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Antibacterial, antibiofilm and antioxidant activity of secondary metabolites of antagonistic bacterial strain *Bacillus endophyticus* PK2 isolated from marine soil

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Abstract

A prime source of diverse antibacterial and anticancer drugs is the bacterial metabolites produced by marine organisms. In this research, we isolated the antagonistic marine bacterial strain *Bacillus endophyticus* PK2 from seashore soil samples and assessed its antibacterial and antioxidant properties of crude secondary metabolites of strain PK2. The potent antagonistic bacterial isolate PK2 was discovered using 16S rRNA sequencing analysis. The secondary metabolites from strain PK2 were extracted by centrifugation with ethanol, and the agar well diffusion method was used to evaluate them for antibacterial activity against respiratory tract bacterial pathogens. This study demonstrated that the secondary metabolites of PK2 effectively inhibited the growth of respiratory tract bacterial pathogens and effectively hindered their biofilm formation. Moreover, established effective concentration-dependent DPPH radical scavenging activity in *in vitro* conditions. The results of the growth optimization tests indicated that the strain PK2 showed effective growth at temperatures between 30 and 40 °C and a pH range of 7-8. Therefore, upon additional clinical testing, the secondary metabolites of strain *B. endophyticus* PK2 may be exploited to create potent antibiotic and anticancer drugs.

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Introduction

Marine microorganisms are the best source of numerous active metabolic chemicals. The contribution of marine bacteria as novel and abundant sources of physiologically active compounds is crucial. Additionally, it is possible to identify diverse bacteria that produce antibiotics from soil (Srividya *et al.*, 2008; Chandra *et al.*, 2017). Antibiotics, secondary metabolites produced by microorganisms, are frequently chosen as the first line of treatment for bacterial-based microbial infections. Similar to this, just a small number of genera of microorganisms, primarily *Penicillium* sp., *Streptomyces* sp., *Cephalosporium* sp., and *Bacillus* sp. are utilized to create the majority of frequently used antibiotics (Zinsser *et al.*, 1992). These antibiotic-producing microbes are extensively studied to find new types of antimicrobial compounds as alternatives to available antibiotics and combat emerging multidrug-resistant microorganisms.

The *Bacillus* sp. is a member of a phylogenetically distinct group of marine bacterial species. They can tolerate challenging circumstances such as high temperature, pressure, salinity, and pH and are widely distributed in the alkaline environment (Rampelotto *et al.*, 2010). *Bacillus* strains usually require more food and space to grow than their competitor species. Due to the ocean's dilution effect, marine species develop powerful bioactive molecules to repel competitors or avoid predators (Paul *et al.*, 2007; Sayem *et al.*, 2011).

The marine strains differ from their terrestrial counterparts in terms of metabolic behaviors. They may therefore develop distinctive bioactive substances that are not present in their terrestrial equivalents (Jensen *et al.*, 1994; Feling *et al.*, 2003).

For example, the genomic sequencing of the *Bacillus* strains that are extensively spread revealed that around 8% of the genome is used to produce antibiotics (Chen *et al.*, 2007; Kunst *et al.*, 1997). Secondary metabolite groups produced by marine *Bacillus* isolates are structurally diverse and include lipopeptides, polypeptides, macrolactones, fatty acids, polyketides, lipoamides, and isocoumarins. In the current study, we isolated an antibiotic-producing bacteria from soil samples taken from the seashore and used 16S rRNA gene sequencing to identify species. It was discovered that the isolated bacteria belong to the genus *Bacillus*. This study aimed to investigate the antibacterial, antioxidant, and antagonistic activity of secondary metabolites isolated from the marine bacterial strain.

Materials and methods

Sample collection

The seashore soil samples were collected from the tide region of Pudhukuppam (11°31'36.3"N 79°46'00.9"E) coastal region in Cuddalore district, Tamilnadu, India. At a depth of 5–10 cm, soil samples weighing about 10 and 30 g were collected in sterile plastic bags. Obtaining removal of stones and plant remains, the soil samples were sieved through a 0.5 mesh sieve. Soil samples that had been sieved were immediately brought into the lab and kept there for future use in a refrigerator.

Isolation of marine bacteria

The dilution spread plate technique followed by Warcup (1950) was used to isolate the bacteria from a collected soil sample. A soil sample weighing 1g was diluted in 10 ml of 50% seawater (1:1 v/v seawater (30 ppt): distilled water). Serial dilutions were obtained up to 10^{-5}; further, the aliquot of 0.1 mL samples from 10^{-5} dilution was transferred into Nutrient agar plates containing different concentrations of NaCl (10, 20, 30, 40, and 50 %) and incubated for 24 h at 37 °C. After incubation, the plates were observed for the visible colonies on the surface of agar plates.

The total number of colonies was counted using a microbiological colony counter. Further, the morphologically distinct bacterial colonies found on nutrient agar (NA) plates containing 20% NaCl were picked and purified. The purified bacterial isolates were stored in NA slants containing 20% NaCl at 4 °C. the collected bacterial isolates were designated based on the isolation source viz., PK1, PK2, and PK3.

Collection of test bacterial pathogens

The respiratory tract pathogenic bacterial cultures *Haemophilus influenzae* (ATCC- 49247), *Streptococcus pneunoniae* (ATCC - 19615), *Streptococcus pyogens* (ATCC - 49619), *Pseudomonas aeruginosa* (ATCC - 9027) were obtained from the American Type Culture Collection (ATCC), USA, for the study. The acquired cultures were maintained in NA slants.

Screening for antagonistic activity

Using the cross-streaking method, isolated bacterial strains were evaluated for their antagonistic behaviour toward bacterial pathogens. The bacterial pathogens were cross-stretched next to the test bacteria on Muller-Hinton agar (MHA) plates, which had been streaked with a bacterial test strain. The plates were then incubated at 37 °C for 24 hours. To provide the active organism sufficient time to create the metabolites that will penetrate the agar media. After incubation, the plates were checked for visible bacterial pathogen growth inhibition.

Identification of antagonistic bacterial strain

Using a complete DNA extraction kit (OMEGA BioTek, Norcross, GA, USA) in accordance with the manufacturer's instructions, the genomic DNA of antagonistic bacteria, PK2, was obtained. After isolating the genomic DNA, it was amplified at the 16S rRNA V4 region using polymerase chain reaction using universal primers 27F (50-AGAGTTTGATCCTGGCTCAG-30) and 1492R (50-GGTTACCTTGTTACGACTT-30) (PCR). The nucleotide sequences of strain PK2 were discovered and deposited in GenBank (Ass.No: ON873884). The phylogenic tree was created using a neighbor-joining technique after the nucleotide sequences of the strain were compared to known sequences in the NCBI database using MEGA 6.0.

Extraction of secondary metabolites

The bacterial isolate PK2 was chosen for further investigation based on its antagonistic behavior toward bacterial pathogens. A 2000 mL conical flask containing 1000 mL of Nutrient Broth medium with optimum nutrient levels was used to prepare the inoculum of the chosen bacterial isolate. At 30°C, inoculated flasks were incubated for 48 hours while shaken at 200 rpm. After incubation, the culture broth was carefully separated from the supernatant and centrifuged at 10,000 rpm for 20 minutes. In addition, the supernatant was mixed with 100% cold ethanol in a ratio of 13 (v/v) and maintained at 4 °C for 24 hours to precipitate secondary metabolites. The sediments were recovered by centrifugation and purified by washing with Mille Q water, and secondary metabolites pellets were dried at 60 °C.

Assessment of Antibacterial activity

A loopful of cells was subcultured to the nutrient broth and incubated for 24 hours at 37 °C to create the active young cultures for the experiment. To match the turbidity to the 0.5 Mc-Farland standard, or around 1 × 10^6 CFU/ml, the cultures were suspended in the sterile nutritional broth for 24 hours. The test bacterial cultures were swabbed over the Muller Hinton agar in the well-diffusion assay (Kirby-Bauer Method). Then, different amounts of crude secondary metabolite extract from the antagonistic bacteria (100, 150, and 200 µg) and positive control (the common antibiotic ciprofloxacin, 20 µg) were added to the wells. All plates were incubated for 24 hours at 37 °C. After incubation, the plates were checked for visible growth inhibition around the wells. By measuring the inhibition zone, the growth inhibition's diameter was calculated.

Determination of Minimal Inhibitory Concentration (MIC)

The MIC of metabolic extract of strain PK2 against bacterial pathogens was determined in 96 well plates. The metabolite extract was diluted in two-fold dilution from 21.85 to 700 µg and loaded into the wells containing the 100 µL of Muller-Hinton broth, further inoculated with 10 µL bacterial pathogens (for a final concentration of 1 x 10^6 CFU/ml). At 37 °C, the plates were incubated for 24 hours. After incubation, the lowest concentration of the metabolic extract that controlled the visible growth of the tested organisms was determined as MIC.

Biofilm inhibition assay

The antibiofilm experiment was performed to assess the effectiveness of metabolic extracts in preventing biofilm formation (Christensen *et al*., 1985). The 96 wells of flat-bottom polystyrene titer plates were used for the biofilm inhibition assay. After adding 10 lit µL of an overnight pathogenic bacterial culture into the wells containing 100 µL of Muller Hinton broth (MHB), the various concentration of metabolic extract of PK2 (100, 150, and 200 µg) was added to the wells, and further incubated at 37 °C for 24 hours. Following incubation, the well's contents were taken out and washed with 0.2 mL of phosphate buffer saline (PBS), pH 7.2, to eliminate any free-floating bacteria. Crystal violet (0.1% w/v) was used to stain the sessile bacteria's adhesion after sodium acetate (2%) was used to fix it. The deionized water wash removed the bulk of the stain and stored it for drying. Additionally, dried plates were cleaned in 95% ethanol before measuring the optical density at 600 nm with a microtitre plate reader (Thermo). The formula shown below was used to compute the percentage of biofilm inhibition.

$$\text{Percentage of biofilm inhibition} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

DPPH free radical- scavenging activity

The DPPH free radical assay was used to assess the antioxidant activity of the crude metabolic extract of strain PK2. The method described by Brand-Williams (1995) was used to test the DPPH radical scavenging activity. A stable DPPH radical solution containing ethanol was used to react to the sample. 0.3 mL of a DPPH radical solution containing 0.5 mM in methanol, 3 mL of 100% ethanol, and 0.5 mL of sample are added to the reaction mixture. A color change will happen when DPPH reacts with an antioxidant molecule. At absorbance was recorded at 517 nm. A blank mixture comprises 3.3 mL of ethanol and 0.5 mL of the sample. Alcohol (3.5 mL) and a DPPH radical solution were combined to create the control solution of ascorbic acid. The scavenging activity percentage (AA%) was calculated using the following formula.

$$\text{Radical scavenging activity (AA\%)} = \frac{Abs\ control - Abs\ sample}{Abs\ control} \times 100$$

Growth parameter optimization studies

The growth parameter optimization studies were conducted for PK2 strain for better growth performance. The parameters such as pH, and temperature were subjected to single factor optimization experiments. This study including different pH (5.0, 6.0, 7.0, 8.0, and 9.0), and different temperature (10, 20, 30,40, and 50 °C) in nutrient medium. All experimental sets were incubated in a shaker (180 rpm) for 72 hours. All experiments were conducted in triplicate, and the colorimeter determined the optical density.

Results and discussion

Isolation of bacteria

Microbiological research focuses on developing new antibiotics and antimicrobials to fight resistant bacteria to existing antimicrobials. The advent of microorganisms that are resistant to antibiotics poses a serious threat to human life and makes it difficult to control infectious diseases that are brought on by these pathogens (Tabbene *et al*., 2009; El-Baz *et al*., 2021). The collected soil sample was serially diluted and inoculated on nutrient agar plates containing different concentrations of NaCl.

The maximum number of colonies (28.24±1.62 CFU/g x 10^5) were found on the NA plates containing 20% NaCl, followed by the 30% (25.14±1.32 CFU/g x 10^5) and 10% (12.32±0.67 CFU/g x 10^5). Very least or no visible colonies were observed on the NA plates containing 40% and 50% NaCl (Table 1). Three morphologically distinct bacterial colonies were isolated, purified, and stored for further study.

Table 1. Total colony forming units in a collected soil sample (CFU/g x 10^5).

NaCl concentration (%)				
10	20	30	40	50
12.32±0.67	28.24±1.62	25.14±1.32	4.65±0.83	0.43±.02

The values are expressed in the mean ± standard deviation of three replicates.

Antagonistic activity of isolated bacteria strain

To determine whether the isolated bacterial strains were antagonistic to the tested bacterial pathogens. Among the tested bacterial strains, isolate PK2 showed significant growth inhibitory activity against more than 3 examined bacterial pathogens.

Other isolates showed inhibitory activity against one bacterial pathogen or non (Fig. 1A). In the current investigation, we examined soil samples taken from the shoreline for the presence of microorganisms that produce antibiotics. The strain PK2 significantly inhibited the growth of bacterial pathogens. By partial sequencing of the 16S rRNA, we isolated and identified the strain PK2 as *Bacillus endophyticus*. Potential sources of structurally diverse secondary metabolites, such as lipopeptides, polypeptides, macrolactones, fatty acids, polyketides, lipoamides, isocoumarins, and carotenoids, include marine *Bacillus* species. Complex biosynthetic metabolic pathways give rise to the structurally diverse natural compounds produced by marine isolates.

Table 2. Antibacterial activity and Minimal inhibitory concentration of secondary metabolites of strain PK2.

Bacterial pathogens	Zoon of inhibition (mm)				
	100 µg	150 µg	200 µg	Ciprofloxacin (20 µg)	MIC
Haemophilus influenzae	09.39±0.52	11.15±0.35	15.26±0.15	21.28±0.46	37.5±1.6
Streptococcus pneunoniae	-	08.25±0.34	12.34±0.53	19.35±0.43	150.0±3.7
Streptococcus pyogens	-	-	08.44±0.37	19.59±0.57	150.0±3.2
Pseudomonas aeruginosa	08.53±0.73	10.23±0.41	14.38±0.52	20.53±0.73	75.0±3.7

-: No zone of inhibition. The values are expressed in the mean ± standard deviation of three replicates.

There is great potential in several of these bioactive chemicals to create powerful pharmaceutical and agrochemical products. *Bacillus* strains obtained from particular niches (such as a hydrothermal vent, deep sea, pH > 9.0, and salt lakes) may produce beneficial bioactive chemicals because of their genetic capacity to adapt to extreme settings (Li and Vederas, 2009).

Identification of antagonistic bacteria

The nucleotide sequences of the isolated antagonistic bacterial strain were examined for similarities with known sequences using the BLAST search tool. Over 98% identity was found between the derived sequences and the known *Bacillus* sp. sequence. The strain's genus and species names were also determined as *B. endophyticus* (Fig. 1B). Either ribosomal or non-ribosomal processes produce peptide antibiotics that are encoded in the genomes of several *Bacillus* sp. (Khalaf *et al*., 2020; Mannanov and Sattarova, 2020). Although glycopeptide antibiotics have a limited range of activity, they are the last line of defense in the fight against life-threatening infections brought on by Gram-positive human pathogens, including MRSA and *Clostridium difficile* (Binda *et al*., 2014; Garau *et al*., 2009).

Antibacterial activity, MIC and antibiofilm activity of the crude metabolic extract

The metabolic extract of the PK2 strain was examined for the bactericidal activity again the respiratory tract pathogens on solid MHA plates. The significant growth suppression activity was established by the crude secondary metabolites of isolate PK2 according to the concentrations treated. The maximum growth inhibitory activity of metabolic extract of strain PK2 was observed against *Haemophilus influenzae* followed by *Pseudomonas aeruginosa* and *Streptococcus pneunoniae*. However, the metabolites extract of strain PK2 exhibited lesser inhibitory activity against *Streptococcus pyogens* than other tested bacterial pathogens.

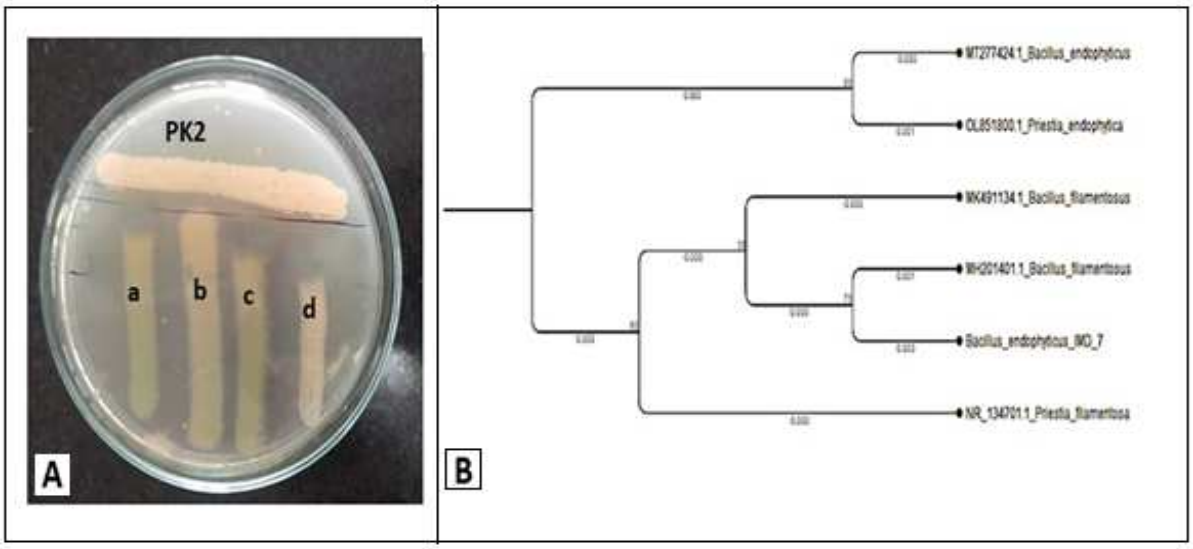

Fig. 1. (**A**) Antagonistic activity of bacterial isolate PK2 against the human respiratory bacterial pathogens (a) *Streptococcus pyogens,* (b) *Streptococcus pneunoniae, (c) Pseudomonas aeruginosa,* and *(d) Haemophilus influenzae* on solid agar medium. (B). Phylogenetic classifications of isolated antagonistic bacterial stain PK2. The tree was constructed by the neighbour-joining method with known bacterial sequences using MEGA 6.0.

The zone of inhibition exhibited by the antibiotic Ciprofloxacin was 18 to 20 mm (Fig. 2). The lowest MIC value, 37.5 1.6 µg/ml, was observed against *Haemophilus influenzae*. The MIC values ranged from 37.5 to 150.0 µg/ml. At a dose of 150.03.2 µg/ml, *Streptococcus pyogens* were first inhibited (Table 2). The isolate PK2 showed effective antibacterial activity in the current study against the tested respiratory tract infections. Against all of the test microorganisms, the crude metabolic extract of the 200 µg *Bacillus endophyticus* strain showed encouraging antibacterial activity.

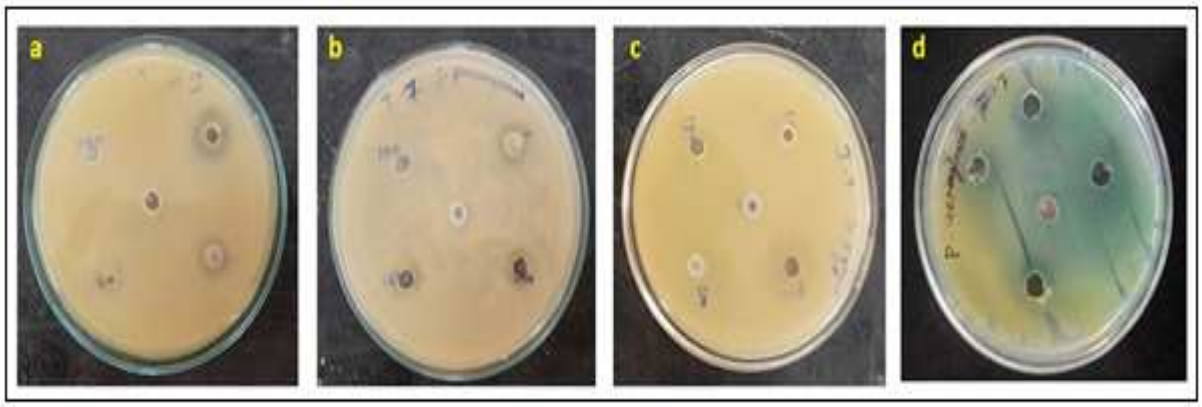

Fig. 2. Antibacterial activity of crude secondary metabolites of PK2 against respiratory bacterial pathogens (a) *Haemophilus influenzae* (b) *Streptococcus pneunoniae,* (c) *Streptococcus pyogens* (d) *Pseudomonas aeruginosa* on solid MHA plates.

At low concentrations, *Streptococcus pyogens* was resistant to the tested crude extract. The lipopolysaccharide barrier for the hydrophobic chemicals and the limited permeability of the bacteria's outer membrane may be responsible for this resistance (Delcour, 2009; Wiener and Horanyi, 2011). The antibiofilm activity of crude metabolic extract of strain PK2 was determined by the 96-well plate method. The crude metabolic extract of strain PK2 exhibited effective antibiofilm activity against selected pathogens in a concentration-dependent way.

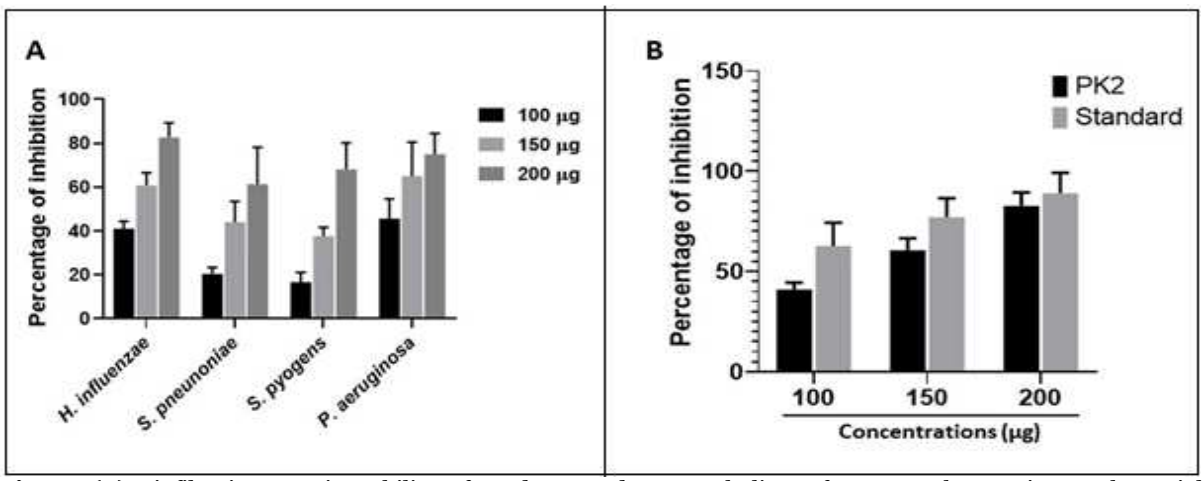

Fig. 3. (A) Biofilm interruption ability of crude secondary metabolites of PK2 on the respiratory bacterial pathogens were tested in 96 well plates. The maximum biofilm inhibition was observed at 200 µg concentration against *Haemophilus influenzae*. (B) DPPH radical scavenging activity of secondary metabolites of PK2. The maximum inhibition was observed at 200 µg. Error bars indicate the mean ± standard deviation of three replicates.

The metabolic extract interrupted the biofilm formation of *Haemophilus influenzae* and *Streptococcus pyogens* more than the other tested bacterial pathogens (Fig. 3A).

Antioxidant activity crude metabolic extract

The crude metabolic extract of strain PK2 showed a concentration-dependent DPPH free radical scavenging activity. It possesses hydrogen donating ability, and the maximum inhibition of 51.6% was observed at 40 µg concentration, which is considerably lesser than the inhibitory activity of 40 µg/ml of standard (Fig. 3B). Previously, Delcour, (2009) reported that the metabolites extracted from *Bacillus* sp showed effective antioxidant activity in *in vitro* condition.

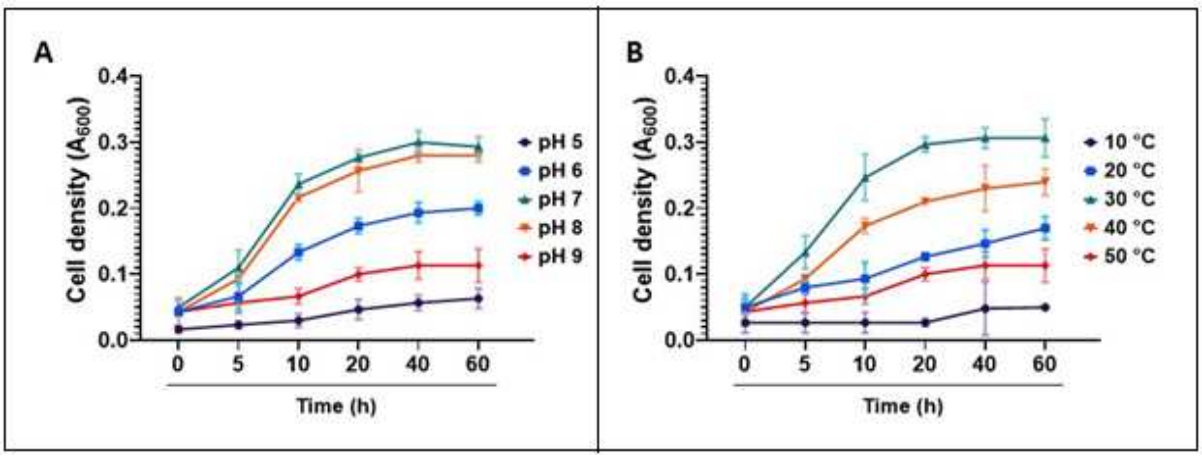

Fig. 4. (A) Effect of different pH on growth of bacterial strain PK2 in liquid medium. The maximum change was observed at the pH of 7.0. (B) Effect of different temperatures on the growth of bacterial strain PK2 in liquid medium. The maximum growth was observed at the temperature of 30 and 40 °C. Error bars indicate the mean ± standard deviation of three replicates.

Effect of different pH and Temperature on bacterial growth

The results show that the strain PK2 had effective growth at pH 6 to 8. The maximum bacterial growth was observed at pH 7, followed by pH 6.0 and pH 8.0. Other tested pH levels have highly reduced the

growth of PK2 strain in a liquid medium. The strain PK2 had effective development at the temperature ranging from 20 °C to 40 °C. Other tested temperature levels have highly reduced the growth of the tested bacterial strains. The maximum bacterial growth was observed at 30 °C, followed by temperatures of 40 °C and 20 °C (Fig. 4).

Conclusion

Bacillus endophyticus KP2, an isolated antagonistic bacterial strain, effectively inhibited a number of bacterial pathogens. The *Bacillus endophyticus* KP2 crude secondary metabolic extract on MH agar plates exhibited effective bactericidal activity against respiratory bacterial pathogens. A crude secondary metabolic extract of *Bacillus endophyticus* PK2 also demonstrated a significant amount of bacterial biofilm inhibition and DPPH radical scavenging ability. The results indicate that the crude secondary metabolic extract of *Bacillus endophyticus* KP2, an antagonistic bacterial strain, has potent antibacterial and antioxidant properties in an *in-vitro* condition. Therefore, following appropriate clinical trials, it could be used to treat infectious diseases.

Conflict of interest

There is no conflict of interest in the current study.

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