International Journal of Molecular Microbiology

ISSN: 2617-7633 (Online)

Research Article 2019 | Volume 2 | Issue 3 | 41-50

Article Info

Open Access

Citation: Iqbal, S., Rahman, H., Begum, F., Sajid, I., Qasim, M., 2019. Characterization and Antibacterial Activity of *Bacillus subtilis* MK-4 Isolated from Southern Area of Pakistan. Int. J. Mol. Microbiol., 2(3): 41-50.

Received: September 13, 2018

Accepted: October 24, 2019

Online first: December 29, 2019

Published: December 31, 2019

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Characterization and Antibacterial Activity of *Bacillus subtilis* MK-4 Isolated from Southern Area of Pakistan

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Abstract:
Antibacterial molecules are generally considered as secondary metabolites produced by bacteria during the stationary phase of their growth, which can kill or inhibit the growth of other bacteria. Nowadays, the unsystematic use of antibiotics has resulted in resistant bacteria. Investigation of new antibacterial metabolites and the identification of unexplored antibacterial exhibiting bacteria are necessary. In this study, the bacterial isolate MK-4 was obtained from the soil of the local habitat (Karak, Pakistan). The isolate MK-4 was preliminarily screened for antibacterial activity against a set of Gram-positive as well as Gram-negative bacterial isolates. Antibacterial activity was evaluated against 9 ATCC bacterial strains including *Staphylococcus aureus* (29213), *Staphylococcus epidermidis* (12228), *Escherichia coli* (25922), *Salmonella typhimurium* (14028), *Shigella flexneri* (12022), *Streptoccocus pneumonia* (6305), *Pseudomonas aeruginosa* (27853), *Klebsiella pneumoniae* (13889) and *Vibrio cholerae* (9459) and 4 clinical multidrug-resistant (*A. buemannii, S. aureus, E. coli* and *P. aeruginosa*). Antibacterial activity was measured as zone of inhibition (ZOI) in mm. Identification of bacterial isolate *B. subtilis* MK-4 was based on 16S rRNA gene sequencing apart from biochemical and morphological characteristics. The isolate was further optimized for growth as well as for antibacterial metabolites production at different pH, temperature and incubation time. The isolate MK-4 showed maximum growth at 30°C, maximum antibacterial activity at 37°C. MK-4 exhibited maximum growth and antibacterial activity at pH 8 after 48 hours incubation time.

Keywords: Soil bacteria, Antibacterial activity, optimization, Bacillus, Pakistan.

INTRODUCTION

The discovery of antibiotics and development of new compounds increased the chances of the survival. In the last few decades due to extensive use of antibiotic, the number of multidrug resistant bacterial strains has increased at an alarming rate (Agaba *et al.,* 2017; Iqbal and Ashraf, 2018). Due to frequent emergence of resistance discovery of new antibacterial molecules are necessary to combat the multi-drug resistant pathogens (Boucher *et al.,* 2010; Yunus *et al.,* 2016; Iqbal and Ashraf, 2019). Despite the necessity, the progress in the development of these antibacterial molecules has been slowdown and the number of approved new antibacterial molecules per year has been slowly decreasing (Donadio *et al.,* 2010). Soil microorganisms produce antibacterial molecules which have been recognized as an important source of new antibacterial agents (Sánchez *et al.,* 2009). Bacteria is the attractive source for antibacterial molecules due to its fast growth rate, minimum space requirement for cultivation and possess almost all the characteristics requirement for its industrial applications (Karbalaei-Heidari *et al.,* 2007). In spite of the toxicity of some antibacterial drugs like polymyxines produced by some Bacillus strains to the mammal's cells they still continued to be in the focus of research for scientists. The number of antimicrobial agents produced by bacilli is about 167, among these 66 are purified from the culture of *B. subtilis*, 23 from *B. brevis* and the remaining are produced by other species of genus Bacillus. *B. subtilis* is the major producer of bioactive compounds such as polyketides (PKs), non-ribosomally peptides (NRPs), and ribosomal peptides synthesized and post translationally modified peptides (RiPPs) (Caulier *et al.,* 2019). Various studies conducted and shown that soil bacteria represent a new and promising source to search for novel antibacterial molecules, although only a small amount of these diverse biological compounds have been studied, continued search may result in new antibiotic (Ravot *et al.,* 2006). The current study was designed to investigate the inhibitory potential of indigenous soil bacterial isolate, against ATCC and human MDR bacterial pathogens. The isolate found was identified as *Bacillus subtilis* showing antibacterial activity. Moreover, different culture parameters for their growth and antibacterial metabolites production were optimized.

MATERIALS AND METHODS

Isolation and maintenance of bacterial culture

Soil samples were collected from the Southern district (Karak, 33°7'12N 71°5'41E) of Khyber Pakhtunkhwa, Pakistan, in sterile bottles with the help of sterile spatula. The crowded plate or soil sprinkle technique was used for the isolation of antibacterial metabolites producing bacteria. The bacterial culture was purified by repeated sub culturing technique on Trypticase soya agar (TSA). All the soil isolates were stored at 4 °C until required.

Screening for antibacterial activities

Antibacterial activities of the purified soil bacterial isolates were performed on Muller Hinton Agar (MHA) against a set of Gram positive as well as Gram negative ATCC bacteria and against some clinical MDR bacterial pathogens. Initially the antagonistic activity was checked by using cross streaking or spot inoculation technique. Subsequently, the antibacterial activity was confirmed by agar well diffusion method and ZOI (Total diameter of inhibitory zone - diameter of agar well = ZOI) was measured in mm (Al-Ajlani *et al.,* 2007). All indicator strains were cultured in TSB for 24 hours at 37 °C. The 0.5 McFarland turbidity standards of indicator bacterial suspensions were used each time for antibacterial activity evaluation. All the antibacterial activity tests were conducted in triplicate. A total of 18 isolates among 49 were found to be producing antibacterial metabolites, among all these MK- 4 exhibited high and broad spectrum of inhibitory

activity and was selected for further studies. The antibacterial activity was evaluated against 9 ATCC bacterial strains including *Staphylococcus aureus* (29213), *Staphylococcus epidermidis* (12228), *Escherichia coli* (25922), *Salmonella typhimurium* (14028), *Shigella flexneri* (12022), *Streptoccocus pneumonia* (6305), *Pseudomonas aeruginosa* (27853), *Klebsella pneumonia* (13889) and *Vibrio cholerae* (9459). All these strains were procured from Kohat University of science and Technology (KUST) Kohat, Pakistan. The clinical multidrug resistant (MDR) strains *Acinetobacter baumannii*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* were obtained from local hospitals in Pakistan. All the indicator bacterial strains were maintained on nutrient agar slants.

Morphological and biochemical characterization of Antibacterial Exhibiting Bacteria (AEB)

The soil isolate MK-4 was identified morphologically (Gram staining, shape, spore staining, and motility) and biochemically (indole production, citrate utilization, methyl red-voges proskauer (MR-VP), oxidase production, catalase production, starch hydrolysis, nitrate reduction and gas production from glucose) according to the Bergey's manual of determinative bacteriology (Holt *et al.,* 1994; Iqbal *et al.,* 2016; Alsohiby *et al.,* 2016; Yunus *et al.,* 2017).

Molecular identification and hierarchical analysis of AEB

Genomic DNA from bacterial isolate MK-4 was extracted as described by Goldenberger et al with a little modification (Goldenberger *et al.,* 1995) briefly overnight bacterial culture was centrifuged at 13000 rpm for 10 minutes and suspended in 570 µl TE buffer, 5 µl of 10 % SDS, 3 µl of 30 µg/ml proteinase K. Mix thoroughly and incubated for 1 hour at 37 0C. The 100 µl of 5 M NaCl and 80 µl of solution were added and incubated for 15 minutes at 65 0C in water bath. Finally the genomic DNA was extracted with the equal volume of chloroform/isoamyl alcohol and centrifuged for 6 minutes. The aqueous phase was transferred to fresh eppendorf tube and DNA was again extracted with phenol/chloroform/isoamyl alcohol. Again aqueous phase was transferred to fresh tube and 0.6 volume isopropanol was added and centrifuged for 10 minutes, the pallet was washed with 70% ethanol and briefly air dried in laminar flow hood. The extracted DNA was re-suspended in 50 µl TE buffer and kept at -20 °C until required. The 16S rRNA was amplified by polymerase chain reaction (PCR) using primers, 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1510R (5'-GGCTACCTTGTTACGA-3') Premix ExTaq (Takara, Japan) as a forward and reverse primers respectively. The PCR was carried out in ABI Veriti PCR Machine (Applied Biosystems, USA) using optimized PCR program. Initial denaturation at 94 °C for 2 minutes, 35 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1.3 minutes and extension at 72 °C for 2 minutes. The final extension was performed at 72 °C for 5 minutes. The Amplified PCR product was sequenced using an ABI Prism dye terminator cycle sequencing reaction kit and an ABI PRISM 377 DNA sequencer (Applied Biosystems) (Felnagle *et al.,* 2007).

The 16S rRNA gene sequence result was BLASTn in NCBI/Ezbiotaxon to identify the isolate MK-4 up to species level. 16S rRNA gene sequences of the related species/strain were also retrieved from Genebank to infer the evolutionary relationship of MK-4 with other related strain. The nucleotides sequence alignment and trimming were performed by using ClustalW and BioEdit respectively. Phylogenetic tree was constructed based on neighbor joining method by using bioinformatics' software MEGA-6 (Tamura *et al.,* 2013).

Optimization of growth and antibacterial metabolites production

The influence of incubation time, pH, temperature and oxygen was evaluated for growth and antibacterial metabolites production

by using TSB growth medium. The isolate MK-4 was cultured in different pH, temperature and in shaking/static incubator. The antibacterial activity 9 ATCC and 4 clinical pathogens and growth (O.D at 600 nm) was determined after different time intervals using UV vis spectrophotometer.

Statistical analysis

All the experiments were performed in triplicate. Data are reported as means ± standard deviation (SD).

RESULTS

Soil samples were collected from Southern area (Karak) of Khyber Pakhtunkhwa, Pakistan. The isolated bacteria were screened for antibacterial activity against ATCC bacterial isolates and MDR clinical bacterial isolates. In the current study a total of 49 bacterial isolates were purified among these 18 (36.73%) exhibited antibacterial activity against either Gram positive or/and Gram negative bacteria. The isolate MK-4 exhibited significantly high antibacterial activity against both ATCC and MDR indicator strains. The isolate MK-4 was identified as *Bacillus* spp. by morphological and biochemical characters. Subsequently the identification was confirmed and identified up to species level by 16S rRNA gene sequencing. The isolate MK-4 is Gram positive rod shaped motile and spore forming and gives irregular, raised, flat, opaque and half-white colony on TSA plate. The biochemical profile shows that MK-4 is ureas, indole, citrate, vogues prauskauar and oxidase negative and catalase and MR positive. A molecular genetics approach was used to identify the bacterial isolate MK-4 at species level. According to BLASTn outcome at NCBI server (https://ncbi.nlm.nih.gov/Blast.cgi), the soil isolate MK-4 is a member of bacillus genus and showed 99% similarity with *Bacillus subtilis* (ABQL01000001). Similar result were obtained when the 16S rRNA gene sequence of the isolate MK-4 was compare with other closely related strains by EZ-taxon server (http://www.ezbiocloud.net/identify).

Phylogenetic tree was constructed to infer the evolutionary relationship of *Bacillus subtilis* MK-4 with other closely related strains by using MEGA 6. The phylogenetic tree result indicates that soil isolate MK-4 and *B.subtilis* sub-specie NCIB3610T accession no. ABQL01000001 diverged from single ancestor (Figure 1).

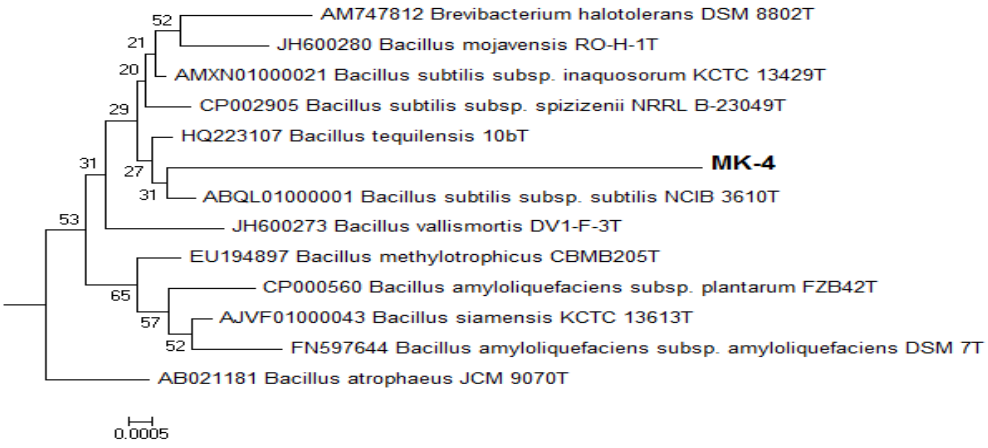

Fig. 1. The Phylogenetic tree showing relationship of isolate MK-4 with other closely related species of the *Bacillus* based on 16S rRNA sequences. This phylogenetic tree was constructed by using the neighbor joining method. Bootstrap values, expressed as percentage of 1000 replications, and are indicated at the node. The scale bar 0.0005 corresponds to substitution per site.

Effect of temperature on growth and antibacterial metabolites production was examined for MK-4. The results indicated that, the optimum temperature for growth and production of antibacterial metabolites is 30 °C. Although the antibacterial activity was remained optimum but slightly decrease in growth rate (O.D) was noted at 37 °C against S. aureus (Figure 2). The isolate MK-4 showed high antibacterial activity against Gram positive indicator strains as compare to Gram negative indicator strains (Figure 3). The isolate MK-4 growth and antibacterial activity was evaluated at different time interval, the maximum antibacterial activity was obtained after 48 hours of incubation (15±0.5 mm) and remained maximum up to 72 hours of incubation against some indicator strains. But gradual decrease of antibacterial activity was observed with the passage of time. While the maximum growth was observed after 48 hours of incubation (Figure 4).

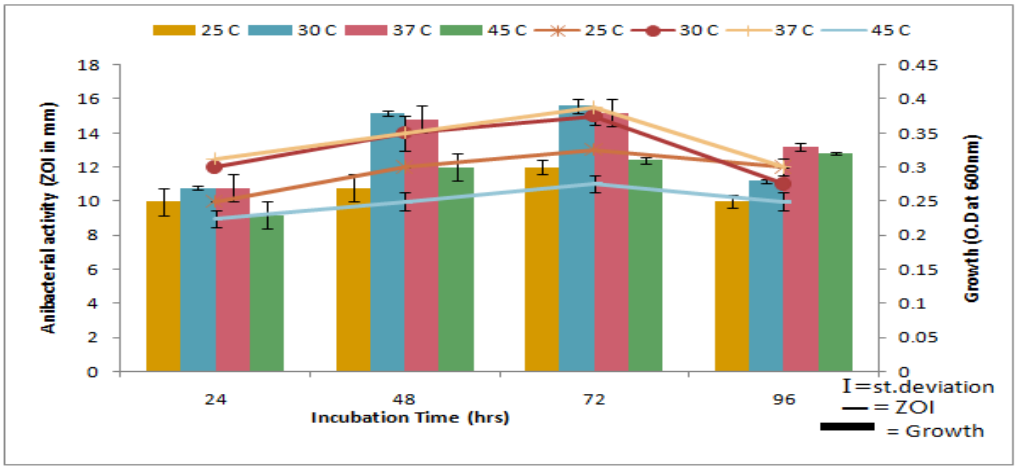

Fig. 2 . Effect of temperature and incubation time on growth and antibacterial activity (against *S.aureus*) of *Bacillus subtilis* MK-4.

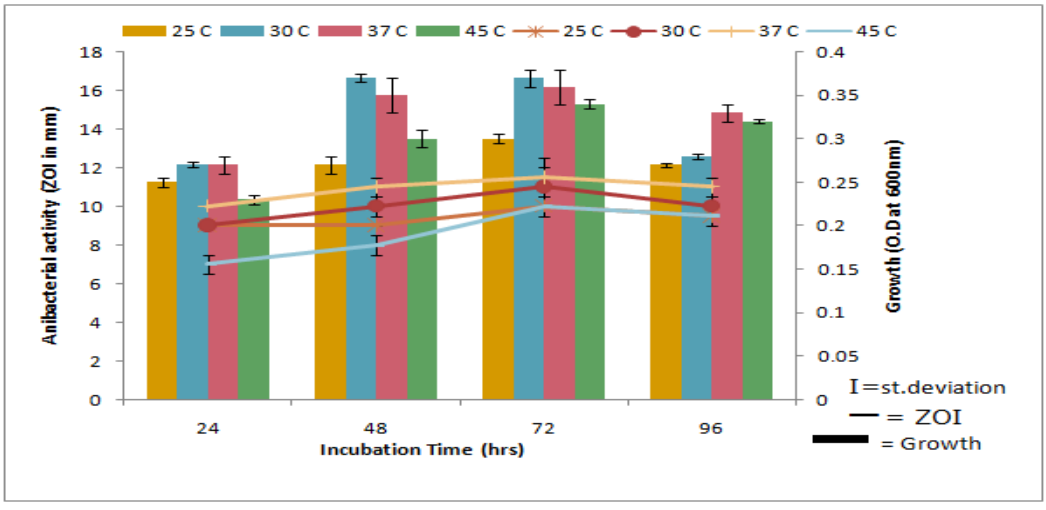

Fig. 3. Effect of temperature and incubation time on growth and antibacterial activity (against *E.coli*) of *Bacillus subtilis* MK-4.

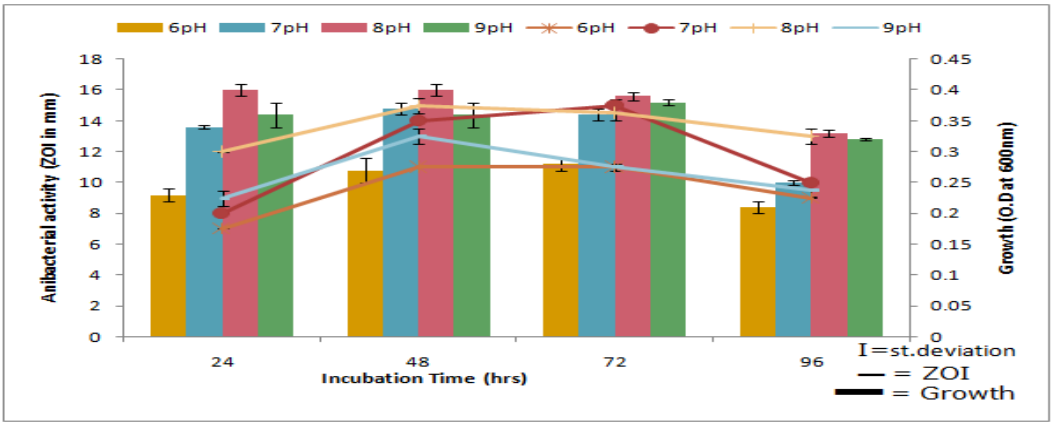

Fig. 4. Effect of temperature and incubation time on growth and antibacterial activity (against *S.aureus*) of *Bacillus subtilis* MK-4.

The effect of different pH on growth and antibacterial metabolites production was studied. The effect of pH from 5 to 9 revealed that the growth and antibacterial activity increased and attained maximum with gradual increase of pH from 6 to 8. Further increase in pH to 9, antibacterial activity decreases against all indicator strains. Best antibacterial activity was observed at pH 8 (Figure 5). The effect of oxygen availability was analyzed and result revealed that maximum growth and antibacterial activity was obtained, when the isolate MK-4 was cultivated in shaking culture (120 rpm) while good antibacterial activity and growth was also obtained in static culture (Figure 6). Antibacterial activity against MDR clinical isolates. *Bacillus subtilis* MK-4 exhibited antibacterial activity against all 4 indicator MDR strains while the maximum activity was noted against E. coli as shown in Figure 7.

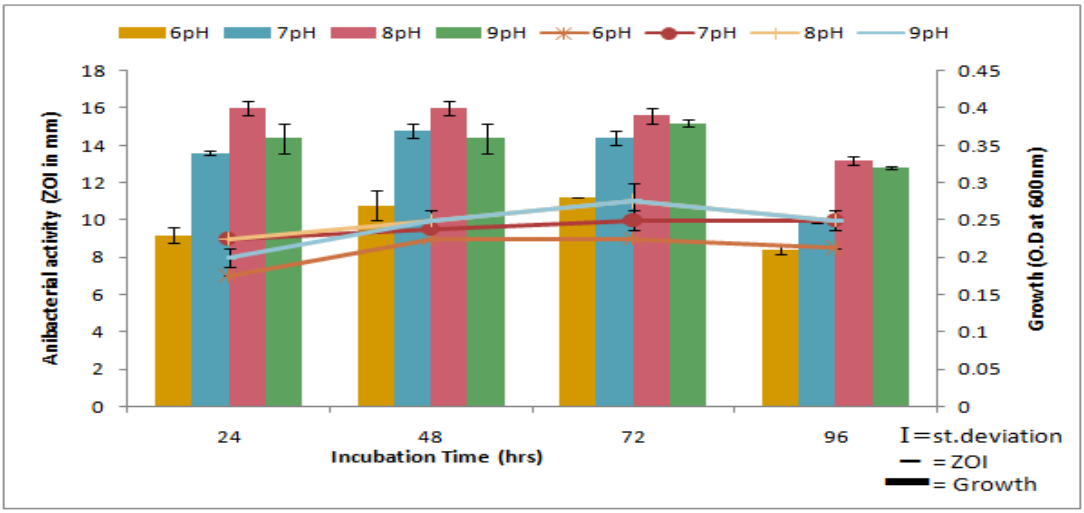

Fig. 5. Effect of temperature and incubation time on growth and antibacterial activity (against *E.coli*) of *Bacillus subtilis* MK-4.

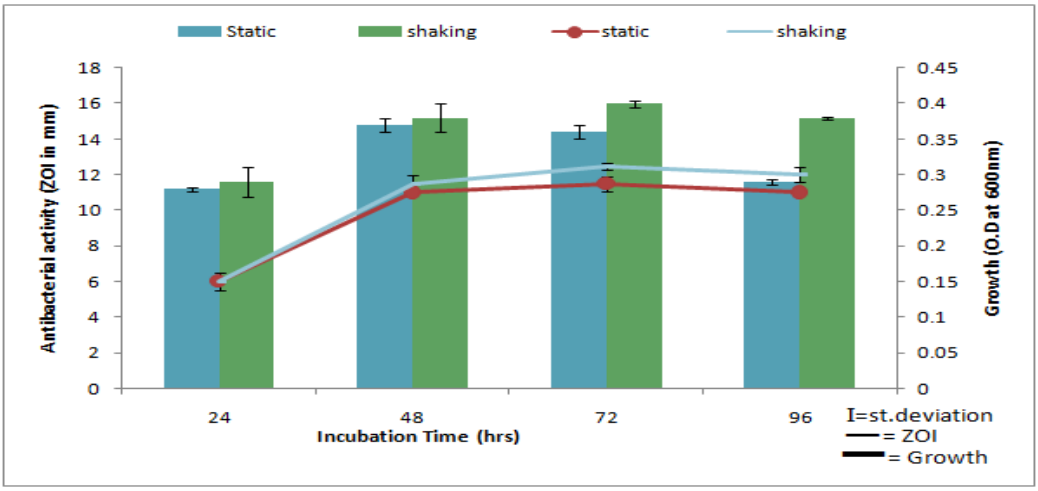

Fig. 6. Effect of shaking in growth and antibacterial activity of *Bacillus subtilis* MK-4.

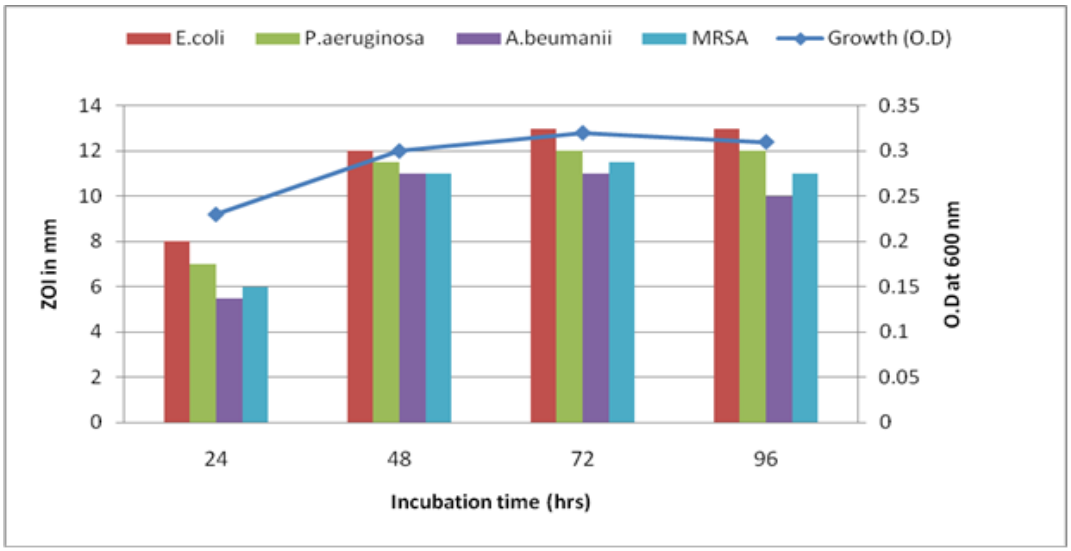

Fig. 7. Growth and antibacterial activity of *bacillus subtilis* MK-4 against MDR clinical isolates.

DISCUSSION

In the last few decades antibiotic resistance has increased at alarming rate all over the world and especially in Pakistan due to the lake of education, and unnecessary prescription. In the last few decades a plenty of unexplored habitats of soils were screened in the world for isolation of antibacterial metabolites producing bacteria. In the current study an unexplored habitats District Karak, Khyber Pakhtunkhwa in Pakistan for soil sampling was targeted to isolate bacterial flora active against wide range of indicator bacterial strains. The soil isolate *Bacillus subtilis* MK-4 producing metabolites was identified, which effectively inhibited the growth of Gram positive and Gram negative ATCC as well as MDR clinical strains. In a similar study conducted in Baida (Jordan), bacterial strains isolated from soil were active

against antibiotic resistant clinical bacterial isolates (Usta and Demirkan *et al.,* 2013). Another study explored the high potential of arid region bacteria that synthesize antibacterial metabolites against gram negative multi drug resistant bacteria strains (Nasfi *et al.,* 2018). The current study results are also correlate with another study conducted in Iran, in which bacteria were isolated from soil samples and showed antibacterial activity against pathogenic multi drugs resistant bacteria (Maleki *et al.,* 2013). The antibacterial exhibiting isolate MK-4 was identified based on phenotypic, biochemical and genotypic (16S rRNA gene sequence) characteristics. BLAST analysis demonstrated a high level of similarity (98%) to the sequence of *B. subtilis* and several other related strains. Ghribi et al. (2012) isolated *B. subtilis* SPB1 (HQ392822) identified by 16S rRNA gene sequence analysis from Tunisian soil exhibited antibacterial activity against a group of multi drug resistant bacteria isolated from clinical specimens. Our results are in accordance with the study conducted by Al-Ajlani and Hasnain in which soil bacterial isolates from Punjab soil (Pakistan) were identified as *B. subtilis* on the basis of morphological, biochemical and 16S rRNA gene sequence analysis and exhibited broad spectrum of antimicrobial activity (Al-ajlani and Hasnain, 2010). The influence of different parameters analyzed on the growth and production of antibacterial metabolites in TSB medium, maximum (15 ± 0.05 mm) zone of inhibition was observed against indicator bacterial strains at pH 8, temperature 30 °C and after 48 hours of incubation time in shaking culture. Yun and his colleagues optimized bioactive metabolites production through response surface methodology and achieved maximum metabolites production at 29.97 °C (Yun *et al.,* 2018).

CONCLUSION

In the current study *Bacillus subtilis* MK-4 isolated from the soil of Karak, Khyber Pakhtunkhwa, Pakistan exhibited prominent broad spectrum of antibacterial activity against 9 ATCC and 4 clinically important pathogens. The TSB medium with pH 8 at 37 0C temperature for 48 hrs was the optimal conditions for antibacterial metabolites production by *Bacillus subtilis* MK-4.

ACKNOWLEDGMENTS

We are grateful to the HoD and all staff members of the department of Microbiology, KUST for providing research facilities.

CONFLICT OF INTEREST

All the authors have declared that no conflict of interest exists.

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