



# Screening, Characterization and Optimization of Bioactive Peptides with Antibacterial Activities Against Multi-Drug Resistant Pathogens, Produced by *Bacillus safensis* Strain MK-12.1

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## Abstract

Antimicrobial resistance in pathogenic bacteria is increasing rapidly. Therefore, investigation of novel therapeutic agents are essential to combat the growing multidrug resistance (MDR) in human pathogens. The current study was intended to identify antibacterial metabolites producing *Bacillus* spp. from the unexplored southern region of Pakistan. The bacterial isolates ( $n = 47$ ) were isolated from soil samples and screened for antibacterial activity against a set of American Type Culture Collection (ATCC) and MDR human pathogenic bacterial strains. Among all isolated *Bacillus* spp. the strain MK-12.1 exhibit promising antibacterial activities against all tested strains. The antibacterial metabolites from MK-12.1 were partially extracted and their molecular weight and toxicity were evaluated. Genomic DNA was extracted from a fresh culture of antibacterial metabolite-producing strain MK-12.1, and the 16S rRNA gene was amplified and sequenced. The growth and antibacterial metabolites production by MK-12.1 was optimized at various pH, temperature, incubation time, and medium. The strain MK-12.1 efficiently inhibit the growth of ATCC as well as MDR pathogenic bacterial strains with the zone of inhibition ranges from 14 to 18 mm. The partially extracted antibacterial compound was characterized and confirmed to be proteinogenic, non-hemolytic, and molecular weight was less than 20 kDa. The isolate MK-12.1 was identified as *Bacillus safensis* and revealed 99% similarity with *B. safensis* strain NBRC. Maximum antagonistic activity and growth of MK-12.1 were observed at pH 8, 30 °C temperature, and 48 h of incubation in a shaking incubator when fermented in an optimized (opt) medium. The current study results indicate that *B. safensis* MK-12.1 produces promising antibacterial compounds that could be a source of new antibacterial leads to fight against MDR bacteria in future.

**Keywords** *Bacillus* spp. antibacterial peptides · Multi-drug resistance · Growth Optimization

## Background

Before the discovery of antibiotics, individuals with bacteremia and septicemia had low chances of survival. However, the availability of antibacterial drugs and the synthesis of new chemotherapy agents upturned this scenario. Recently,

the rise of multi-drug resistance (MDR) in human pathogens, challenging medical practitioners and researchers to develop or discover new antimicrobial agents to combat these organisms (Vivas et al. 2019). Urinary tract infections (UTIs) are prominent infections that occur worldwide, especially in developing countries including Pakistan, where the chances of drug-resistant are much higher due to injudicious use of antibiotics. The majority of UTIs are caused by *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. Unfortunately, these bacteria have been proven as Multidrug resistance (MDR) thus making its treatment ineffective (Shah et al. 2015). Further, the pan-drug resistance in pathogenic bacteria has turned into more serious distress to the community, gaining urgent focus of research to find a more effective chemotherapeutic agent to combat these pathogenic bacteria. Despite the salient need, the progress of developing new antibiotics is slow

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and the increase of newly approved antibiotics has steadily decreased in the last few decades (Vivas et al. 2019). Therefore, to treat the infections, especially in hospitals where resistance immediately may become life-threatening, the discovery of new antibacterial compounds is becoming more urgent (Dutescu and Hillie 2021). Previously several antibiotics such as penicillin, vancomycin, and tetracycline, have been discovered from environmental microorganisms. Among bacteria *Actinomycetes* and *Bacillus* spp. are known to produce a plethora of secondary metabolites that have potent antibacterial activities (Yahya et al. 2021; Selim et al. 2021). In terms of bioactivity, *Actinomycetes* have been extensively studied as compared to *Bacillus*. Bacilli are ubiquitous, endospore-forming, rod-shaped Gram-positive bacteria that are of great importance due to their ability to produce a broad spectrum of antibacterial metabolites. These bacteria are extensively used in industrial processes for different enzyme production like proteases and also used as plant growth-promoting bacteria. They produced diverse bioactive metabolites that may be used to control various pathogens (Abdelghani et al. 2021).

The current study was therefore conducted to explore the indigenous soil bacteria with broad spectrum antibacterial metabolites that could be used to combat MDR pathogens causing nosocomial infections.

## Methods

### Soil Sampling and Bacterial Isolates Cultivation

Soil samples ( $n=9$ ) were collected aseptically from various regions of district Kohat (33.5638° N, 71.4656° E), Pakistan at the depth of 3–8 cm. The samples were serially diluted, 200  $\mu$ l from each dilution were spread over Tryptic Soy Agar (TSA) plate and incubated at 37 °C for 48 h. A single bacterial colony was obtained by repeated sub-culturing on TSA (Oxide, UK). Fresh pure bacterial cultures were deep-frozen in 20% glycerol and maintained at -80 °C until required.

### Screening for Antibacterial Metabolites Production

The purified bacterial isolates ( $n=47$ ) were preliminarily examined for antibacterial metabolites production against indicator strains by cross-streaking method (Williston et al. 1947). Subsequently, the antagonistic activity of the Cell-Free Supernatant (CFS) from MK-12.1 culture was determined against a set of indicator ATCC (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Salmonella typhimurium*, *Shigella flexneri*, *Klebsiella pneumoniae*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, and *Escherichia coli*) (Table S1) as well as clinical MDR bacterial strains (*Acinetobacter baumannii*, *Staphylococcus*

*aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*) by agar well diffusion assay on Muller Hinton Agar (MHA) plate (Iqbal et al. 2021b). The Petri plates were maintained at 37 °C for 24 h and Zone Of Inhibition (ZOI) was measured in mm. The MDR bacterial indicator strains were previously collected (Ullah et al. 2016) from local tertiary care hospitals and the resistant pattern was determined according to Clinical and Laboratory Standards Institute (CLSI) 2015 protocols.

### Optimization of Growth and Antibacterial Activity of Selected Strains

Various culture conditions including temperature, culture medium, aeration, pH, and incubation time were optimized for antibacterial compounds production and growth of the strain MK-12.1. In order to assess the impact of pH and temperature, 100 ml TSB and 1 ml standardize inoculum of strain MK-12.1 was poured in Erlenmeyer flasks. The pH of flasks containing 100 ml media and 1 ml inoculum were adjusted as 6, 7, 8, 9 and incubated at various temperature, i.e., 25, 30, 35, 44 °C for 48 h. For culturing medium optimization the standard inoculum was added to sterile media, i.e., Luria–Bertani (LB) broth, Brain Heart Infusion (BHI) broth, Nutrient broth (NB), Tryptic soy broth (TSB), and Optimized (OPT) medium with a few modifications (Table S2). To evaluate the impact of incubation time on bioactive metabolites production, the inhibitory activity of CFS was determined at different time intervals (every 24 h, for 96 h) against test bacterial strains. The influence of aeration on biomass and antibacterial metabolites synthesis was studied by culturing the producer isolate in shaker culture (80, 120, 160, 200 rpm) and static culture for 48 h. The antagonistic potential was determined by agar well diffusion assay and bacterial biomass was estimated by UV–vis spectrophotometer (600 nm). For each experiment, 5 ml fresh standardized (1 McFarland) culture of strain 12.1 was used as the initial inoculum.

### Antibacterial Metabolites Production and Partial Purification

Antibacterial exhibiting strain MK-12.1 overnight fresh culture was inoculated in Erlenmeyer flasks containing 500 ml modified OPT medium, maintained at 30 °C for 48 h as shaking culture. Subsequently, the culture of MK-12.1 was spun at 6000 rpm for 15 min. Further, the supernatant was passed via a 0.45  $\mu$ m pore size membrane to remove all the cell debris. The supernatant was neutralized using 1 M NaOH and 10% HCl. The bioactive compounds were partially extracted from the supernatant using ethyl acetate (1:1), and dried in a rotary evaporator under reduced pressure (Iqbal et al., 2021a, b). Vancomycin (30  $\mu$ g) and

ethyl acetate were used as positive and negative control, respectively.

### Determination of Minimum Inhibitory Concentration (MIC)

The MIC of partially purified metabolites from strain MK-12.1 was determined against indicator strains using a two-fold serial dilution technique (Kowalska-Krochmal and Dudek-Wicher 2021). The dried antibacterial metabolites extract was dissolved in Dimethyl sulfoxide (DMSO) at 100 mg/ml concentration. Initially, 1 ml of extract (100 mg/ml) was poured into tube no. 1 and serially diluted all test tubes (1–10) containing 1 ml of TSB. Then, 10 µl of test bacterial strain culture (final concentration equal to 0.5 McFarland) was dispensed into all tubes (1–12). The second last tube (11) containing DMSO and last tube (12) containing antibiotic streptomycin (100 µg) was used as a negative and positive control, respectively. In order to determine the MIC of positive control (streptomycin), 100 µg was dissolved in 1 ml sterile distilled water, and the same two fold dilution method was performed. The lowest amount of partially extracted antibacterial compounds that entirely restrict the proliferation of indicator strains were defined as MICs.

### Characterization of Antibacterial Metabolites

To estimate the molecular weight of antibacterial metabolites, the partially purified antibacterial metabolites were dispersed on a dialysis membrane having a pore size < 20 Kilo Dalton (kDa). All the setup was kept on an agitator for 18 h at 25 °C and subsequently, antibacterial activity was determined. To confirm the nature of antibacterial metabolites, the partially purified metabolites were mixed with proteinase K, and later on, antagonistic activity was assessed. The temperature stability of antibacterial metabolites was evaluated by heating the partially purified metabolites from 20 to 121 °C for 20 min and antagonistic activity was evaluated against indicator strains. Hemolytic activity was determined on a blood agar plate. The antibacterial metabolites (100 µg/ml) were aseptically dispensed into the pre-made agar well and the plates were maintained at 37 °C for 24 h.

### Identification of Soil Isolate MK-12.1

Initially, the antibacterial metabolites producing isolate was tentatively identified by colony morphology, Gram reaction, and biochemical testing as per Bergey's manual of determinative bacteriology. Subsequently, the soil isolate MK-12.1 was molecularly identified based on 16S rRNA gene sequencing. Total genomic DNA was extracted using phenol:chloroform method with a little modification (Wright et al. 2017) and the aforementioned gene was amplified by

9F (GAGTTTGATCCTGGCTCAG) and 1510R (GGC TACCTTGTTACGA) as forward and reverse primers. In PCR initial denaturation was performed at 94 °C for 120 s., annealing temperature was 52 °C for 90 s. and extension time was 2 min at 72 °C, and then the reaction was repeated 35 times, then a final extension was performed at 72 °C for 10 min. The amplified PCR product was separated on 0.9% agarose gel. The amplified product was recovered and purified using PCR purification kit (Roche, Germany) and sequenced using the di-deoxy chain termination method (Macrogen, Korea). The obtained sequence was BLASTn in NCBI (<https://www.ncbi.nlm.nih.gov/BLAST/>) and Eztaxon (<https://www.ezbiocloud.net/identify>) databases. The 16S rRNA gene of the related strains were downloaded from Ezbiocloud. Multiple sequence alignments were performed using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). The phylogenetic tree was constructed based on the neighbor-joining method using a computational tool MEGA-X (Kumar et al. 2018). The reliability of the phylogenetic trees was determined using the bootstrap technique on 1000 replicates.

## Results

### Soil Sampling and Plate Count

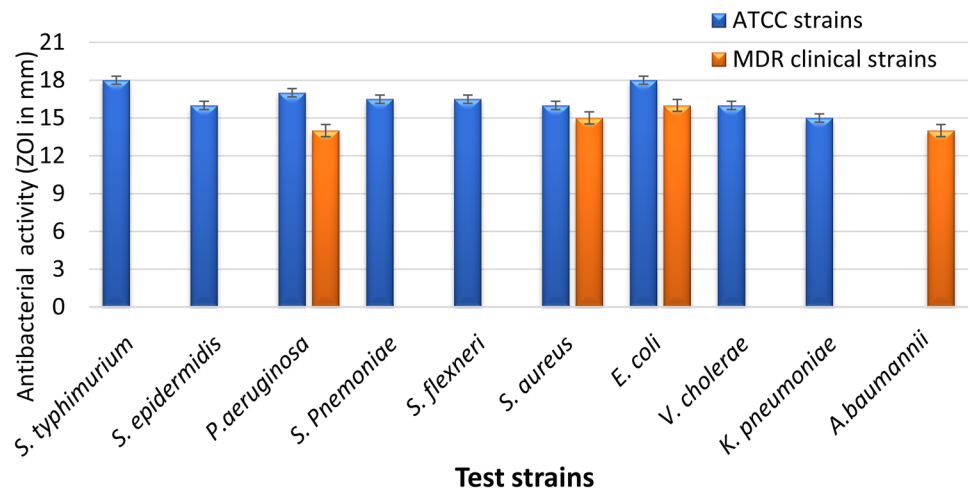
The soil samples were collected from various localities including hilly areas, lawns, gardens, industrial areas, open cultivated fields, riverbanks, stream banks, rhizosphere, and ponds surrounding area. Plate count on TSA ranged from  $4 \times 10^5$  to  $7 \times 10^5$  CFU/g of soil samples. A minimum count of  $4 \times 10^5$  CFU/g was obtained for the mill effluent soil sample, Kohat.

### Antibacterial Activity

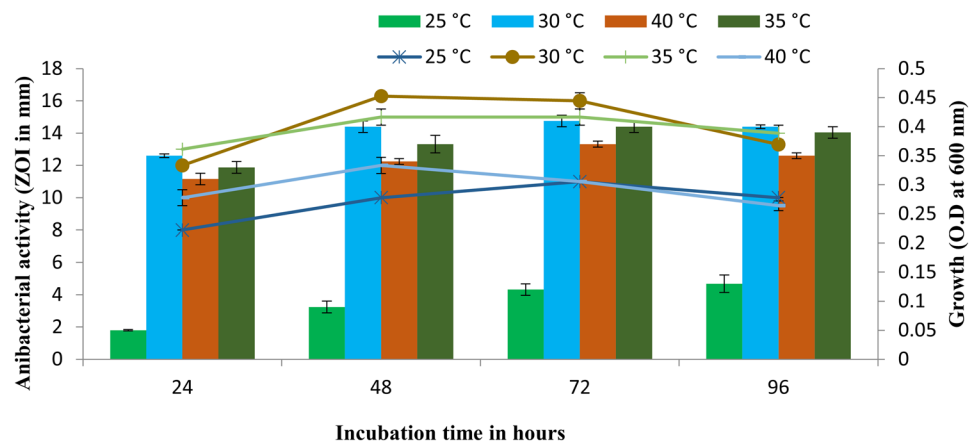
Among all 47 soil isolates, 12 isolates (25.53%) exhibited antagonistic activities either against Gram-positive bacterial strains or Gram-negative bacterial strains or both (Figure S1). Among these, three (25%) isolates designated as MK-9, MK-10 and MK-32, showed antagonistic activity only against Gram-positive test bacterial strains while nine isolates (75%), exhibited antagonistic activity against both Gram-positive as well as Gram-negative bacterial strains (data not shown here). One isolate *B. safensis* MK-12.1 exhibited promising antibacterial activity against all test strains (Fig. 1).

The partial extract of *B. safensis* MK-12.1 efficiently inhibits the growth of *S. typhimurium* (ZOI = 18 + 0.5 mm) as compared to other test strains such as *K. pneumoniae* (ZOI = 15 mm).

**Fig. 1** Antagonistic activities of *B. safensis* strain MK-12.1 against indicator strains

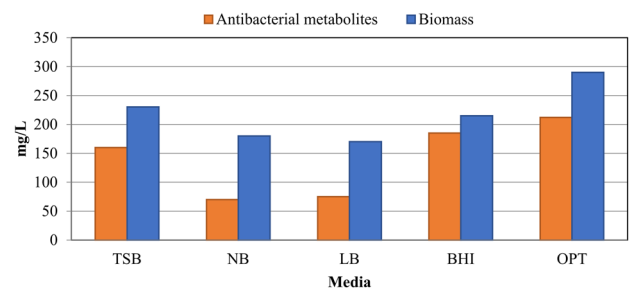


**Fig. 2** Optimization of incubation time and temperature for growth and bioactive compound production by *B. safensis* MK-12.1



### Optimization of Growth and Antibacterial Metabolites

Maximum antibacterial activity of CFS was achieved at 30 °C with 18 mm ZOI followed by 35 °C with 17 mm ZOI. On the other hand, lower antagonistic activity was observed at 25 and 40 °C, indicating that the soil isolate *B. safensis* MK-12.1 produces the highest amount of antibacterial metabolites at 30 °C (Fig. 2). The current study result revealed that nitrogen-rich media, i.e., BHI and TSB enhanced the growth and production of antibacterial metabolites production as compared to a simple nutrient broth medium. The CFS collected from OPT medium exhibited 19 mm ZOI, followed by BHI and TSB medium which exhibited 18 mm ZOI each against indicator strain. On the other hand, a high amount of biomass was obtained by cultivating the strain MK-12.1 in OPT medium followed by TSB and BHI medium, indicating that nutrients have a considerable effect on antibacterial metabolites production (Fig. 3). The BHI and TSB culture media are rich in carbon and nitrogen sources as compared to the



**Fig. 3** Culturing medium optimization for *B. safensis* MK-12.1 growth and bioactive compounds production. Trypticase soy broth: TSB, Nutrients broth: NB, Luria Bertani LB, Brain heart infusion: BHI and Optimized: OPT

nutrient broth as previously demonstrated earlier (Morais and Suárez 2016).

The effect of pH on antibacterial metabolite production was evaluated by cultivating the strain 12.1 at various pH ranges from 6–9. Maximum ZOI against indicator strains was obtained at pH 8, while lower ZOI was recorded when

the strain was cultured at 6, 7, and 9 pH. These results indicate that pH 8 favors the synthesis of antibacterial metabolite. Similarly, maximum growth was also observed at pH 8 after 48 h of incubation (Fig. 4).

### Partially Purification and Characterization of Bioactive Compounds

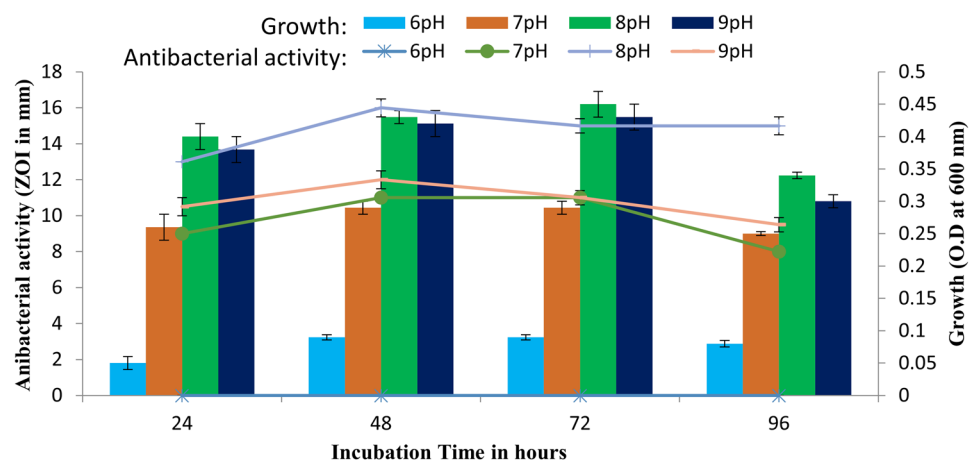
The partially purified bioactive compounds exhibited increased antibacterial activity against the test organism as compared to CFS. The molecular weight of antibacterial metabolites was estimated as > 20 kDa. The antibacterial metabolites were treated with proteinase K lost their antibacterial activity validating that it is a protein in nature. When the CFS was heated from 20–121 °C for 20 min,

the antagonistic activity against test strains decreased at 45–80 °C and denatured when heated up to 100 °C. The sensitivity of antibacterial metabolites to proteinase K and high temperature confirm that it is protein in nature. Hemolytic activity of the partially purified extract was evaluated and no activity was observed against human erythrocytes.

### Determination of MIC

The MIC of partially purified bioactive compounds against ATCC bacterial strains ranged from 2.35–4.66 mg/ml. While against local MDR bacterial strains the MIC ranged from 3.12 to 6.25 mg/ml as shown in Table 1.

**Fig. 4** Optimization of pH for growth and bioactive compounds production by *B.safensis* MK-12.1



**Table 1** Minimum Inhibitory Concentration (MIC) values (mg/ml) of the partially extracted antibacterial metabolites from *B. safensis* MK-12.1, against various test bacterial strains

S.no	Test bacteria	Strain no	MIC	
			MK-12.1 extract (mg/ml)*	Streptomycin (+ive control) µg/ml*
	<i>Salmonella typhimurium</i>	ATCC 29,213	3.12 ± 0.125	50 ± 0
	<i>Staphylococcus epidermidis</i>	ATCC 12,228	3.12 ± 0.125	25 ± 0
	<i>Pseudomonas aeruginosa</i>	ATCC 6305	4.66 ± 0.144	25 ± 0
	<i>Streptococcus pneumonia</i>	ATCC 14,028	2.35 ± 0.132	25 ± 0
	<i>Shigella flexneri</i>	ATCC 12,022	3.12 ± 0.125	25 ± 0
	<i>Staphylococcus aureus</i>	ATCC 13,889	3.12 ± 0.144	50 ± 0
	<i>Escherichia coli</i>	ATCC 9459	2.35 ± 0.132	25 ± 0
	<i>Vibrio cholerae</i>	ATCC 27,853	3.12 ± 0.125	50 ± 0
	<i>Klebsiella pneumoniae</i>	ATCC 25,922	4.66 ± 0.144	50 ± 0
	<i>Acinetobacter baumannii</i>	Local clinical MDR	6.25 ± 0	50 ± 0
	<i>Staphylococcus aureus</i>	Local clinical MDR	3.12 ± 0.12	25 ± 0
	<i>Escherichia coli</i>	Local clinical MDR	3.12 ± 0.12	25 ± 0
	<i>Pseudomonas aeruginosa</i>	Local clinical MDR	3.12 ± 0.14	50 ± 0

\*Data values are the mean of three replicates (n = 3) followed by SD (±)

## Morphological, Biochemical, and Molecular Identification of Strain MK-12.1

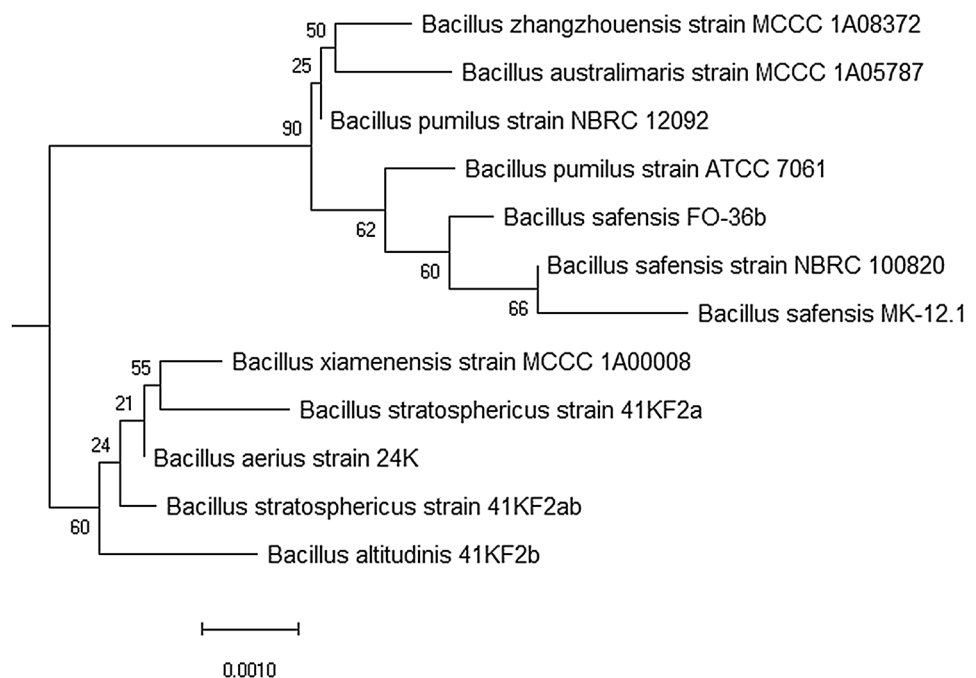
The colony morphology of *B. safensis* MK-12.1 was irregular in shape, with an undulant margin, raised elevation, light yellow in color, and opaque opacity. The phenotypic analysis showed that the strain MK-12.1 is Gram-positive, rod-shaped, motile, and spore-forming bacilli. Biochemical results indicate that *B. safensis* MK-12.1 is positive for ureas, catalase, oxidase production, and MR test while negative for indole, citrate, and VP test. The 16S rRNA gene of strain 12.1 was successfully amplified and > 1200 bp amplified product was obtained (Figure S2). The 16S rRNA gene sequence of strain MK-12.1 has been submitted to GenBank (NCBI) database under the accession number MN519460. The 16S rRNA gene sequence BLASTn analysis with other related strains showed maximum similarity (99.73%) with *B. safensis* strain NBRC 100,820. The phylogenetic tree indicates the close relation of strain MK-12.1 with other *B. safensis* strains and shared a clade with *B. safensis* NBRC and *B. safensis* FO-36b (Fig. 5).

## Discussion

The prevalence of MDR bacteria has increased at an alarming rate in the past decades. Therefore, it is an urgent need to investigate new drugs to combat these MDR pathogenic bacteria. In the present study, environmental samples were collected from the unexplored region in terms of bioactive metabolites producing bacteria. A total of 12 (25.53%) out

of 47 bacterial isolates exhibited antibacterial activity in primary screening. This result indicates a relatively low number of antibacterial strains previously reported (Mutaz Al-Ajlani and Hasnain 2010). Previously CFS of *L. plantarum* was evaluated against pathogenic *E. coli* and 26 mm ZOI was recorded (Kaewchomphunuch et al. 2022). Recently, CFS was collected from *Lactobacillus paracasei* strain CH88 efficiently inhibits the growth of *Gardnerella vaginalis* (Moon et al. 2022). The results of the current study are fascinating as the bioactive compounds synthesized by strain MK-12.1 exhibited promising antibacterial activity against both ATCC and MDR clinical pathogens. According to the current study result, vancomycin showed 24 mm ZOI against MDR clinical strains which is higher than partially purified metabolites from the soil isolates. However, it is expected that further complete purification of the antibacterial metabolites might be comparable to vancomycin. The current study result corroborates with the earlier, where *B. subtilis* strain RS10 was isolated from soil and exhibit potent antimicrobial activity against ATCC indicator strains including *S. aureus* (14 mm ZOI) (Iqbal et al. 2021a). Optimization for biomass and bioactive compound synthesis revealed that CFS of MK-12.1 culture exhibited maximum antibacterial with 18.6 mm ZOI at 30 °C followed by 37 °C with 18 mm of ZOI. While lowest inhibitory activity was noted at lower and higher temperature, which indicate that the optimum temperature for *B. safensis* MK-12.1 is 30 °C and temperature fluctuations play a major role in bacterial growth and production of metabolites. These results match with the other study in which *Lactobacillus* spp. yield highest amount of bacitracin at 25 °C and 30 °C (Malheiros

**Fig. 5** Evolutionary analysis of *B. safensis* strain MK-12.1 with related *Bacillus* species. Phylogenetic tree was generated based 16S rRNA with neighbor-joining method using MEGA X



et al. 2015). Temperature significantly affects the antibacterial metabolite production and may inactivate it to some level. In the earlier investigation, *Bacillus* sp. was found to produce antimicrobial compounds that are stable at high temperature such as 75 °C. A slight loss of activity (20%) was observed at 80 °C (Chalasanani et al. 2015). As compared to general-purpose media such as nutrient broth medium, the nitrogen-rich BHI and OPT media enhance the growth and bioactive compounds synthesis. The ZOI of CFS taken from OPT culture medium was higher (25 mm) than the nutrient broth (15 mm), demonstrating the significant role of nutrients in the production of antibacterial metabolites. The OPT, BHI and TSB medium is rich in terms of carbon and nitrogen source as compared to the nutrient broth medium as conferred in an earlier study (Holzapfel 2002). The MDR Gram-positive strains such as *S. aureus* are often causing UTIs and nosocomial infections and confer resistant to antibiotics by modifying its teichoic acid in the cell wall with D-alanine as a result of increased MIC. The result of the current study is worth observing that the MIC values (mg/ml) against MDR strains did not increase and are comparable to other tested bacterial strains. In previous study, the MIC values against different indicator strains were determined and ranges from 0.3 to 16 µg/ml (for MRSA 5) in BHI and CA-MHB to 120 µg/ml (Chalasanani et al. 2015). The antibacterial activity of extracted bioactive compounds from *B. safensis* MK-12.1 is impeded at high temperature and it did not exhibit hemolytic activity on the blood agar.

In conclusion, the current study results showed that the *B. safensis* strain 12.1 isolated from the southern region in Pakistan has potential for the discovery of novel antibacterial compounds. The strain 12.1 exhibits promising antibacterial activities against both ATCC as well as local clinical MDR strains. The partially extracted bioactive compounds were determined to be protein in nature and have a molecular size of less than 20 kDa. The broad spectrum of antibacterial activities are either due to the broad spectrum of bioactive compound production or, to several bioactive compounds with various potencies. Therefore, further analysis is required to purify and elucidate their structures using various analytical techniques.

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## Declarations

**Conflict of Interest** The authors declare no competing interests.

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