rotary shaker at 120 rpm, at ambient room temperature (22-28°C). Bacterial growth was monitored by measuring the turbidity of a culture at the beginning and every 24 hours untill 144 hours using a spectro-

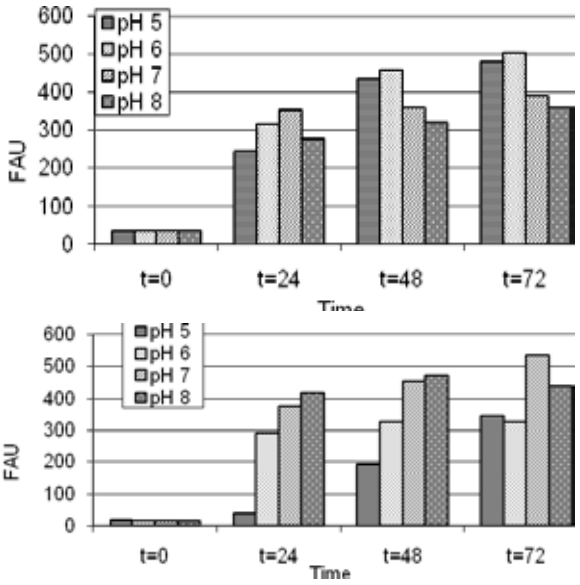

Fig. 3. Effect of various pH levels on the growth of *P. alvei* and *B. subtilis* in a stationary series.

Table 1. Changes in the pH of the media after 72 hours of incubation

P. alvei	Before incubation	pH 5	pH 6	pH 7	pH 8
	After incubation	pH 6.77	pH 7.00	pH 7.04	pH 7.35
B. subtilis	Before incubation	pH 5	pH 6	pH 7	pH 8
	After incubation	pH 5.14	pH 5.53	pH 6.72	pH 7.15

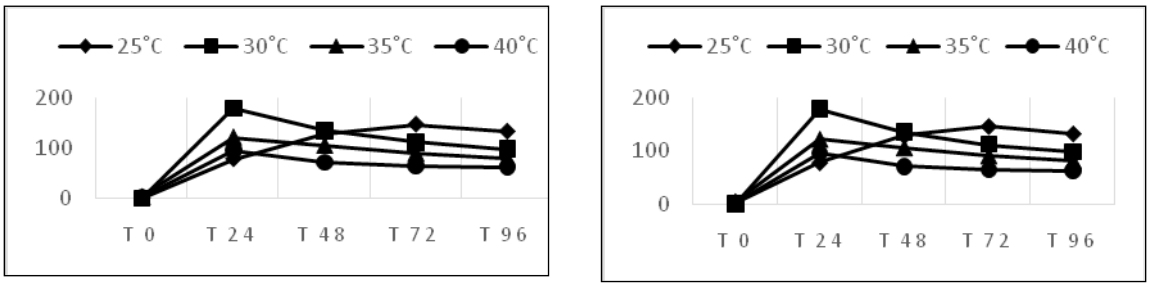

Fig. 4. Effect of temperature and time of incubation on the growth of *P. alvei* and *B. subtilis*.

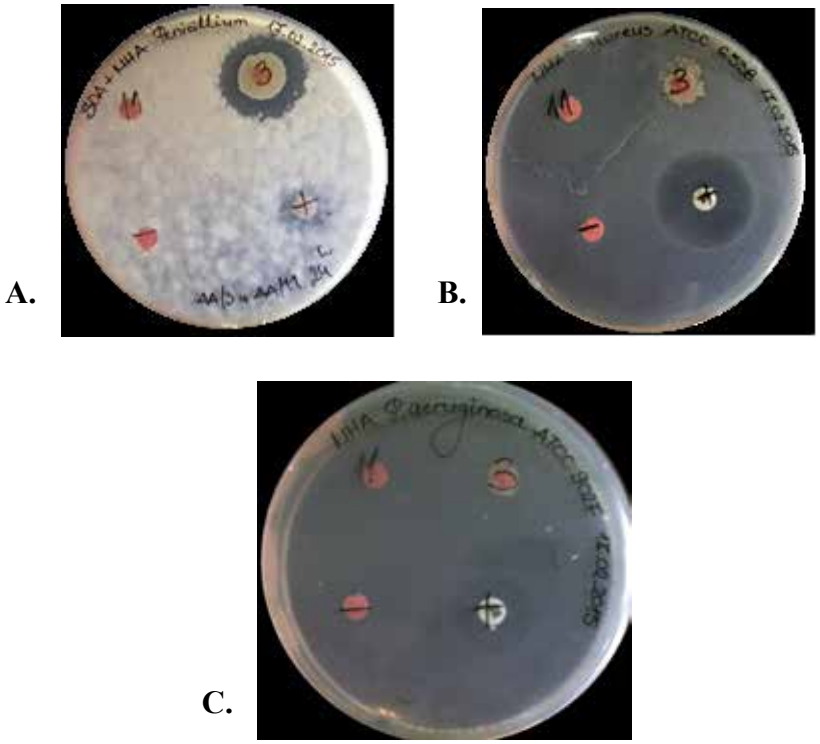

Fig. 5. Diffusion agar method for determining **A**) the antifungal activity of *P. alvei* against *Penicillium* sp. **B**) the antibacterial activity of *B. subtilis* against *S. aureus* ATCC 6538. **C**) *P. alvei* and *B. subtilis* show no antibacterial activity against *P. aeruginosa* ATCC 9027.

Table 2. Antibacterial activity of *P. alvei* and *B. subtilis* against different bacterial species

Diameter of zone of inhibition (mm)				
Test organism	*P. alvei*	*B. subtilis*	Control (10 µg gentamicin)	Control (sd H_2O)
E. coli ATCC 8739	15 bs[1]	25 bc[2]	13 bc 27 bs	0
B. subtilis ATCC 6633	14 bc	8 bc	28 bc	0
P. aeruginosa ATCC 9027	0	0	17 bc	0
S. aureus ATCC 6538	15 bc	40 bc	26 bc	0
M. luteus	20 bc	0	26 bc	0

1- bactericidal effect; 2- bacteriostatic effect

Table 3. Antifungal activity of *P. alvei* and *B. subtilis* against different fungal species

Diameter of zone of inhibition (mm)				
Test organism	*P. alvei*	*B. subtilis*	Control (10 µg cycloheximide)	Control (sd H_2O)
C. albicans ATCC 10231	20 fc[1]	0	0	0
S. cerevisiae ATCC 9763	15 fc	0	46 fc	0
A. niger ATCC 16404	18 fc	0	12 fs[2]	0
Cucumber mold	27 fc	0	0	0
Tangerine mold	17 fc	0	11 fs	0
Penicillium sp.	24 fc	0	14 fs	0

1- fungicidal effect; 2- fungistatic effect

photometer (Lovibond, Photometer MaxiDirect) to determine transmission or absorbance.

The influence of pH on bacterial growth was tested using the determined optimal medium at pH ranging from 5-8. pH was adjusted before autoclaving. For each pH, two Erlenmeyer flasks with 50 ml medium were prepared, autoclaved, then inoculated with 1.5 ml of inoculum of 24-hour-old cultures and incubated stationary at 25 °C. The turbidity of a culture was measured before autoclaving and every 24 hours untill 72 hours, after which the pH of the medium was again registered in order to observe media pH changes.

The effects of temperature and incubation period on the bacterial growth were studied at 25, 30, 35 and 40°C under different incubation periods (0, 24, 48, 72 and 96 hours) using determined optimal medium and determined optimal pH. For each culture, eight tubes with 7 ml medium were prepared, inoculated with 70 µl 24-hour-old culture isolates and incubated at four different temperatures. The turbidity of a culture was measured before autoclaving and every 24 hours untill 96 hours.

Results

Isolation of microorganisms with antimicrobial potential

In the course of screening for novel substances with antimicrobial potential from rotten apples compost samples collected from the composting plant in Resen, Macedonia, two bacterial colonies (AA3 and AA11) were isolated and screened using MHA medium. The isolated cultures were sub-cultured on MHA slants and preserved in a refrigerator at +4°C and on glass beads at -20°C. Further screening was done to determine the bioactivity of the isolates.

Identification of microorganisms

Morphological studies showed that the isolates are Gram-positive, sporulating, rod shaped bacteria (Fig. 1). Alignment of the 16S rRNA sequences of the 2 bacterial species revealed identity of 99% to the genus Bacillus. Isolates AA3 and AA11 were identified as *Paenibacillus alvei* and *Bacillus subtilis*, respectively. Inoculated on MHA, *P. alvei* produced large, circular, rough, white-yellowish colonies with irregular margins and *B. subtilis* produced very small, circular, convex and smooth, white in color colonies. The spores of *B. subtilis* and *P. alvei* are smooth, spherical and green in color using the Schaeffer and Fulton staining method.

Optimization of various parameters for bacterial growth

Maximum growth of *P. alvei* in a stationary and rotary shaker series was observed in MHB after 24 and 96 hours of incubation, respectively (Fig. 2). Maximum growth of *B. subtilis* in a stationary and rotary shaker series was observed in MHB after 48 and 24 hours of incubation, respectively (Fig. 2).

Regarding the optimal pH of the growth medium according to the results of the previous experiment, maximum growth for *P. alvei* was observed at pH 6, and for *B. subtilis* at pH 7 after 72 hours of incubation (Fig. 3). After 72 hours of incubation, changes in the pH of the media inoculated with *P.*

alvei and *B. subtilis* were observed (Table 1).

The effects of temperature and incubation period on the bacterial growth of *P. alvei* and *B. subtilis* were studied in MHB at pH 6 and pH 7, respectively. The results showed that the optimal temperature for growth of *P. alvei* is 30 °C and the optimal incubation period is 24 hours. The optimal temperature for growth of *B. subtilis* is 35 °C and the optimal incubation period is 48 hours (Fig. 4).

Preliminary screening for antimicrobial activity

The isolated bacterial strains from rotten apples compost were screened for secondary metabolites with antimicrobial activity by diffusion agar method under conditions which showen as optimal in previous experiments. *Paenibacillus alvei* showed potential antifungal activity against all tested yeasts and molds, and antibacterial activity. Fig. 5A shows the potential antifungal activity of *P. alvei* against *Penicillium* sp. *Bacillus subtilis* showed potential antibacterial activity against some Gram-positive and Gram-negative bacteria, but no antifungal activity. Fig. 5B shows antibacterial activity of *B. subtilis* against *S. aureus* ATCC 6538. Fig. 5C shows that *P. alvei* and *B. subtilis* have no antibacterial activity against *P. aeruginosa* ATCC 9027. *P. alvei* and *B. subtilis* also exhibited a potent antimicrobial activity against a wide range of both Gram-negative and Gram-positive bacteria and fungi (Table 2 and Table 3).

Discussion

Soil is a prosperous source of microorganism that produces a wide range of antibiotics including peptide antibiotics (Awais *et al.,* 2008; Janabi, 2006). The development of antibiotics resistance and lesser safety margins provoked scientists to search for antimicrobial agents with modified properties and maximum activity. *Bacillus* species have been considered as extremely useful microorganisms for producing antimicrobial agents (Amin *et al.,* 2012). *Bacillus* species are aerobic or facultative anaerobic, sporulating, rod-shaped, Gram-positive bacteria (Ali Janabi *et al*., 2006; Graumann, 2007). Some species may turn Gram-negative with age (Baron, 1996). The *Bacillus* species are known for the synthesis of secondary metabolites with remarkable diversity both in structure and function (SiloSuh *et al*., 1994). The *Bacillus* species are most popular for producing peptide antibiotic compounds such as polymyxin, colistin and circulin (Katz and Demain, 1997). In this study we have also identified a Bacillus strain with strong antimicrobial activity.

The identified *B. subtilis* shows potent antibacterial activity. The most sensitive strain to its antibacterial activity was *S. aureus* ATCC 6538, against whom *B. subtilis* forms a 40 mm inhibition zone. Awais *et al.* (2007) studied the inhibitory effects of a *Bacillus* sp. isolate against two pathogenic strains of *Micrococcus luteus* ATCC 10240 and *Staphylococcus aureus* ATCC 6538 and determined an 18 mm inhibition zone for these two indicator strains. In our study, *B. subtilis* showed no antifungal activity, but antimicrobial studies of Moshafi *et al*. 2015 determined its ability to inhibit *C. albicans, A. flavus* and *A.niger* as well.

The other identified strain with antibacterial activity was *P. alvei. P. alvei* are Gram-positive, rod-shaped, motile, spore-forming and catalase-positive bacteria (Najafi *et al.,* 2011). The first report of antimicrobial peptide production by these bacteria was by Anandaraj *et al*. 2009, who isolated a strain from fermented tomato fruit and detected two antimicrobial peptides, Paenibacillin P and Paenibacillin N. *P. alvei* has potent antibacterial and antifungal activity and showed the highest zones of inhibition against *M. luteus* (20 mm) and cucumber mold (27 mm). The antibacterial activity of *P. alvei* AN5 against different bacterial strains was confirmed by Alkotaini *et al.,* (2013) and Al-Obaidy (2010). These studies showed that *P. alvei,* isolated from rhizosphere soil of garden flowers, had a wide range of antifungal activities toward different kinds of pathogenic fungi.

In order to achieve rapid and unambiguous identification of the strains that showed the highest antimicrobial activity, molecular methods of identification were preferred over culture-based methods. Determinative bacteriology based on culture-based methods involves time-consuming isolation, cultivation and characterization of phenotypic traits, which is often not discriminatory and can take days to weeks for unambiguous identification. The 16S rRNA gene, universally present in all bacteria, has both highly conserved and more variable domains, which makes it an ideal target for studying phylogenetic relationships and obtaining precise and reliable identification.

In this study different experiments were performed to determine the most favorable conditions for growth of *B. subtilis* and *P. alvei*. The highest turbidity of *P. alvei* and *B. subtilis* in a stationary series and on a rotary shaker incubation was observed in MHB. The observed increasing turbidity in the NYD medium as a function of the incuba-

tion period may be due to the high concentration of sugars in the medium (10 gL^{-1} glucose). Glucose initially inhibited the growth of the cells and then as the cells adapted and began to exploit sugars, their concentration decreased and subsequently, the bacterial cells growth increased. The optimal pH for *P. alvei* and *B. subtilis* was determined to be pH 6 and pH 7, respectively, after 72 hours of incubation. Amin *et al.* (2012) studied the effect of pH on zones of inhibition produced by *Bacillus* sp. GU 057 and the widest inhibitory zone was reported at pH 8 after 48 hours of incubation. The media pH changes during the incubation suggest that bacteria have an ideal buffer system and they have the ability to adjust the pH which is optimal for them. Alkotaini *et al*. (2013) showed that *P. alvei* AN5 have high stability against pH ranges. According to the obtained results from this study, the optimal growth temperature for *P. alvei* is 30 °C during the 24 hours of incubation and the optimal growth temperature for *B. subtilis* is 35 °C during the 48 hours of incubation. The result from Amin *et al.* (2013) showed that 48 hours of incubation at 40 °C have greatest effect on the growth and secondary metabolites (antibiotics) synthesis of *B. subtilis*. Alkotaini *et al*. (2013) showed that *P. alvei* AN5 have high heat stability and formed great zones of inhibition at 90 °C.

The results presented here pave the way for future research of the compounds responsible for the antimicrobial activity of the isolated strains of *P. alvei* and *B. subtilis*. In addition, testing for the activity of these strains over a broader range of patogenic microorganisms, including resistance testing and possible clinical application, are projected for future research.

References

Alkotaini, B., N. Anuar, A. A. H. Kadhum, A. A. A. Sani (2013). Detection of secreted antimicrobial peptides isolated from cell-free culture supernatant of *Paenibacillus alvei* AN5. *J. Ind. Microbiol. Biotechnol*. **40**: 571–579.

Al-Obaidy, M. O. (2010). Isolation and identification of wide-spectrum antifungal bacteria. *Raf. Jour. Sci*. **21**(3): 1-10.

Amin, A., A. M. Khan, M. Ehsanullah, U. Haroon, F. M. S. Azam, A. Hameed (2012). Production of peptide antibiotics by *Bacillus* sp. GU 057 indigenously isolated from saline soil. *Braz. J. Microbiol*. **43**(4): 1340-1346.

Anandaraj, B., A. Vellaichamy, M. Kachman, A. Selvamanikandan, S. Pegu, V. Murugan (2009). Co-production of two new peptide antibiotics by a bacterial isolate *Paenibacillus alvei* NP75. *Biochem. Biophys. Res. Commun.* **379**: 179–185.

Awais, M., A. Ali Shah, A. Hameed, F. Hasan (2007). Isolation, identification and optimization of bacitracin produced by *Bacillus* sp. *Pak. J. Bot.* **39**: 1303-1312.

Awais, M., A. Pervez, S. Qayyum, M. Saleem (2008). Effects of glucose, incubation period and pH on the production of peptide antibiotics by *Bacillus pumilus*. *Afr. J. Microbiol. Res.* **2**: 114-119.

Baron, S. (1996). Medical Microbiology. 4th ed. Texas, TX, USA: University of Texas Medical Branch.

Demain, A. L., Y. Aharonowitz, J. F. Martin (1983). Metabolite control of secondary biosynthetic pathways. in: L. C. Vining (Ed.), Biochemistry and Genetic Regulation of Commercially Important Antibiotics. Addison-Wesley, London, pp. 49-67.

Devasahayam, G., W. M. Scheld, P.S. Hoffman (2010). Newer antibacterial drugs for a new century. *Expert. Opin. Investig. Drugs.* **19**: 215–234.

Graumann, P. (2007). *Bacillus*: Cellular and Molecular Biology. 1st ed. Caister Academic Press.

Hasegawa, S., N. Kondo, F. Kodama (1991). Suppression of Fusarium wilt of adzukibean by rhizosphere microorganisms. *Pest. Alter.* 477-486.

Holt, J. G., N. R. Krieg, P. H .A. Sneath, J. T. Staley, S. T. Williams (1994). Bergey`s Manual of Determinative Bacteriology. 9th Ed. Williams and Wilkins, Baltimore. 518-537.

Janabi, A. A. H. (2006). Identification of Bacitracin produced by local isolate of *Bacillus licheniformis*. *Afr. J. Biotechnol.* **5**(18): 1600-1601.

Katz, E., A. L. Demain (1997). The peptide antibiotics of *Bacillus*, chemistry, biogenesis, and possible functions. *Bacteriol. Rev.* **41**: 449-474.

Kaur, S., R. M. Vohra, M. Kapoor, Q. K. Beg, G. S. Hoondal (2001). Enhanced production and characterization of a highly thermostable alkaline protease from *Bacillus* sp. P-2. *World. J. Microb. Biot.* **17**: 125-129.

Lee, Y.K., M. Senthilkumar, J.H. Kim, K. Swarnalakshmi, K. Annapurna (2008). Purification and partial characterization of antifungal metabolite from *Paenibacillus lentimorbus* WJ5. *World. J. Microb. Biot.* **24**(12): 3057- 3062.

Li, J., Y. Qian, Z. Li-hua, Z. Shu-mei, W. Yu-xia, Z. Xiao-yu (2009). Purification and characterization of a novel antifungal protein from *Bacillus subtilis* strain B29. *J. Zhejiang. Univ. Sci. B.* **10**(4): 264-272.

Moshafi, H. M., H. Forootanfar, A. Ameri, M. Shakibaie, G. Dehghan-Noudeh, M. Razavi (2011). Antimicroabial activity of *Bacillus* sp. strain FAS 1 isolated from soil. *Pak. J. Pharm. Sci.* **24**(3): 269-275.

Najafi, A., M. Rahimpour, A. Jahanmiri, R. Roostaazad, D. Arabian, M. Soleimani, Z. Jamshidnejad (2011). Interactive optimization of biosurfactant production by *Paenibacillus alvei* ARN63 isolated from an Iranian oil well. *Colloids Surf B.* **82**: 33-39.

Papagianni, M. (2003.) Ribosomally synthesized peptides with antimicrobial properties: biosynthesis, structure, function, and applications. *Biotechnol. Adv*. **21**: 465–499.

Rizk, M., T. Abdel-Rahman, H. Metwally (2007). Screening of antagonistic activity in different Streptomyces species against some pathogenic microorganisms. *J. Biol. Sci.* **7** (8)**:** 1418-1423.

Shetty, R. P., K.S. Buddana, B. V. Tatipamula, V. V. Y. Naga, J. Ahmad (2014). Production of polypeptide antibiotic from *Streptomyces parvulus* and its antibacterial activity. *Braz. J. Microbiol.* **45**(1): 303-312.

SiloSuh, L. A., B. J. Lethbridge, S. J. Raffel, H. He, J. Clardy, J. Handelsman (1994). Biological activities of two fungi-

static antibiotics produced by *Bacillus cereus* UW85. *Appl. Environ. Microbiol*. **60**(6): 2023-2030.

Vincent, W. A., G. Priestly (1975). Large-Scale production of enzymes. Techniques in Fermentation. Handbook of Enzyme Biotechnology Ellis Horwood Limited, England, pp. 27-54.