

Optimization of Culture Medium for the Production and Partial Purification and Characterization of an Antibacterial Activity from *Brevibacillus laterosporus* Strain EA62

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ALEV USTA AK¹, ELIF DEMIRKAN^{1*}, MURAT CENGIZ², TUBA SEVGI¹, BEHICE ZEREN¹, MAOULIDA ABDOU¹,

¹Uludag University, Faculty of Arts and Sciences, Biology Department, Bursa, 16059, Turkey

²Uludag University, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, Bursa, 16059, Turkey

*Address for correspondence to: edemirkan@uludag.edu.tr

Abstract

The present study was performed to report the bacterial identification, optimization of culture conditions and characterization of a novel antibiotic substance from a *Bacillus* sp. EA62 strain isolated from soil. The EA62 strain was identified based on 16S rRNA analysis. The new isolate EBD 9-1 showed 100% sequence identity with *Brevibacillus laterosporus*. The antibacterial activity of the new substance was examined against five pathogenic bacteria and was observed to be the most effective against *Escherichia coli*. An agar diffusion assay was performed to evaluate the antibacterial activity of the substance. The effects of some nutritional (amino acid, carbon, nitrogen and metal sources) and physical factors (pH and temperature) and incubation time on the antibiotic activity were studied. Antibiotic activity in basal medium reached the maximum levels after 72 h of incubation. The best antibiotic activity was obtained in the presence of glucose as a carbon source, yeast extract as a nitrogen source, glutamic acid as an amino acid source and $MgSO_4 + CaCO_3$ as metal ion sources. For physical parameters, the best results were obtained at 37°C, pH 7.0. The antibiotic substance was partially purified, and the estimated molecular weight was 6.3 kDa. The minimum inhibitory concentration (MIC) values determined against five pathogen bacteria were >256 µg/ml. The substance was identified by thin-layer chromatography, and its R_f value was measured as 0.04 cm.

Keywords: *Brevibacillus*, antimicrobial substance, optimization, partial purification.

1. Introduction

Antibiotics are molecules that inhibit microbes, both bacteria and fungi, from growing ('static' antibiotics) or kill them outright ('cidal' antibiotics). The study of antibiotics began with the discovery of penicillin by Fleming and its subsequent introduction into therapy (M. SCHAECHTER & al. [1]). Antibiotics are chemotherapeutic agents that can be extracted from plants or obtained from secondary metabolites produced during the idiophase of microbial growth. Secondary metabolites are synthesized by a wide variety of pathways, and both the specific genetic makeup of the producing strains and different environmental conditions can affect their activity. These molecules are primarily produced by microorganisms that live in soil. Majority of antibiotics are produced by *Penicillium*, *Streptomyces*, *Cephalosporium*, *Micomonospora* and *Bacillus* (H. ZINSSER [2]). *Bacillus* species are one of the largest sources of bioactive natural products, which exhibit a wide range of antibiotic activities and are produced as low molecular weight polypeptides by ribosomal or nonribosomal mechanisms. There are 167 antibiotics known to be produced by Bacilli, including 66 derived from *B. subtilis*, 23 from *B. brevis* and the remainder from other *Bacillus* species. *Brevibacillus laterosporus* was previously classified as *Bacillus laterosporus* (O. SHIDA [3]). The primary

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 and loloatin A), *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). The genus *Brevibacillus* was established in 1996 by the genetic reclassification of strains that were previously allotted to the *Bacillus brevis* group. *B. brevis* was first described and reclassified [4] as a member of the novel genus *Brevibacillus*, along with nine other species (O. SHIDA & al. [3]; W. MIGULA & al. [4], N. TAMEHIRO & al. [5]), among which *B. laterosporus* has a great potential to produce polyketides, nonribosomal peptides, toxins, parasporal crystalline, extracellular protease and lipopeptide antibiotics (X. HUANG & al. [6] ; K. DESJARDINE & al. [7] ; J.E. SMITH. [8]). Over 4000 antibiotics have been identified from different microorganisms, but only 50 have been commercially used to treat human, animal and plant diseases (J.E. SMITH [8]). The number of antibiotic-resistant bacterial strains has increased, partly due to the misuse of antibiotics, resulting in serious health challenges in hospital settings. Thus, the discovery of new potential antibiotic-producing microorganisms has become an important goal. Many species, such as *Streptomyces*, *Bacillus* and *Penicillium* have been studied continuously for their ability to produce antibiotics (A. USTA & al. [9]). Fermentation parameters, such as the incubation time, temperature, pH, aeration, nutrient concentrations and nutrient combinations can be modified to obtain better and more stable antibiotic activity, and the selection of media components and their optimum levels are crucial for the production of secondary metabolites in microbial systems (P.A. JOSE & al. [10]). In our previous study, we screened antibiotic-producing *Bacillus* species from soil samples, and the most promising *Bacillus* strain was named *Bacillus* sp. EA62 (A. USTA & al. [9]). In the present study, we performed a 16S rRNA gene analysis of the identified strain and tested the effect of various nutritional and physical factors on the antibiotic activity of this bacterium. Furthermore, an antibiotic was partially purified and assessed for MIC values.

2. Material and Methods

Materials

Bacillus sp. EA62, which was isolated in our previous study, was used in the current study (A. USTA & al. [9]). Antibiotic activity was evaluated against *Escherichia coli* (ATCC 25212), *Enterococcus faecalis* (ATCC 29212), *Salmonella typhimurium* (ATCC 14028), *Yersinia enterocolitica* (ATCC 9610) and *Staphylococcus aureus* (ATCC 25923).

Methods

16S rRNA gene sequencing and analysis

For the bacterial identification and phylogenetic analysis, genomic DNA was extracted from strain EA62 using a Qiagen Blood & Tissue kit (Qbiogene, Montreal, PQ, Canada) according to the manufacturer's protocol. The 16S rRNA gene amplification was performed using the universal primers 27F (5'AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'ACGGCTACCTTGTTACGACTT-3'). The amplicons were sequenced with an automated ABI 3100 Genetic Analyzer (Applied Biosystems, Foster city, CA, USA). The resulting sequences were compared with the GenBank database (NCBI) using BLAST (S.F. ALTSCHUL & al., [11]). The 16S rRNA gene sequence of strain EA62 was aligned with those of other *Bacillus* species using CLUSTALW (J.D. THOMPSON & al., [12]). A phylogenetic analysis was performed using MEGA 6.0 (K. TAMURA & al., [13]), and a tree was obtained using the neighbor-joining method with related organisms (N. SAITOU & al. [14]).

Production medium and extraction of antibiotic activity

The liquid medium was prepared (S. ILIC & al [15]), yeast extract was used instead of soybean in this medium. The growth medium was composed of 15 g/L glucose, 1 g/L yeast extract, 0.5 g/L MgSO₄, 3 g/L CaCO₃ and 0.5 g/L (NH₄)₂HPO₄. The medium pH was adjusted to 7.0. *Bacillus* sp. EA62 was incubated at 37 °C on an orbital shaker at 150 rpm for 96 h. After every 24 h, samples were withdrawn and filtered through a 0.2 µm filter, and the filtrate was subsequently tested for activity.

Determination of antibiotic activity

The antibiotic activity of samples was determined following the agar well diffusion method (K.S. SEN & al [16]). Briefly, the turbidity of test-strain cultures grown for 24 h was adjusted to a 0.5 McFarland turbidity standard. A sterilized cotton swab was dipped in the diluted cultures and spread over the surface of the nutrient agar. Then, wells (7 mm in diameter) were made in the inoculated agar medium under sterile conditions, and 100 µl of the filtered sample was pipetted into the wells. The agar plates were incubated at 37 °C for 24 h, after which the diameter of the zone of inhibition was measured in millimeters. Bacterial biomass was determined by measuring optical density at 600 nm.

Effect of nutritional factors on antibiotic activity

Various carbon sources, such as sucrose, maltose, potato starch and wheat bran (1.5% w/v) were evaluated for their effect on antibiotic activity by replacing glucose in the growth medium. In addition, the effects of various nitrogen sources on antibiotic activity were assessed, including organic (corn steep liquor, tryptone and skim milk at 0.1% w/v) and inorganic sources ((NH₄)₂NO₃ and (NH₄)₂SO₃ at 0.05% w/v). These nitrogen sources were used to replace the organic and inorganic source available in the medium. In addition, different amino acids, including alanine, phenylalanine, valine, tyrosine, lysine, histidine, cystine, arginine, glutamic acid (1% w/v) were tested to obtain the best amino acid as a nitrogen source. The culture medium was supplemented with CaCl₂, FeSO₄, LiSO₄, NaCl, CaCO₃, MgSO₄, and MnSO₄ (0.05% w/v) in place of MgSO₄ and CaCO₃. The overnight cultures with an OD₆₀₀=0.3 were inoculated at 10%.

Effects of temperature and pH on the antibiotic activity

Physical parameters such as temperature and pH were evaluated for their influence on antibiotic activity in basal medium. The effect of temperature was evaluated by incubating the reaction mixtures at different temperatures (30, 40, 45, 50 and 55 °C) in growth medium at the optimal pH of 7.0. The pH of the culture medium was evaluated in the range of 4.0–9.0 to test its effect on antibiotic activity by the isolate.

For statistical analysis, the standard deviation for each experimental result and student's t-test were calculated using Microsoft Excel. All of the assays were carried out in triplicate. The bars correspond to standard deviation.

Partial purification of antibiotic substance

The crude antibiotic preparation partially purified by ammonium sulfate precipitation at 80% (w/v) saturation at 4 °C. After centrifugation at 10,000 rpm for 20 min at 4 °C. The precipitate was suspended in 20 mM phosphate buffer (pH 6.8) and dialyzed against the same buffer at 4 °C overnight to remove the ammonium sulfate. After dialysis, the antibiotic activity was evaluated against *E. coli* as a test bacterium.

SDS-PAGE, Thin-layer chromatography-bioautography

The molecular weight of partially purified substance was analyzed using SDS-PAGE (12.5%) according to the Laemmli method (U.K. LAEMMLI & al [17]). TLC bioautography was performed by modification of the original procedure described (S.K. PANDA & al. [18]). Standard antibiotics (streptomycin and bacitracin) and the partially purified antibiotic (10 µl)

were applied 2 cm from the base of the silica plate (Merck, silica gel 60 F254). After drying, the plate was developed using chloroform:methanol (9:1) as a solvent. Then, the TLC plate was dried to completely remove the solvents (110 °C for 30 min.). Twenty milliliters of soft agar (1%) containing 5 mL of cultured bacterial inoculum was spread onto the TLC plate under sterile conditions, and the plate was incubated at 37 °C for 24 h. A bacterial inoculum in soft agar was overlay on the dried TLC plate under aseptic conditions, and the overlaid plate was incubated at 37 °C overnight. Antibacterial activities were observed as a transparent zones on the TLC plate, and the Rf values of the zones were calculated. The retention factor, or Rf, is defined as the distance the compound migrated divided by the distance the solvent migrated.

Determination of minimum inhibitory concentration (MIC)

Bacterial strains of *Salmonella typhimurium* (ATCC 14028), *Staphylococcus aureus* (ATCC 25923), *Yersinia enterocolitica* (ATCC 9610), *Enterococcus faecalis* (ATCC 29212), *E. coli* (ATCC 25922) were used. Broth microdilution testing was performed to determine the minimum inhibitory concentrations (MICs) of ampicillin, gentamicin, and the new antimicrobial compound according to the guidelines of the Clinical Laboratory Standards Institute (CLSI [19]). The bacterial cultures were prepared in Mueller–Hinton broth at 37 °C for 20 h. All compounds were dissolved in DMSO. Freshly prepared stock solutions were sterilized using 0.20 µm single-use filter units (Minisart, Sartorius Stedim Biotech). Dilutions ranging from 0.008 to 256 µg/mL were prepared in Mueller–Hinton broth, and inoculated with a density equivalent to 0.5 McFarland turbidity were added to tubes containing the dilutions of the compounds. After incubation at 37 °C for 20 h, the MICs were determined as the minimum concentration of the compound that inhibited growth of the organism. The optical densities of the cultures were measured at a wavelength of 595 nm (iMark, Bio-Rad). All MIC determinations were performed in duplicate.

3. Results and Discussion

In this study, a phylogenetic tree based on the 16S rRNA gene sequences from *Bacillus* strains showed that the new isolate (EA62) shared 100% sequence identity with *Brevibacillus laterosporus* strain SMC57 (Figure 1). Thus, it was named *Brevibacillus laterosporus* EA62.

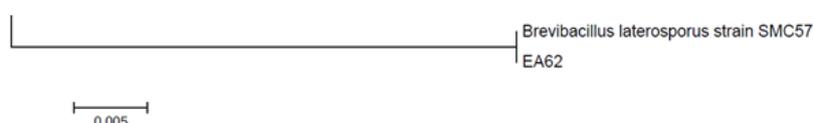


Figure 1. Phylogenetic relationship of the 16S rRNA sequences of *Bacillus* sp. EA62 with other type strains of *Bacillus*. Bar, 0.005 substitutions per nucleotide position.

The novel isolate EA62 was evaluated for the optimal time required to produce the antibiotic in liquid medium, and the maximum level of antibiotic activity occurred at 72 h (Figure 2).



Figure 2. The effect of incubation time of *Brevibacillus laterosporus* EA62 on the antibacterial activity of newly identified substance.

The maximum antibiotic activity was obtained at the end of log phase (exponential phase). The *B. laterosporus* EA62 metabolite showed maximum antimicrobial activity against *Escherichia coli*, *Yersinia enterocolitica*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Enterococcus faecalis*, with zones of inhibition of 19, 17, 15, 15 and 15 mm, respectively. The

biomass of EA62 was 1.07 at 72 h. Further studies were carried out with *Escherichia coli* as a test bacterium, using 72 h as a growth time.

Antibiotic formation typically occurs during the late growth phase of the antibiotic-producing microorganism. The temporal nature antibiotic formation has genetic factors, but the expression is greatly affected by environmental conditions (S.N. KUMAR & al [20]). The biosynthesis of secondary metabolites is directly related to culture conditions (A.L. DEMAINE & al [21]), including the biomass present during the activity phase and the duration of the incubation periods (B.E. ELIAS & al [22]).

The activity of antibiotic metabolites has been known to be influenced by media components and culture conditions, such as carbon sources, nitrogen sources, metal ions, pH, and temperature, which vary from organism to organism (B. RUIZ & al [23]). In particular, carbon and nitrogen sources in the media play a very crucial role in the antibacterial activity of antimicrobial substances by a given bacterium.

In this study, the maximum antibiotic activity from *B. laterosporus* EA62 was observed when glucose was used as carbon source, followed sucrose, starch and maltose. No antibiotic activity was observed using wheat bran, but a higher growth rate was observed (Figure 3).

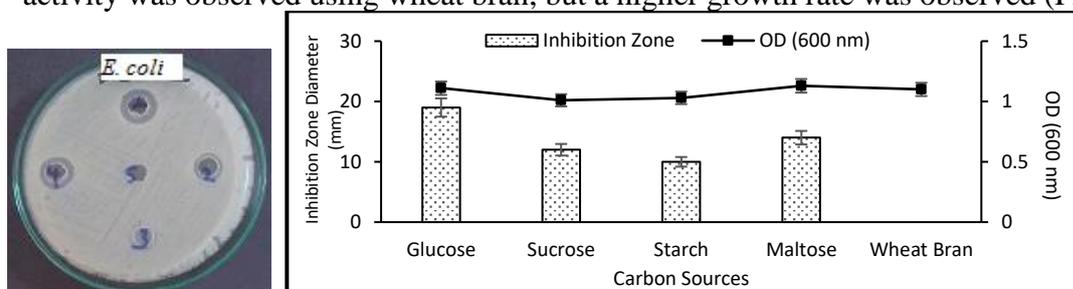


Figure 3. Effect of different carbon sources on the antimicrobial activity of the newly identified substance against *E. coli*. 1. Glucose, 2. Sucrose, 3. Starch, 4. Maltose, 5. Wheat Bran on petri plates.

In general, a quickly metabolized substance such as glucose is responsible for catabolic repression, but in some cases it is also reported to enhance antibiotic activity (T. FUKUDA & al [24]). Similar results have also been reported in other studies. Antibiotic activity by *Bacillus* sp. (R.D. JOSHI & al [25]), *Bacillus cereus* NB-4 and *Bacillus cereus* NB-5 has been reported to be induced by glucose. However, glucose repressed the activity of antimicrobial substances by *Bacillus subtilis* NB-6 (M. NASSER & al [26]). Starch was previously reported to be the best carbon source, followed by dextrose, for *Brevibacillus laterosporus* strain BPM3 (R. SAIKIA & al [27]). The antibiotic activity of strain EA62 was repressed by wheat bran. Different carbon sources, such as maltose P. SINGHA [28], dextrose (H. LINDA & al [29]), arabinose (S.K. KADIRI & al [30]), fructose (S.N. KUMAR & al [20]), and mannitol (T. ABDELGHANI & al [31]) have been reported to be suitable for the activity of antibiotic substances in different microorganisms. These different results shows that the optimal carbon source must be identified for maximal yield of the antibiotic of interest in different bacteria.

The antibacterial activity of antibiotics is significantly influenced by different nitrogen sources. In this study, yeast extract and tryptone alone appeared less efficient at supporting the antibacterial activity of the newly identified antibiotic substance, and activity was not observed in the presence of the other nitrogen sources, although bacterial growth was detected. Thus, there was no direct relationship between the growth of the organism and antibiotic formation. The yeast extract and $(\text{NH}_4)_2\text{SO}_3$ in basal medium were tested separately, resulting in less antibiotic activity was obtained than combined with yeast extract. This result showed that the combination of organic and inorganic nitrogen sources is required for the activity of antibiotics (Figure 4).

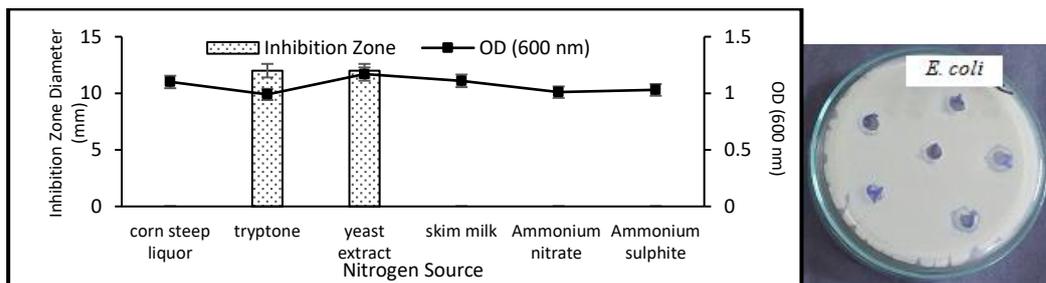


Figure 4. Effect of different nitrogen sources on the antimicrobial activity of the newly identified substance against *E. coli*. 1. Corn steep liquor, 2. Tryptone, 3. Yeast extract, 4. Skim milk, 5. Ammonium nitrate, 6. Ammonium sulfate on petri.

It was previously reported that antimicrobial activity was considerably decreased when nitrogen sources alone were used in the fermentation media (S.N. KUMAR & al [20]). Similar results were observed for antibiotic activity in batch cultures of *Bacillus laterosporus* ST-1 when grown in the presence of yeast extract in glucose broth (S. KALPANA & al [32]). Some studies have reported that organic or inorganic nitrogen sources influence enzyme activity. Malt extract proved to be the best nitrogen source, and lower activity was observed in medium containing yeast extract (H. LINDA & al [29]). It was reported that $(\text{NH}_4)_2\text{HPO}_4$ and yeast extract were adequate nitrogen sources for antibiotic activity by *Streptomyces kanamyceticus* M27 (A. PANDEY & al [33]).

The use of certain amino acids as a nitrogen source can inhibit the synthesis of secondary metabolites (JF. MARTIN & al [34]). Therefore, some amino acids were tested in the growth medium in this study. Glutamic acid has little effect on antibiotic activity, while phenylalanine, valine, cysteine and arginine resulted in no antibiotic being produced (Figure 5). When glutamic acid alone was used, less antibiotic activity was observed, but when glutamic acid was added with other nutrients in the basal medium, a slight increase in antibiotic activity was observed. Some studies have reported positive effects of amino acids on antibiotic activity. Asparagine was determined to be the most suitable nitrogen source for bacitracin activity, and the best source was also determined to be tyrosine and methionine.

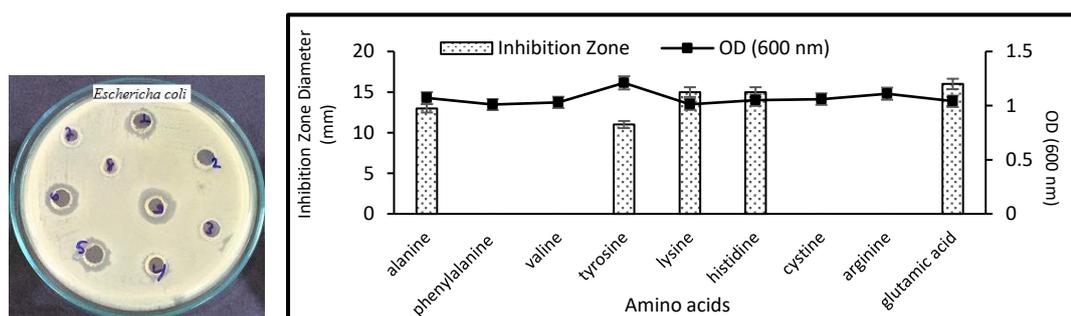


Figure 5. Effect of different amino acid sources on the antimicrobial activity of the newly identified substance against *E. coli*. 1. Alanine, 2. Phenylalanine, 3. Valine, 4. Tyrosine, 5. Lysine, 6. Histidine, 7. Cystine, 8. Arginine, 9. Glutamic acid.

Some metal ions can be stabilize enzyme-substrate complexes participating in antibiotic biosynthesis, and metals are needed by enzymes in other metabolic pathways. In this study, a small effect was only observed for MgSO_4 among of tested the metal ions, no antibiotic activity was obtained in the presence of the others, but good growth was detected (Figure 6). Antibiotic activity was considerably decreased when metal ion sources alone were used in media. This showed that a synergistic positive effect of MgSO_4 and CaCO_3 on the activity of antibiotic

compounds occurred. The addition of $MgSO_4$ increased iturin A activity by *Bacillus amyloliquefaciens* B128 (H.Y. LIN & al [35]; K. OCHI & al [36]), indicating that that rare earth metals and trace metals, notably manganese, zinc and iron, may trigger the activity of various secondary metabolic pathways. This effect was also recorded for manganese, and a slight increase in the antibiotic concentration was recorded for Cu, whereas the addition of Zn lowered the antibiotic activity (M.A. HASSAN & al [37]). Both our results and those of other studies have shown that the antibiotic activity pathways of different microorganisms are very different.

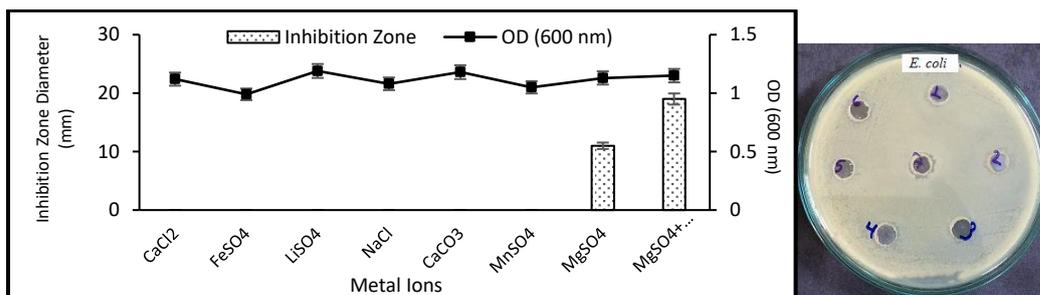


Figure 6. Effect of different metal ions on antimicrobial activity of the newly identified substance against *E. coli*. 1-CaCl₂, 2-FeSO₄, 3-LiSO₄, 4-NaCl, 5-CaCO₃, 6-MnSO₄, 7-MgSO₄ on petri plates.

The effect of temperature on antibiotic activity was determined and the maximum antibiotic activity was obtained at 37 °C, although 40 °C was also effective at promoting antibiotic activity, as the observed inhibition zone was 15 mm. The antibiotic activity decreased at lower and higher temperatures (Figure 7).

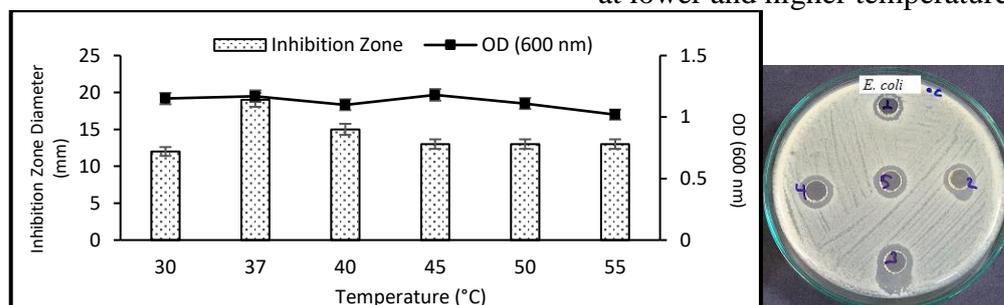


Figure 7. Effect of temperature on the antimicrobial activity of the newly identified substance against *E. coli*. 1-30 °C, 2-40 °C, 3-45 °C, 4-50 °C, 5-55 °C on petri plates, 37 °C (control).

The maximum antibiotic activity was obtained at an initial pH of 7.0. At lower and higher pH values the activity of the antibiotic was reduced. However, at pH 8.0 a 14 mm inhibition zone was observed. Growth was decreased at low pH values but increased at high pH values (Figure 8).

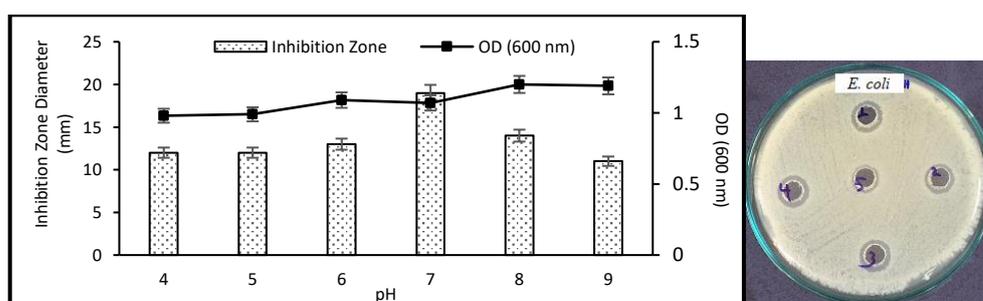


Figure 8. Effect of pH on the antimicrobial activity of the newly identified substance against *E. coli*. 1-pH 4.0, 2-pH 5.0, 3-pH 6.0, 4-pH 8.0, 5-pH 9.0 on petri plates, pH 7.0 (control).

A similar result for temperature was previously reported, but the antibiotic showed maximum activity at pH 8.0 (S.S. BISHT & al [38]). Although the maximum antibiotic activity occurred at a low temperature (30 °C) and at pH 7.0 (S. KALPANA & al [32]), antibiotic activity was achieved at temperatures as high as 40 °C and at pH 7.0 (R.D. JOSHI & al [39]). The antibiotic substance from *Brevibacillus laterosporus* EA62 was partially purified by ammonium sulfate precipitation and dialysis. A gel assay analysis showed that the partially purified substance preparation had three bands, with molecular weights of 6.3, 25 and 58 kDa. (Figure 9A). A bioautography test of the substance was also performed, and its homogeneity was confirmed. In the bioautography test, antibiotic activities of streptomycin, bacitracin and the partially purified antibiotic substance were observed, and their Rf values were determined to be 0.25, 0.08 and 0.04, respectively (Figure 9B).

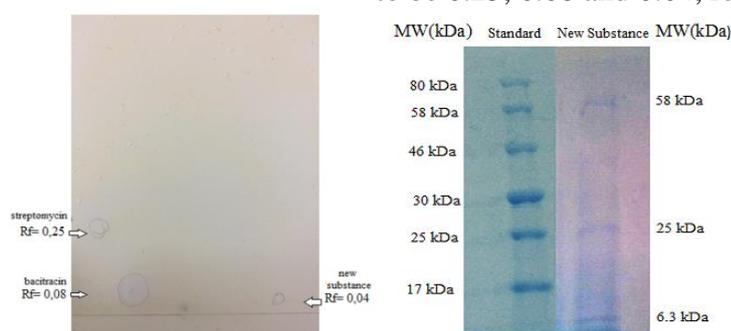


Figure 9. A. Molecular weight of the partially purified antibiotic substance. B. Bioautography assay showing the zone of inhibition against *E. coli*. I; Streptomycin, II; Bacitracin, III; the partially purified antibiotic substance of EA62.

The estimated molecular weight of the partially purified substance was 6.3 kDa. When compared with antibiotic substances of other *Brevibacillus* sp. strains, the observed molecular weights were between 1-12 kDa. The low molecular weight observed for this substance may indicate that it is synthesized nonribosomally. Similar to our results, low molecular weights of antibiotic substances were observed in other studies. *B. laterosporus* also secretes short peptides with broad antibiotic spectra, such as loloatin (A S.A. KRACHKOVSKII & al [40]). *Brevibacillus laterosporus* strain A60 can also produce a short linear peptide, also known as a pseudopeptide (J. ZHAO & al [41]). The molecular masses of peptides were reported for *Brevibacillus borstelensis* AG1 (12 kDa) (N. SHARMA & al [42]), *B. subtilis* (1 kDa) (G.H. EBRAHIMPOUR & al [43]), and *Bacillus* sp. (1083 Da) (M.L. TEIXEIRA & al [44]).

P. BHARTI & al [45] reported that *Burkholderia gladioli* OR1 produced at least three antistaphylococcal compounds (Rf values: 0.24, 0.45, and 0.59) and two anti-Candida compounds (Rf values: 0.62 and 0.76). Rf values of an antimicrobial compound from *Bacillus* sp. (S.S. BISHT & al [38]) and *Brevibacillus laterosporus* strain BPM3 (R. SAIKIA & al [27]) were determined to be 0.26 and 0.22, respectively. In addition, three lipopeptide antibiotics were identified as fengycin (Rf:0.09), iturin A (Rf:0.3) and surfactin (Rf:0.7) from *Bacillus subtilis* (D. ROMERO & al [46]).

The antibacterial activities of the new antimicrobial compound and some licensed antimicrobials against various bacterial strains are shown in Table 1, as estimated by the MIC. Five bacterial species were used in this study. MIC values of the antibiotic compound determined against five test bacteria were $>256 \mu\text{g/ml}$. Some *B. laterosporus* strains exhibit a broad-spectrum antimicrobial activity against various bacteria. New *B. laterosporus* isolates are continuously being collected worldwide and efforts are being conducted to purify and characterize novel bacterial enzymes and compounds for biotechnological exploitation.

However, the mechanism of action of many compounds are still undetermined and further investigations are necessary (L. RUIU & al [47]).

Table 1. Minimum Inhibitory Concentration (MIC) for the newly identified antibiotic substance.

	Minimum Inhibitory Concentration (MIC) for new antibiotic substance				
	<i>S. typhimurium</i>	<i>S. aureus</i>	<i>Y. enterocolitica</i>	<i>E. faecalis</i>	<i>E. coli</i>
Ampicillin	>256 µg/mL	>256 µg/mL	>256 µg/mL	2 µg/mL	>256 µg/mL
Gentamicin	2 µg/mL	2 µg/mL	4 µg/mL	16 µg/mL	1 µg/mL
New Substance	>256 µg/mL	>256 µg/mL	>256 µg/mL	>256 µg/mL	>256 µg/mL

4. Conclusion

Many antibiotics have been isolated from a variety of microorganisms, and studies on this subject continue to identify new antibiotics that are effective against pathogenic microorganisms. Natural products from bacteria have been used to develop pharmaceutical drugs that are widely used to fight bacterial infections. Natural products represent the traditional source of new drug candidates.

In this study, a novel antibiotic substance produced by *B. laterosporus* EA62, which was isolated from soil, was observed to be active against some pathogens. The effects of nutritional and physical factors on antibiotic activity were examined. The antibiotic substance was partially purified and some features of this substance were revealed. The antibiotic produced by EA62 may have potential applications for the pharmaceutical industry.

References

1. M. SCHAECHTER, *Encyclopedia of microbiology*, 3th ed., Academic Press, 2009.
2. H. ZINSSER, Antimicrobial agents. 9th chapter. In: H. ZINSSER, W.K. JOKLIK, H.P. WILLETT, D. B. AMOS, C. WILFERT, *Zinsser Microbiology*, Prentice Hall International, UK, 1988, pp.128-160.
3. O. SHIDA, H. TAKAGI, K. KADOWAKI, K. KOMAGATA, Proposal for two new genera, *Brevibacillus* gen. nov. and *Aneurinibacillus* gen. nov. *Int. J. Syst. Bacteriol.* 46:939–946 (1996).
4. W. MIGULA, *Das System der Bakterien*, G. FISCHER, Jena, 1900.
5. N. TAMEHIRO, H.Y. OKAMOTO, S. OKAMOTO, M. UBUKATA, M. HAMADA, H. NAGANAWA, Bacilysocin, a novel phospholipid antibiotic produced by *Bacillus subtilis* 168. *Antimicrob Agents Chemother.* 46:315–320 (2002).
6. X. HUANG, B. TIAN, Q. NIU, J. YANG, L. ZHANG, K. ZHANG, An extracellular protease from *Brevibacillus laterosporus* G4 without parasporal crystals can serve as a pathogenic factor in infection of nematodes. *Res. Microbiol.* 156:719–727 (2005).
7. K. DESJARDINE, A. PEREIRA, H. WRIGHT, T. MATAINAHO, M. KELLY, R.J. ANDERSEN, Tauramamide, a lipopeptide antibiotic produced in culture by *Brevibacillus laterosporus* isolated from a marine habitat: structure elucidation and synthesis. *J. Nat. Prod.* 70:1850–1853 (2007).
8. J.E. SMITH. *Biotechnology*. 5th ed. Cambridge University Press, 2009.
9. A. USTA AK. E. DEMIRKAN, The effect of growth parameters on the antibiotic activity and sporulation in *Bacillus* spp. isolated from soil. *J. Microbiol. Biotech. Food. Sci.* 2(5):2310-2313 (2013).
10. P.A. JOSE, K.K. SIVAKALA, S.R.D. JEBAKUMAR, Formulation and statistical optimization of culture medium for improved activity of antimicrobial compound by *Streptomyces* sp. JAJ06. *Int. J. Microb.* 1–9 (2013).
11. S.F. ALTSCHUL, W. GISH, W. MILLER, E.W. MYERS, D.J. LIPMAN, Basic local alignment search tool. *J. Mol. Biol.* 215:403–410 (1990).
12. J.D. THOMPSON, D.G. HIGGINS, T.J. GIBSON, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nuc. Acids Res.* 22:4673–4680 (1994).
13. K. TAMURA, J. DUDLEY, M. NEI, S. KUMAR, MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24:1596–1599 (2007).
14. N. SAITOU, M. NEI, The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 14:406–425 (1987).
15. S. ILIC, S. KONSTANTINOVIĆ, V.B. VELJKOVIĆ, D.S. SAVIĆ, G.D. GOJGIĆ CVIJOVIĆ, The impact of different carbon and nitrogen sources on antibiotic activity by *Streptomyces hygroscopicus* CH-7. *Curr. Res. Technol. Ed. Top. Appl. Microbiol. Microb. Biotechnol.* 1337-1342 (2010).

16. K.S. SEN, .FS. HAQUE, C.S. PAL, Nutrient optimization for activity of broad spectrum antibiotics by *Streptomyces antibioticus* Str. 15.4. *Act. Microbiol. Hung.* 42:155-162 (1995).
17. U.K. LAEMMLI, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:680–685 (1970).
18. S.K. PANDA, L.P. PADHI, G. MOHANTY, Antibacterial activities and phytochemical analysis of Cassia fistula (Linn.) leaf. *Journal of advanced pharmaceutical technology & research*, 2(1), 62 (2011).
19. CLSI (Clinical Laboratory Standards Institute), Approved standard M7-A6. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 6th edn. Clinical Laboratory Standards Institute, Wayne (2003).
20. S.N. KUMAR, J.V. SIJI, R. RAMYA, B. NAMBISAN, C. MOHANDAS, Improvement of antimicrobial activity of compounds produced by *Bacillus* sp. associated with a *Rhabditid* sp. (entomopathogenic nematode) by changing carbon and nitrogen sources in fermentation media. *J. Microbiol. Biotech. Food. Sci.* 1(6):1424-1438 (2012).
21. A.L. DEMAIN. Pharmaceutically active secondary metabolites of microorganisms. *Appl. Microbiol. Biotechnol.* 52:455– 463 (1990).
22. B.E. ELIAS, S. SAID, S. ALBUQUERQUE, M.T. PUPO, The influence of culture conditions on the biosynthesis of secondary metabolites by *P. verrucosum* Dierck. *Microbiol. Res.* 161:273–280 (2006).
23. B. RUIZ, A. CHÁVEZ, A. FORERO, Y. GARCÍA-HUANTE, A. ROMERO, M. SÁNCHEZ, Activity of microbial secondary metabolites: Regulation by the carbon source. *Crit.Rev.Microbiol* 36(2):146-167 (2010).
24. T. FUKUDA, A. MATSUMOTO, Y. TAKAHASHI, H. TOMODA, S. OMURA, Phenolic acids A and B, new Potentiators of antifungal miconazole activity produced by *Streptomyces* sp. K03–0132. *J. Antibiot.* 58: 252- 259 (2005).
25. R.D. JOSHI, V.S. HAMDE, A.M. UMRİKAR, S.S. KULKARNI, M.A. BHATE, Studies on activity of peptide antibiotic by thermotolerant *Bacillus* sp. *Int. Multidiscip. Res. J.* 2(6):30-33 (2012).
26. M. NASSER, EL-BANNA. Effect of carbon source on the antimicrobial activity of the air flora. *World J. Microbiol. Biotechnol.* 21:1451–1454 (2005).
27. R. SAIKIA, K. SINGH, D.K. ARORA, Suppression of *Fusarium*-wilt and charcoal rot of chickpea by *Pseudomonas aeruginosa* RsB29. *Ind. J. Microbiol.* 44:10-14 (2004).
28. P. SINGHA, M.G. ROYMON, Antibiotic potential of soil actinomycetes under influence of physical and nutritional parameters. *Indian J. Sci. Res.* 13(2):203-207 (2017).
29. H. LINDA, AL-GHAZALI, R. OMRAN, Optimization of medium composition for antibacterial metabolite activity from *Streptomyces* sp. *Asian J. Pharm. Clin. Res.* 10(9): 381-385 (2017).
30. S.K. KADIRI, N.S. YARLA, Optimization of antimicrobial metabolites activity by *Streptomyces fradiae*. *Int. J. Pharm. Sci.* 7:223–225 (2016).
31. T. ABDELGHANI, Activity of antibacterial and antifungal metabolites by (*S.albovinaceus*) strain no.10/2 and media optimization. *Am. Int. J. Bio.* 5(1):1-24 (2017).
32. S. KALPANA, A.I. BAGUDO, A.A. ALIERO, Effect of inhibitory spectrum and physical conditions on the activity of antibiotic substance from *B. laterosporus* ST-1. *Niger J. Microbiol.* 24(1): 2134-2139 (2010).
33. A. PANDEY, A. SHUKLA, S.K. MAJUMDAR, Utilization of carbon and nitrogen sources by *Streptomyces kanamyceticus* M 27 for the activity of an Antibacterial antibiotic. *Afr. J. Biotechn.* 4(9):909,910 (2005).
34. JF. MARTIN, AL. DEMAIN, Fungal development and metabolite formation. In: The Filamentous Fungi. Eds. Smith JE, Berry DR, Arnold, London. 3: 426-450 (1978).
35. H.Y. LIN, Y.K. RAO, W.S. WU, Y.M. TZENG, Ferrous ion enhanced lipopeptide antibiotic iturin A activity from *Bacillus amyloliquefaciens* B128. *Int. J. Appl. Sci. Eng.* 5: 123–132 (2007).
36. K. OCHI, T. HOSAKA, New strategies for drug discovery: activation of silent or weakly expressed microbial gene clusters. *Appl. Microbiol. Biotechnol.* 97(1): 87-98 (2013).
37. M.A. HASSAN, M.Y. EL-NAGGAR, W.Y. SAID, Physiological factors affecting the activity of an antimicrobial substance by *Streptomyces violatus* in batch cultures. *Egypt J. Bio.* 3:1-10 (2001).
38. S.S. BISHT, B. PRAVEEN, A. PANDA, V. RAJAKUMAR, Isolation, purification and characterization of bacitracin from *Bacillus* sp. *Int. J. Pharm. Pharm. Sci.* 3(4): 136-138, (2011).
39. R.D. JOSHI, V.S. HAMDE, A.M. UMRİKAR, S.S. KULKARNI, M.A. BHATE, Studies on activity of peptide antibiotic by thermotolerant *Bacillus* sp.. *Int. Multidis. Res. J.* 2(6):30-33 (2012).
40. S.A. KRACHKOVSKII, A.G. SOBOL, T.V. OVCHINNIKOVA, A.A. TAGAEV, Z.A. YAKIMENKO, Isolation, biological properties, and spatial structure of antibiotic lolotain A. *Russ. J. Bioorg. Chem.* 28:269– 273 (2002).

41. J. ZHAO, L. GUO, H. ZENG, X. YANG, J. YUAN, H. SHI, Y. XIONG, M. CHEN, L. HAN, D. QIU, Purification and characterization of a novel antimicrobial peptide from *Brevibacillus laterosporus* strain A60. *Peptides*. 33:206–211 (2012).
42. N. SHARMA, A. GUPTA, N. GAUTAM, Characterization of bacteriocin like inhibitory substance produced by a new strain *Brevibacillus borstelensis* AG1 isolated from ‘Marcha. *Brazi. J. Microbiol.* 45(3):1007-1015 (2014).
43. G.H. EBRAHIMIPOUR, Z. KHOSRAVIBABADI, H. SADEGHI, A. ALIAHMADI, Isolation, partial purification and characterization of an antimicrobial compound, produced by *Bacillus atrophaeus*. Jundishapur *J. Microbiol.* 7(9): e11802 (2014).
44. M.L. TEIXEIRA, A.D. ROSA, A. BRANDELLI, Characterization of an antimicrobial peptide produced by *Bacillus subtilis* subsp. *Spizezinii* showing inhibitory activity towards *Haemophilus parasuis*. *Microbiol.* 159:980–988 (2013).
45. P. BHARTI, V. ANAND, J. CHANDER, I.P. SINGH, T. V. SINGH, R. TEWARI, Heat stable antimicrobial activity of *B. gladioli* OR1 against clinical drug resistant isolates. *Indian J. Med. Res.* 135:666-671 (2012).
46. D. ROMERO, A. DE VICENTE, R.H RAKOTOALY, S.E. DUFOUR, J.W. VEENING, E. ARREBOLA, et al. The iturin and fengycin families of lipopeptides are key factors in antagonism of *Bacillus subtilis* toward *Podosphaera fusca*. *Mol. Plant. Microbe. Interact.* 20:430-440 (2007).
47. L. RUIU, *Brevibacillus laterosporus*, a pathogen of invertebrates and a broad-spectrum antimicrobial species. *Insects.* 4(3):476–492 (2013).