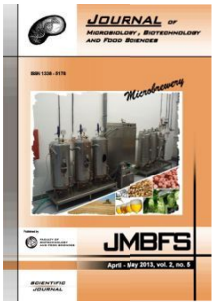

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THE EFFECT OF GROWTH PARAMETERS ON THE ANTIBIOTIC ACTIVITY AND SPORULATION IN *BACILLUS* SPP. ISOLATED FROM SOIL

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ABSTRACT

Fifty-two *Bacillus* strains, which were isolated from different soil samples, were screened for antibiotic properties. The *Bacillus* strains were checked for antibacterial properties by the cross-streak method against 5 test pathogens, and 25 *Bacillus* strains had an effect on the test microorganisms. One strain of *Bacillus*, which exhibited the largest inhibition zone (25 mm) against *Shigella sonnei*, was named *Bacillus* sp. EA62. The antibacterial activity from *Bacillus* sp. EA62 was tested in six different culture media against *Shigella sonnei* using the agar well diffusion method. The best activity medium was selected and used for further studies. The influence of the incubation period, pH, and different glucose and nitrogen concentrations on the antibacterial activity was studied. The optimal conditions for the strongest antibiotic activity were found to be 72 hours (18 mm), pH 7.5 (23 mm), 3% glucose (25 mm), and 0.3% nitrogen concentration (23 mm). Additionally, the relationship between the antibiotic activity and sporulation was investigated. Accordingly, it was determined that the increase of the activity paralleled sporulation.

Keywords: *Bacillus*, screening, antibiotic activity, sporulation

INTRODUCTION

Antibiotics have been used against infectious bacteria and fungi for over 50 years. Antibiotics are used as antitumor agents, immunosuppressive agents, hypocholesterolemic agents, enzyme inhibitors, antimigraine agents, and antiparasitic agents.

Antibiotics first became widely available in the 1940s with the use of penicillin and sulfonamides. Since that time, the pharmaceutical industry has developed more than 100 varieties of these drugs, with 150 million prescriptions being written for antibiotics annually in the United States alone. This growth in antibiotic use has been paralleled by the ability of bacteria to resist being killed by these agents, which has resulted in a steady decline in the number of effective antibiotics each year **(Levy, 1998)**. The emergence in recent years of numerous strains of pathogenic microorganisms that have developed resistance to a range of formerly efficacious antibiotics constitutes a serious threat to public health. The fact that certain nosocomial pathogens are already resistant to all available antibiotics and are therefore essentially untreatable dramatically demonstrates the need for completely new types of antibiotics **(Conlon *et al.*, 2006)**. Antibiotic activity is a feature of several kinds of soil bacteria and fungi and may represent a survival mechanism whereby organisms can eliminate competition and colonize a niche **(Jensen and Wright, 1997; Talaro and Talaro, 1996)**.

Bacillus species are one of the largest sources of bioactive natural products, exhibiting a wide range of antibiotic activities. There are 167 antibiotics produced by bacilli, including 66 derived from *B. subtilis*, 23 from *B. brevis* and the remainder from other species of the genus *Bacillus*. The main antibiotic producers of this genus are *B. brevis* (e.g., gramicidin and tyrothricin), *B. cereus* (e.g., cerexin and zwittermicin), *B. circulans* (e.g., circulin), *B. laterosporus* (e.g., laterosporin), *B. licheniformis* (e.g., bacitracin), *B. polymyxa* (e.g., polymyxin and colistin), *B. pumilus* (e.g., pumulin), and *B. subtilis* (e.g., polymyxin, difficidin, subtilin, mycobacillin, and bacitracin).

Several research reports that in bacilli, the polypeptide antibiotics produced affect spore formation directly or indirectly **(Vitkovic and Sadoff, 1975; Sarkar *et al.*, 1977; Demain and Piret, 1978; Marahiel *et al.*, 1979; Piret *et al.*, 1983)**, and antibiotic activity by *Bacillus* begins at the early stages of the stationary phase, which coincides with the beginning of the sporulation process.

Secondary metabolites are synthesized by a wide variety of pathways, and both the particular genetic make up of the producing strains and different environmental conditions can affect their production. Fermentation parameters, such as the incubation time, temperature, pH and available nutrients, can be modified to expand the range of the secondary metabolites produced **(Furtado *et al.*, 2005)**. Therefore, the purpose of this study was to isolate new, potentially antibiotic-activity bacteria from soil samples, optimize different parameters for antibiotic activity, and determine the relationship between antibiotic activity and sporulation.

MATERIAL AND METHODS

Isolation of potential antibiotic-activity bacteria

Soil samples were collected from various cities of Turkey and were examined for the potential presence of antibiotic-activity bacteria. The test microorganisms, including *Shigella sonnei* (ATCC 9290), *Salmonella typhimurium* (ATCC 14028), *Yersinia enterocolitica* (ATCC 9610), *Klebsiella pneumoniae* (ATCC 13883), and *Staphylococcus aureus* (ATCC 25923), were inoculated onto nutrient agar plates. The soil samples were sprinkled on the lawn of test microorganisms. The plates were incubated at 37°C for 24 hours. After the incubation, the plates were analyzed for the inhibition zones surrounding the bacterial colonies.

Identification of *Bacillus* species

The isolated strains were identified by their morphological and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology **(Buchanan and Gibbons, 1974)**.

Cross-streak Method

The preliminary screening was performed using the cross-streak method to test for antibiotic activity against *Shigella sonnei* (ATCC 9290), *Salmonella typhimurium* (ATCC 14028), *Yersinia enterocolitica* (ATCC 9610), *Klebsiella pneumoniae* (ATCC 13883) and *Staphylococcus aureus* (ATCC 25923). The isolated *Bacillus* strains were streaked across one third of the plate, and the plate was incubated at 37°C for 24 h. After the *Bacillus* grew, the test bacteria were streaked perpendicular to the *Bacillus*, and the plate was incubated at 37°C for another 24 h. The microbial inhibition was evaluated by determining the diameter of the inhibition zones.

Extraction of antibiotic compounds

To select the best media for antibiotic activity, the *Bacillus* isolates were inoculated into six different culture media **(Paulus and Gray, 1964; Hanlon *et al.*, 1982; Haddar *et al.*, 2007; Awais *et al.*, 2010; Carvalho *et al.*, 2010; Ilić *et al.*, 2010)** and incubated at 30°C in an orbital shaker at 150 rpm for 96 hours. After every 24 hours, samples were taken and filtered through a 0.2 µm filter. The filtrate was transferred to a sterile Eppendorf tube and stored at 4 °C.

Agar well diffusion method

The agar well diffusion method was used to check the culture for the antibiotic properties **(Sen *et al.*, 1995)**. A test culture of *Shigella sonnei* was prepared by diluting a 24 h culture suspension in 0.9% saline to achieve an absorbance of 0.3 at 600 nm as determined by a spectrophotometer. A sterilized cotton swab was dipped in the diluted cultures and spread over the surface of the nutrient agar. Wells were made in the inoculated agar medium under sterile conditions (7 mm in diameter), and 100 ml of the filtered sample was pipetted into the wells. The agar plates were incubated at 37 °C for 24 hour. The diameter of the zone of inhibition was measured in mm.

Optimization of conditions for antibiotic activity

The incubation period (0-96 h.), pH of the medium (6.0-8.0), glucose concentration (0,5-3%) and nitrogen concentration (0.1-0.6 %) were optimized for the maximal activity of a antibiotic by the *Bacillus* sp.

Determination of sporulation

The relationship between antibacterial activity and sporulation was investigated. From 24 h to 96 h, the *Bacillus* strain was grown in the selected medium. Every 24 h, samples were prepared by heating 4 mL of the culture broth to 75 °C for 15 min. The number of spores in the suspension was determined by plating a serial dilution of the suspension on nutrient agar. After incubation, the total number of colony forming unit (CFUs) on the plate was determined.

RESULTS AND DISCUSSION

Soil samples are rich sources of antibiotic-producing organisms. *Bacillus* species are aerobic, sporulating, rod-shaped bacteria that are ubiquitous in nature. *Bacillus* is a large genus with many members. There are many species of *Bacillus* that can produce a wide variety of antibiotics, including bacitracin, polymyxin, and colistin. For maximal antibiotic activity, suitable parameters are optimized experimentally, i.e., time, temperature, pH, and media, because changes in external pH and the concentration of nutrients in the medium affect many cellular processes, such as the regulation of the biosynthesis of secondary metabolites **(Datta and Kathary, 1993; Sole *et al.*, 1994, 1997)**. The present study was conducted to evaluate the properties of antibiotics from newly isolated *Bacillus* species from the soil and to optimize parameters, such as media, incubation time, pH, and glucose (as a carbon source) and yeast extract (as a nitrogen source) concentrations, that affect of the antibiotic activity by *Bacillus* sp. EA62. In total, 52 *Bacillus* species were identified (Tab 1) according to Bergey's Manual of Determinative Bacteriology **(Bunchanan and Gibbons, 1974)**.

Table 1 Morphological and biochemical tests for identification of *Bacillus* species

Test	Result
Shape	Rods
Gram staining	+
Spore formation	+
Motility	+
Starch hydrolysis	+
Catalase	+
Indol Production	-
Nitrate Reduction	+
Gas Production from Glucose	-
Acid Production from Glucose	+

The *Bacillus* species were screened for their antibiotic activity by the cross-streak method. The preliminary screening was conducted using 5 test pathogens. The results indicate that 25 *Bacillus* strains had an effect on the test microorganisms used. One *Bacillus* isolate showed the largest inhibition zone, 25 mm, against *Shigella sonnei*. This *Bacillus* isolate, named EA62, was selected for further studies.

The highest antibiotic activity was obtained with the medium described by Ilić et al. among six different culture media, and this medium was selected for further studies. The maximum inhibition zone was observed at 72 hour (optimized) against *Shigella sonnei* (18 mm). A gradual increase in the inhibition zone was observed over 24, 48 and 72 hours, but after 72 hour, a decrease in antibiotic activity was observed against *Shigella sonnei*.

The antibiotic activity by *Bacillus* sp. EA62 was not in parallel with cell growth. *Bacillus* sp. EA62 produced an antibiotic during the end of its exponential growth phase. Similar results have also been reported by other researchers **(Hasan *et al.*, 2009; Kalpana *et al.*, 2010)** after 72 hours of incubation. However, another researcher **(Awais *et al.*, 2007)** found that the maximum zone of inhibition (14 mm) against *M. luteus* was obtained at 48 hours. It has been reported that bacitracin production by *Bacillus licheniformis* ATCC 14580 was observed only during the phase of rapid growth **(Haavik, 1975)**. However, according to another study **(Egorov *et al.*, 1986)**, the maximal efficiency of bacitracin synthesis coincides with the end of the exponential growth phase and the onset of sporulation in the case of *B. licheniformis*. In submerged fermentation, 20-hours-old vegetative inoculum gave the maximum yield of bacitracin by *B. licheniformis* **(Yousaf, 1997)**. It was determined that the concentration of the inhibitory compound was high at 24 hours in the post-exponential phase and reached a maximum at 48 hours in the stationary phase for *Bacillus subtilis* MZ-7 **(Muaaz *et al.*, 2007)**. Several researchers observed that most antibiotics are secondary metabolites, and their synthesis starts at the point where the active growth phase ends and the organism enters the stationary phase **(Hosoya *et al.*, 1998)**. Generally, in *Bacillus*, the time of antibiotic activity is between 24-72 hours of incubation. The time at which the maximum antibiotic activity occurs changes, depending on the particular species of *Bacillus*. This phenomenon may be observed because different species have different metabolic pathways.

At different glucose concentrations (0.5-3%), the growth and antibiotic activity by *Bacillus* sp. EA62 varied. The bacterial biomass and antibiotic activity reached maximal levels in the medium containing 3% glucose. The highest zone of inhibition was approximately 25 mm (Fig 1).

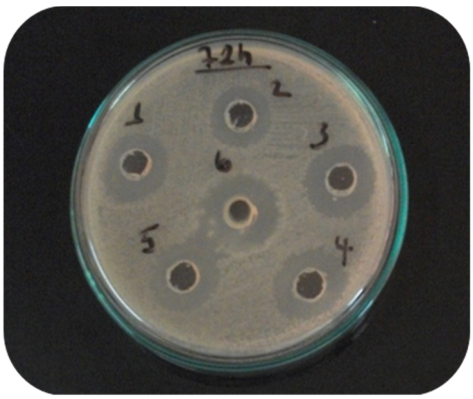

Figure 1 Antibiotic activity of *Bacillus* sp. EA62 against *Shigella sonnei* after 72 hours at different glucose concentrations, as shown by zone of inhibition (1: 19 mm, 2: 18 mm, 3: 20 mm, 4: 21 mm, 5: 20 mm)

Researchers have reported different results from these leves. The effect of glucose concentration on the activity of antibiotic in a free culture, as well as in an immobilized state (polyacrylamide gel), has been previously studied, and the maximum zone of inhibition (32 mm) was produced by *B. pumilus* against *M. luteus* at 72 hours in a 3% glucose, while a maximum zone (24 mm) against *S. aureus* was obtained in 3% glucose at 72 hours **(Hasan *et al.*, 2009)**. It has been reported that better neomycin production by *Streptomyces marinensis* NUV-5 cells immobilized in calcium alginate can be achieved with 3% w/v maltose **(Srivivasulu *et al.*, 2003)**. A maximum zone of inhibition (26 mm) was produced by *B. pumilus* against *M. luteus* in 5% glucose, while *B. subtilis* produced a maximum zone of inhibition (19 mm) in 1% and 5% glucose at 96 hours **(Awais *et al.*, 2007; Muhammad *et al.*, 2009)**. It has been determined that a maximum zone of inhibition is produced against *Micrococcus luteus* at 48 hours in 2% glucose, and the antibiotic activity increased gradually as the glucose concentration was increased **(Awais *et al.*, 2007)**. It has been reported that the effects of easily degradable carbon sources on antibiotic activity is controlled by the catabolite repression of enzymatic processes; the presence of the carbon sources at high concentrations decreased or inhibited the enzymatic synthesis of the polypeptide antibiotic **(Haavik, 1974)**.

Media supplemented with different concentrations of yeast extract (0.1-0.6 %) as a nitrogen source had varying effects on the growth of EA62 and the activity of its antibiotic. The best concentration of yeast extract for the activity of the antibiotic was found to be 0.3%. The inhibition zone was approximately 23

mm (Fig 2). There was no significant difference in the effect of high and low concentration of yeast extract.

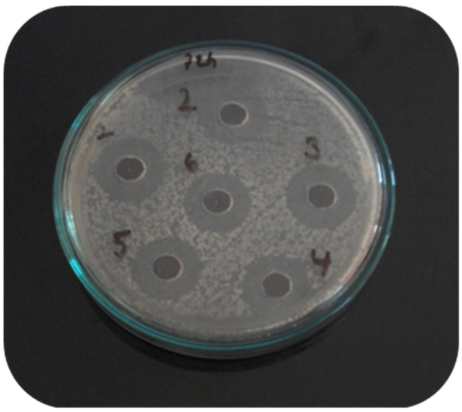

Figure 2 Antibiotic activity of *Bacillus* sp. EA62 against *Shigella sonnei* after 72 hours at different nitrogen concentrations, as shown by zone of inhibition (1: 21 mm, 2: 21 mm, 3: 23 mm, 4: 22 mm, 5: 22 mm, 6: 21 mm)

It has been reported that the concentration of the nitrogen source (glutamic acid) affects the activity of antibiotics because an increased concentration of glutamic acid increased the activity of antibiotics **(Muhammad *et al.*, 2009)**. The authors of this study observed that the maximum zone of inhibition against *Micrococcus luteus* and *Staphylococcus aureus* was produced at 48 hours in 1.5 % glutamic acid. It has also been reported that glutamic acid at a concentration of 0.01 M in minimal medium yielded maximal activity of the same antibiotic by *B. licheniformis* **(Hanlon and Hodges, 1981)**. The results of our study indicated an increase in antibiotic activity in medium containing 3% glucose and 0.3% yeast extract. Several researchers have obtained maximal bacteriocin activity by supplementing normal MRS media with 1 % glucose and 1% peptone **(Ogunbanwo *et al.*, 2003)**.

Studies that have investigated the effects of different concentrations of carbon and nitrogen sources on the activity of antibiotics show different results. The results depend on the particular type of microorganism used and its interaction with components of the medium because differences in biosynthetic pathways can affect the activity of antibiotics.

Changes in the external pH affect many cellular processes, such as the regulation of the biosynthesis of secondary metabolites **(Sole *et al.*, 1997)**. The effect of pH on antibiotic activity was studied by adjusting the initial pH range (6.0, 6.5, 7.0, 7.5 and 8.0) of the activity medium. The optimal pH for maximal antibiotic activity from *Bacillus* sp. EA62 was 7.5, and the zone of inhibition was 23 mm at this pH (Fig 3).

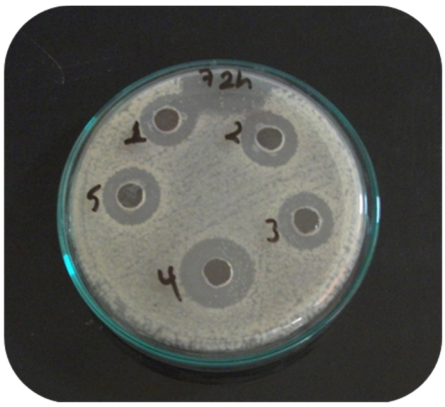

Figure 3 Antibiotic activity of *Bacillus* sp. EA62 against *Shigella sonnei* after 72 hours at different pH values, as shown by zone of inhibition (1: 17 mm, 2: 18 mm, 3: 18 mm, 4: 23 mm, 5: 17 mm)

Acidic and basic conditions did not show a negative effect on the activity of antibiotics. It was previously concluded that antibiotic substances in bacteria, in general, are produced over a pH range of 6.0 – 7.5 **(Chiba *et al.*, 1999)**. It has been reported that *Bacillus* pumilus SAF1 (cell-free extract) showed increased inhibition (30 mm) against *M. luteus* and (32 mm) *S. aureus* after 72 hours of incubation at pH 7 when it was immobilized in a polyacrylamide gel **(Hasan, 2009)**. It has previously been reported that a pH of 7.8 and 8 yielded maximal activity of bacitracin **(Anker *et al.*, 1947)**. However, a strain of *Bacillus subtilis* with activity against *Proteus vulgaris* within the pH range of 5.7 to 6.8 has been isolated **(Iglewski and Gerhart, 1978)**. Conversely, it has been reported that a pH of 7.0 and 7.5 yielded the maximal inhibition zone (32 mm) **(Kalpana *et al.*, 2010)**.

Bacillus antibiotics are generally produced at the early stages of the sporulation process **(Hasan *et al.*, 2009)**. Therefore, the relationship between antibiotic synthesis and sporulation of *Bacillus* sp. EA62 was investigated. At 24, 48 and 72 hours of incubation, the increase in sporulation was paralleled by antibiotic activity. However, at 96 hours of incubation, while antibiotic activity decreased, sporulation increased (Tab 2). At 96 hours, the bacteria were in the death phase, and in this phase, sporulation will naturally increase. Therefore, an indirect relationship may exist between antibiotic synthesis and sporulation. It had been determined that antibiotic activity is regulated in coordination with the physiological changes occurring during sporulation, and antibiotic biosynthesis usually begins at the end of the log phase and the beginning of the stationary phase **(Katz and Demain, 1977)**.

Table 2 Determination of sporulation

Time (hours)	Colony counts (cfu/ml)	Inhibition zone (mm)
24	$7{,}65x10^5$	12
48	$9{,}33x10^5$	14
72	$10{,}9x10^5$	18
96	$12{,}6x10^5$	12

A study of the biological role of antibiotics during sporulation found that in most instances, the antibiotics were produced at the same point in the growth cycle and under conditions conducive to the beginning of sporulation **(Nakano and Zuber, 1990)**. A study of mutant strains incapable of sporulation found that the mutant bacteria did not exhibit antibiotic activity **(Schaeffer, 1969)**. This can be explained by the existence of parallel regulatory pathways. However, this hypothesis was not confirmed because other scientists did not find a correlation between sporulation and the activity of antibiotics **(Modest *et al.*, 1984)**. This result agrees with previous reports on the activity of bacillomycin L **(Chevanet *et al.*, 1986)** and bacitracin **(Hanlon and Hodges, 1981)**. The optimal incubation conditions for spore do not match the ones that promote antibiotic activity.

CONCLUSION

The newly isolated *Bacillus* sp. EA62 strain showed potent antibiotic activity against *Shigella sonnei*. Because of the inhibitory spectrum of the antibiotic produced by *Bacillus* sp. EA62, it may have potential applications for the pharmaceutical industry.

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