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Bioprospecting secondary metabolites with antimicrobial properties from soil bacteria in high-temperature ecosystems

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Abstract

Background The ongoing emergence and spread of drug-resistant pathogens necessitate urgent solutions. Natural products from bacterial sources are recognized as a promising source of antibiotics. This study aimed to isolate and characterize soil microorganisms from extremely hot environments and to screen their secondary metabolites for antibacterial activity.

Methods Bacterial isolates were identified using standard culture techniques. Primary and secondary screenings for antimicrobial activity were conducted using the Modified Kirby-Bauer antibiotic susceptibility test against five bacterial species. Based on the efficacy of antimicrobial activity against these target pathogens, the isolate *Pseudomonas sp*. strain ASTU00105 was selected for further characterization through whole genomic sequencing. Secondary metabolites were analyzed using GC–MS, and antioxidant activities were also evaluated.

Results A total of 76 isolates were identified, and their secondary metabolites were tested against *Escherichia coli*, *Salmonella typhi*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Candida albicans*. Seventeen isolates (22.37%) exhibited antimicrobial activity. Isolate ASTU00105 exhibited the highest activity against all the test organisms and was selected for further analysis. Whole-genome sequencing using the Nanopore MinION sequencer revealed that strain ASTU00105 belonged to the genus Pseudomonas with the highest similarity (95.97%) to *Pseudomonas stutzeri*, and designated as *Pseudomonas sp*. strain ASTU00105. Upon Average Nucleotide Identity (ANI) analysis, the strain exhibited 87.81% sequence similarity with genes of the closest type strain, suggesting its novelty and distinctiveness within the Pseudomonas genus. The genomic analysis of the isolated strain revealed 6 biosynthetic gene cluster (BGC) genes dispersed throughout the entire genome, which are implicated in the synthesis of antimicrobial secondary metabolites. The major chemical compounds detected in the EtAc extracts as detected by gas chromatography-mass spectrometry (GC–MS) were phenol, 2,5-bis (1,1-dimethylethyl) (36.6%), followed by 1,2-Benzenedicarboxylic acid, diethyl ester (12.22%), Eicosane (9.71%), Dibutyl phthalate (3.93%), and 1-Dodecanol (2.34%).

In conclusion *Pseudomonas sp.* strain ASTU00105 exhibited the greatest potential for producing secondary metabolites with significant antimicrobial activity.

Keywords Antimicrobial activities, *Pseudomonas*, *Secondary metabolites*, *Gas chromatography-mass spectrometry*

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Introduction

The emergence and spreading of drug resistant pathogenic bacterial infection has long become significant global public health concern [1]. Most pathogenic strains have developed resistance to the standard antibiotics due to the inappropriate or misuse of antibiotics over an extended period [2]. Antibiotic resistance frequently renders existing drugs ineffective, highlighting the critical need for novel antibiotics. What is even more worrisome is the fact that the production and commercialization of new antibiotics for public health use has been limited for the last 50 years. Walsh, 2003 [3] noted that very limited classes of antibiotics were introduced between the years 1962 and 2000, and can be referred to as a period an innovation gap. This situation creates a serious gap in the treatment of pathogenic bacterial infections, particularly drug-resistant ones and hence raising concerns about a potential return to the pre-antibiotic era, in which millions die from such infections. The World Health Organization (WHO), has recognized this problem and formulated strategies to enhance research and innovation for the development of new antibiotics through research and development schemes [3].

Natural products derived from environmental microorganisms play a crucial role in the treatment of bacterial infections and remain the most promising sources of future antibiotics. In the past, the majority of antibiotics used to combat pathogenic bacterial infections, although most have currently lost and/or are losing their efficacy due to antimicrobial resistance (AMR), were discovered from microorganisms [4, 5]. A vast number of bacteria producing a wide range of primary and secondary metabolites such as enzymes, antibiotics, and novel compounds have been identified and extensively studied by various research groups in the fields of human health, agriculture, and animal health. Due to the untapped potential of these microorganisms in producing valuable secondary compounds, there are several ongoing efforts to discover new microbial species capable of synthesizing distinctive compounds. In light of this, in recent times, unexplored environments such as extreme desert regions, volcanic areas, high-salinity water bodies, sea sediments, and pristine forests are being investigated to identify novel bacteria with the potential to produce secondary metabolites with antimicrobial activities [6, 7].

In the current study, we aimed to identify a bacterial isolate from extremely hot environment of Ethiopia that produces secondary metabolites with antibacterial activity against pathogenic bacteria and fungi, as well as to characterize the antimicrobial potential of these secondary metabolites. To this end, we identified a novel *Pseudomonas sp.* strain ASTU00105 that exhibited antimicrobial activity against *Escherichia coli*, *Salmonella typhi*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Candida albicans*. In silico analysis revealed the presence of six secondary metabolite biosynthesis gene clusters containing potential compounds with antimicrobial activity. Additionally, the EtAc extract of the isolate was characterized using GC–MS, which identified phenol, 2,5-bis(1,1-dimethylethyl) (36.6%) as the major compound, followed by 1,2-benzenedicarboxylic acid, diethyl ester (12.22%), eicosane (9.71%), dibutyl phthalate (3.93%), and 1-dodecanol (2.34%).

Methodology

Sample collection

Soil samples (n = 15) were collected from the Afar region (Daloli, Alalobad, Amedela, Afrera) and Adama (Table 1). During the sample collection, the top layer of the soil was carefully removed and soil samples were obtained from a depth of 5 cm. Around 20–30 gm of soil was aseptically collected in sterile polyethylene bags. Subsequently, the collected soil samples were transported to the Microbiology laboratory of the Institute of Pharmaceutical Sciences (IPS) and then dried under a shade.

Bacterial isolation

The standard serial dilution technique was used to isolate the bacteria from the soil samples. One gram of soil was mixed with 10 ml of sterile water and a series of dilutions ranging from 10^{-1} to 10^{-5} were prepared. A diluted

Table 1 The geo-coordinates of soil sample collection sites

SN	Soil sample collection site	Sample site code	No of samples	Coordinate's locations		Elevations (m.a.s.l)
				Latitude	Longitude	
1	Daloli	DL	3	14.214281	40.309214	− 92
2	Alalobad	AL	4	11.622245	41.013788	400
3	Amedela	AM	3	14.115998	40.348176	− 124
4	Afrera	AF	2	13.220131	40.874831	− 110
5	Adama	AD	3	8.564107	39.290471	1669

sample of 100 µL was then inoculated onto nutrient agar plates supplemented with nystatin 40 µg/mL (Sigma-Aldrich, Steinheim, Germany) to prevent fungal overgrowth. After an incubation period of 2–3 days at 37 °C, individual colonies were selected and transferred aseptically to fresh nutrient agar plates to obtain pure cultures. The isolated bacterial strains were then stored in 2 ml Eppendorf tubes containing 20% glycerol and nutrient broth at − 80 °C until required for further experiments.

Primary screening

Primary screening was conducted using the Modified Kirby Bauer antibiotic susceptibility test (agar plug diffusion method). In this method, 6 mm of bacterial isolates, grown for 2–3 days on nutrient plates, were placed in boreholes of equal diameter created on plates seeded with test pathogens (*E. coli* ATCC 25922, *A. baumannii* ATCC 19606, *S. typhi* ATCC 26531, *S. pyogenes* ATCC 12204, *S. aureus* ATCC 25923 and one pathogenic fungus (*C.albicans* ATCC 10231) at (McFarland standards of 0.5). The plates were then incubated at 37 °C for 24 h, and the zones of inhibition were determined. Isolate (ASTU00105) which showed better activity were selected for secondary screening and further analysis.

Secondary screening

The screening of secondary metabolites of the isolates was conducted by agar well diffusion method as described by Magaldi et al., [8]. Briefly, isolates exhibiting inhibitory activity against the tested pathogens during the primary screening were individually cultured in Mueller–Hinton broth (MHB) under shaking conditions at a temperature of 28 ± 2 °C. After 7–9 days of culture, the cell density was adjusted to an optical density (OD) of 2.5 at 600 nm using a UV/Visible spectrophotometer (Mecasys, Republic of Korea, Model: Optizen 2120UV) The culture was then transferred to 50 ml Falcon tubes and centrifuged at 10,000 × g for 5 min. The resulting supernatant, representing the cell-free culture filtrate, was collected and stored at 4 °C. The inhibitory activity of the crude extract from each isolate was evaluated using the agar well diffusion method. The test pathogens were grown overnight in MHB until reaching a 0.5 McFarland turbidity standard. Subsequently, 100 µl of the test pathogen was evenly spread on MHB agar plates. Using a sterile cork-borer, well (6 mm) were created in the agar plate, and loaded with 50 µl and 100 µl of the crude extract from ASTU00105 isolate that had strong antibacterial activities, with Ciprofloxacin (10 µg/mL) used as a positive control for bacteria, amphotericin B (8 µg/ml) as a positive control for fungi, and dimethyl sulfoxide (DMSO) solvent as a negative control. After 48 h of incubation, the antibacterial activity of the culture filtrate was determined by measuring the zone of inhibition surrounding the wells and mean (± SD) were calculated. The isolates (ASTU00105) which showed the most promising activity was selected for further analysis.

Gram stain, biochemical and growth characteristics of ASTU00105

Overnight incubated isolates were spread onto the slide and heat-fixed. Then, the slide was flooded with crystal violet for 1 min, followed by rinsing with running water. Next, the slide was immersed in a gram of iodine solution for 1 min and washed with alcohol. Finally, the slide was flooded with safranin for 30 s, washed with water, dried, and observed under oil immersion. For the biochemical tests, a loopful of pure test isolate culture was poured into Luria Bertani Broth (LB) and cultured for 4 days at 30 °C. After growth, approximately 100 µl of the culture suspension was used for biochemical tests following the standard protocols described by Masi et al., [9]. These included several biochemical, motility, and enzymatic tests such as triple sugar iron (TSI), MR-VP, sulfur, indole, motility (SIM) test, catalase, citrate, urease test, carboxymethylcellulose (CMC), starch hydrolysis, gelatin hydrolysis, and casein hydrolysis. Optimum growth conditions was determined with respect to the carbon sources) and nitrogen sources as well as incubation temperatures, pH levels and NaCl concentrations as previous described [10].

Extraction of secondary metabolites

Solvent extraction (ethyl acetate) of the bioactive metabolite from ASTU00105 isolate, which exhibited broad-spectrum activity in primary screening, was performed using submerged fermentation, following the method described by Kibret et al. [11] with some modifications. Briefly, each fresh culture (0.5 mL) was transferred into 500 mL conical flasks containing 200 mL of LB broth. The transfer was conducted under sterile conditions and the flasks were placed in a shaking incubator at 30 ± 2 °C for 13 days until the OD value at 600 nm reach 3.6 (stationary phase) using a UV/Visible spectrophotometer (Mecasys, Republic of Korea, Model: Optizen 2120UV). After fermentation, the supernatant was collected by centrifugation at 10,000 rpm for five minutes. The supernatant containing the bioactive metabolite was further filtered using Whatman No. 1 filter paper. The filtrate was collected and mixed at a 1:1 ratio with an equal amount of ethyl acetate. The organic solvent phase containing the bioactive compound was separated from the aqueous phase using a separatory funnel. The solvent phase was collected and evaporated in a vacuum rotary evaporator at 90 rpm and 60 °C following the method described by Sarika et al. [12]. The completely dried residues from

each isolate were weighed separately using a balance, dissolved in DMSO, and stored in small vials at 4 °C for further antimicrobial activity determination and analysis.

Minimum inhibitory concentration (MIC)

Values were determined in triplicate using broth micro dilution assays in a 96-well microtiter plate. The EtAc extract was diluted in 10% DMSO to obtain the stock solution of the extract, and the serially diluted as 500 μg/mL, 250 μg/mL,125 μg/mL, 62.5 μg/mL, and 31.25 μg/mL solutions. One hundred microliters of the extract were introduced into a well containing 90µL of Mueller–Hinton Broth (MHB) or Sabouraud Dextrose Broth (SDB), and 10µL of inoculum (at 1×10^6 CFU/mL for bacteria and 1×10^5 spores/mL for yeasts) which were added to obtain a final concentration range of 500 to 31.25 µg/mL, and plate was incubated for 24 h at 3 °C for bacteria and 48 h at 30 °C for *C. albicans*. The value of MIC was defined as the lowest of concentration of the extract that induced no visible growth when compared with the negative control. Cultures with no visible growth were then placed on appropriate media. The MBCs and MFCs were calculated as the concentration that prevented growth of more than 99.9% of microorganisms after incubation for 24 h at 28 or 37 °C.

Minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC)

To determine MBC or MFC, 10 µL samples were taken from each well where no growth of microorganisms was observed. These samples were then placed on Mueller–Hinton Agar or Sabouraud Dextrose Agar (SDA) plates and incubated for 24 h at 28 or 37 °C. The lowest concentration that showed no growth after subculturing was identified as the minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC), depending on the type of microorganism. The negative control consisted of broth with 20 µL of DMSO. The entire assay was performed triplicate to ensure accuracy.

Antibiofilm assay

Antibiofilm activity of the extract was conducted only for bacteria, according to the method modified by Getahun et al., [13]. Briefly, varying concentrations of the extract, ranging from MIC (31.25–500 µg/mL), were mixed with bacterial culture media at an initial turbidity of 1.5×10^8 CFU/mL. The mixtures were then incubated for 48 h at 37 °C. The planktonic cells were removed by gently washing with sterile phosphate-buffered saline (PBS), and the adherent cells were stained with 1% crystal violet (CV) for 10 min and washed with PBS to remove the excess stain. After air drying, the CV bound to the biofilm was solubilized with 33% glacial acetic acid. A control without any treatment was set up using similar procedures for comparison to quantify the adherent cells. The absorbance of the CV solution was measured using a UV spectrophotometer at OD590 (Mecasys, Republic of Korea, Model: Optizen 2120UV).

Antioxidant activities

The antioxidant activity test was performed by using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging assay as described by Fahmy et al., [6]. A 0.1 mM DPPH solution was prepared in methanol and kept in the dark for 30 min to complete the reaction. Equal volume of extract (50 µg/mL, 150 µg/mL, 250 µg/mL, 350 µg/mL, 450 µg/mL, 550 µg/mL or vitamin C (2–10 µg/mL) mixed with a methanol solution of DPPH (0.2 mM) and incubated in the dark. The same concentrations of ascorbic acid were used as a standard, and the sample-free DPPH solution was used as a negative control. After mixing 1 mL of DPPH solution with 3 mL of prepared samples, the mixture was incubated at room temperature in a dark place for 30 min, and the absorbance (Ab) was measured at 517 nm. The %radical scavenging activity (RSA) was calculated according to the following formula:

$$\%RSA = \frac{\text{Ab Blank} - Ab\,sample}{Ab\,control} \times 100$$

Gass chromatography—mass spectrometry (GC–MS) analysis

The EtAc extracts of ASTU00105 isolate were subjected to GC–MS analysis using an Agilent Technologies GC system (model 7890 B) coupled with an Agilent Technologies MS system (model 5977A network). The GC system was equipped with an HP-5MS column (30 m×0.25 mm internal diameter (i.d.) and 0.25 µm film thickness). Helium was used as the carrier gas, and the injection/purge time was set at 1.0 min with no splitting. A solvent delay of 4 min was employed. The flow rates and total runtime were varied for different analyses. The mass spectra were acquired in electron impact mode with an ionization energy of 70 eV and scanning in the range of 33 to 550 m/z. To identify the volatile compounds, present in the EtAc extracts, the obtained mass spectra were compared with those of the compounds in the NIST11 GC–MS library database.

Sequencing of the strain using nanopore MinION sequencing

High-quality genomic DNA of ASTU00105 isolate was extracted using the QIAamp® BiOstic® Bacteremia DNA Kit (Qiagen, Germany). The concentration and purity of the DNA were assessed using a NanoDrop and Qubit (Thermo Scientific, USA). The purified DNA was sent to

the Department of Health Technology and Informatics laboratory of Hong Kong Polytechnic University (Hong Kong) for library preparation and sequencing. DNA libraries were prepared using the Oxford Nanopore Rapid Barcoding 96 V14 (SQK-RBK114.96) kit. Briefly, fifty nanograms (200 ng) of genomic DNA were barcoded. The barcoded target sample was pooled with another 11 barcoded samples following the manufacturer's protocol. Nanopore adapters were then ligated to the pooled samples and library was prepared and processed for sequencing. The prepared library was loaded and sequenced on MinION Mk1C nanopore sequencing machine according to the manufacturer's instructions.

Genome assembly, annotation, and phylogenetic analysis

Genome de novo assembly was performed with Canu assembler v.1.8 using the default parameters [14]. The quality analysis of the assembly was performed with Racon [15]. The complete *Pseudomonas* sp. strain ASTU00105 genome was uploaded to the Bacterial and Viral Bioinformatics Resource Center (BV-BRC) web server (https://www.bv-brc.org/) for gene prediction and functional annotation with the RASTtk pipeline [16]. For phylogenetic analysis, the closest reference and representative genomes were identified using Mash/MinHash [17]. Global protein families (PGFams) were selected from these genomes to determine the phylogenetic placement of this genome.

Biosynthetic gene cluster identification

Secondary metabolite-related Biosynthetic gene cluster (BGC) were identified using online tool antiSMASH version v7.0 [18].

Results

Primary screening results of an antibiotic-producing organism

A total of 76 isolates were identified and tested against five pathogenic bacteria and one fungal pathogen to assess their antimicrobial potential. From the overall isolates, 17 (22.37%) isolates exhibited antimicrobial activity. Among the bioactive isolates screened, the highest proportion, accounting for 12 (70.58%) isolates, displayed antagonistic activity against *S. pyogenes*. The organisms with the lowest inhibition were *S. typhi*, which were inhibited by 5 (29.41%) of isolates. Isolates IPS001, IPS040, IPS050, and ASTU00105 showed a zone of inhibition against all the microbes investigated in this study. It is worth noting that isolate ASTU00105 exhibited the highest inhibitory activity against.

all the tested pathogens (Table 2).

Secondary screening of *Pseudomonas sp.* strain ASTU00105

Based on the strong inhibitory signal observed in the primary screening, ASTU00105 was selected for secondary screening. The secondary screening was conducted using the well diffusion technique (Figs. 1 and 2). The

Table 2 Primary screening of bacterial isolates against selected pathogenic bacteria and fungus, 2023

Isolate ID	*E. coli*	*A. baumanii*	*S. typhi*	*S. pyogenes*	*S. aureus*	*C. albicans*
IPS001	15.67 ± 1.5	12.67 ± 0.57	15.67 ± 0.57	16.67 ± 0.57	15.67 ± 0.57	15.3 ± 0.57
IPS002	15.67 ± 1.15	0	0	14.67 ± 0.57	13.67 ± 0.57	12.0 ± 1.00
IPS003	14.3 ± 1.15	13.67 ± 0.57	0	0	0	13.3 ± 0.57
IPS004	14.0 ± 2.00	0	0	12.67 ± 1.5	0	0
IPS005	0	3.67 ± 6.35	0	0	0	0
IPS006	0	0	0	12.0 ± 1.00	13.67 ± 0.57	0
IPS007	0	12.0 ± 1.00	0	13.67 ± 0.57	12.3 ± 0.57	0
IPS008	0	14.0 ± 0.00	0	0	0	0
IPS009	13.3 ± 0.57	12.0 ± 1.70	12.3 ± 0.57	0	0	0
IPS020	0	0	0	12.0 ± 1.00	12 ± 1.00	12.0 ± 0.00
IPS030	0	0	0	13.3 ± 0.57	0	0
IPS040	16.0 ± 1.00	14.3 ± 0.57	15.3 ± 0.57	16.67 ± 0.57	15.67 ± 0.57	14.0 ± 0.00
IPS050	15.67 ± 1.5	12.67 ± 0.57	15.67 ± 0.57	16.67 ± 0.57	15.67 ± 0.57	15.3 ± 0.57
IPS060	15.67 ± 1.15	0	0	14.67 ± 0.57	13.67 ± 0.57	12.0 ± 1.00
IPS070	14.3 ± 1.15	13.67 ± 0.57	0	0	0	13.3 ± 0.57
ASTU00104	0	0	0	13.67 ± 1.32	0	14.0 ± 1.00
ASTU00105	21.00 ± 0.89	30.10 ± 0.58	21.0 ± 0.00	20.67 ± 0.57	19.67 ± 0.40	15.70 ± 1.70
Ciprofloxacin*	24.67 ± 0.57	21 ± 0.00	21.3 ± 0.57	22.67 ± 0.57	23.67 ± 0.57	0
Amphotericin B*	0	0	0	0	0	19.67 ± 0.57

*Ciprofloxacin (10 µg/mL) for bacterial pathogens and /Amphotericin B (8 µg/mL) for *C. albicans* used as positive controls

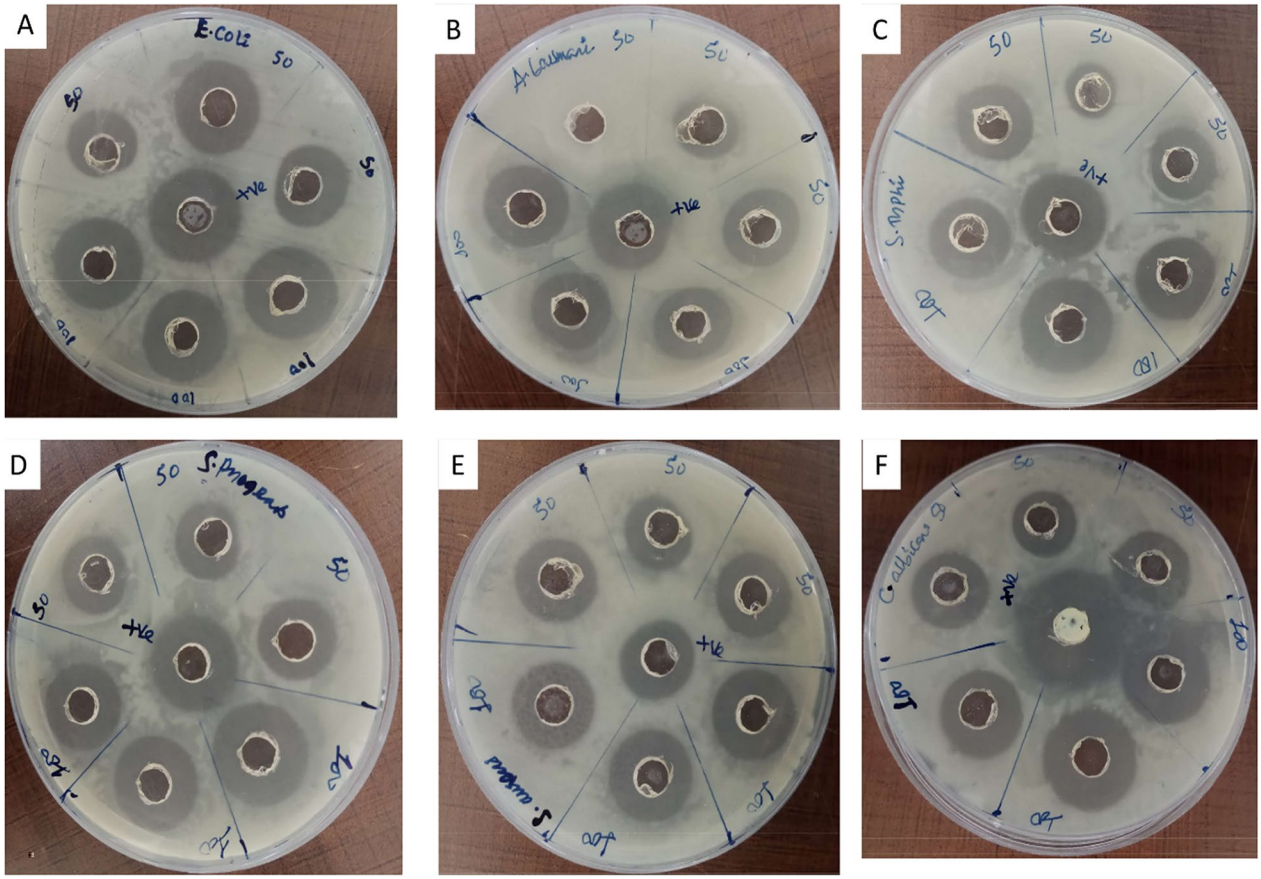

Fig. 1 In vitro antimicrobial activity of EtAc extract of ASTU00105 as determined by well-against **A** *E. coli* **B** *A. baumanii* **C** *S. typhi* **D** *S.pyogenes* **E** *S.aureus* **F** *C. albicans*

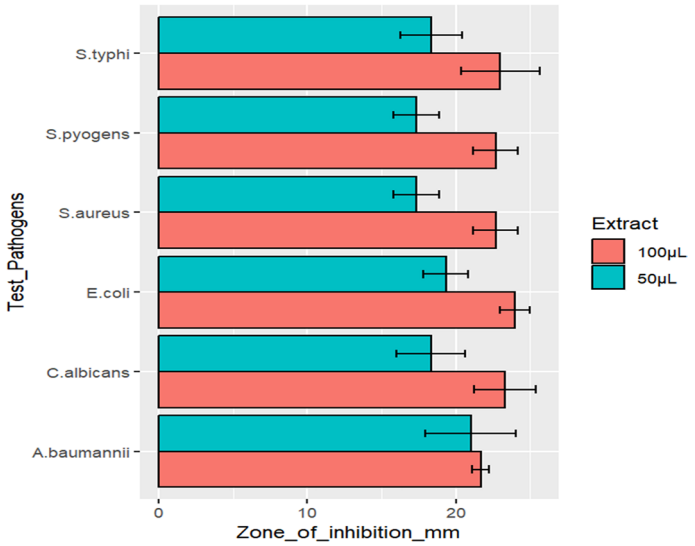

Fig. 2 The inhibition zone (mm) of ASTU00105 extract during the secondary screening using well disc diffusion. *Ciprofloxacin (10 µg/mL) for bacterial pathogens and /Amphotericin B (8 µg/mL) for *C. albicans* were used as positive controls

Table 3 Minimum Inhibitory Concentration, Minimum Bactericidal Concentration, Minimum Fungicidal Concentration of EtAc-extract of ASTU00105

Tested pathogens	MIC (µg/mL)	MBC (µg/mL)	MFC (µg/mL)	MBC/MIC (µg/mL)	MFC/MIC (µg/mL)
E. coli	62.5	125		2	
A.baumani	62.5	125		2	
S.typhi	125	250		2	
S. pyogens	31.25	62.5		2	
S. aureus	31.25	62.5		2	
C. albicans	62.5		125		2

EtAc extract at 50 and 100 µl demonstrated the highest inhibitory zone against the test pathogens. EtAc extract of 50 µl and 100 µl showed a zone of inhibition ranging from 17.33 ± 1.53 to 21.00 ± 3.00 mm and 21.33 ± 0.58 to 26.67 ± 2.08 mm, respectively. Except for *A.baumani* and *C.albicans*, the inhibitor zone of the extract at 100 µl was greater than the positive control in the test pathogens.

Minimum inhibitory concentration (MIC) and minimum bactericidal or fungicidal concentration (MBC/MFC) of EtAc of ASTU00105 extract

The broth dilution technique was employed to investigate the MIC of EtAc-extract of ASTU00105. The overall MIC values ranged from 125 to 31.25 µg/mL against the tested pathogens. The extract exhibited lower MIC against gram-positive bacteria, including *S. pyogens* (31.25 µg/mL) and *S. aureus* (31.25 µg/mL), while higher MIC values were observed in gram-negative bacteria: *E. coli* (62.5 µg/mL), *A. baumani* (62.5 µg/mL), *S.typhi* (125 µg/mL), and the fungus: *Candida albicans* (62.5 µg/mL). A control using 10% DMSO showed no activity against the test pathogens (Table 3). The MBC/MFC of the EtAc extract ranged from 62.5 to 250 µg/mL. Notably, *S. typhi* exhibited higher MIC and MBC values, suggesting potential resistance of this bacterium to the extract (Table 3).

Antioxidant activity

The antioxidant activity of the extract was evaluated using the DPPH free-radical scavenging assay, which is based on the principle of reducing DPPH by hydrogen donors to diphenyl picrylhydrazine. The extract exhibited significant antioxidant activity over a dose range of 50–550 µg/mL as shown in Table 4.

Biofilm inhibition

The biofilm inhibition assay of the extract was conducted in 96-well plates. The higher doses of the extract significantly inhibit the biofilm formation of the test pathogen compared to the negative control (without the extract). The inhibition power of the extract decreases as the concentration of the extract decreases and reaches the minimum inhibitor power at 31.25 µg/mL (Fig. 3).

Biochemical and growth characteristics of ASTU00105

The strain ASTU00105 is a gram-negative, rod-shaped aerobic bacterium which is capable growing well in MHB media (supplementary Fig. 2) and reaching the stationary phase on the average within 7 days. It produced catalase, and hydrolyzed carboxymethyl cellulose, starch and gelatin. Among the carbon sources tested, the strain utilized lactose and sucrose as the carbon sources (Table 5). The detailed effect of physical–chemical factors on growth of strain ASTU00105 presented in the supplementary materials.

Whole genome sequencing, assembly, and annotation and phylogenetic analysis

Whole-genome sequencing was conducted using the Oxford Nanopore MinION sequencer. The FASTQ sequence data were submitted to the BV-BRC web server [19], for genome assembly and further analysis. The assembled genome consisted of two contigs, with a total length of 4,604,890 bp and an average G + C content of 63.89%. Figure 4A shows a circular graphical display of the distribution of the genome annotations of

Table 4 Antioxidant activity of ethyl EtAc extract from the ASTU00105 isolates with DPPH

Concentrations (µg/mL)	EtAc (%RSA)	Standard Control (Ascorbic acid + DPPH (%RSA)
50	50.2	84.5
150	58.5	86.7
250	68.5	89.3
350	77.5	91.3
450	83.5	92.7
550	89.9	93.6

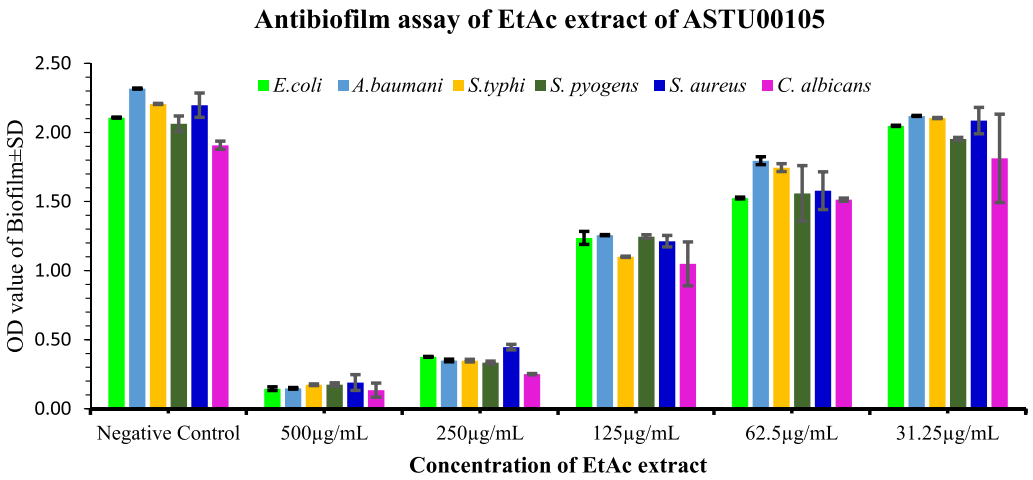

Fig. 3 The antibiofilm activity of EtAc-extract of ASTU00105

Table 5 Biochemical and physiological characteristics of ASTU00105

Biochemical test	ASTU00105
Catalase	+
Citrate	−
Urease test	+
Glucose	−
Lactose	+
Sucrose	+
Gas and H2S	−
Methyl red	+
Voges-Proskauer	+
Mot	+
Indole	−
Carboxymethylcellulose (CMC)	+
Starch hydrolysis	+
Gelatin hydrolysis	+
Casein hydrolysis	+

ASTU00105. Analysis of the sequence data showed that the proteins/gene of specific biological processes or structural complexes were clustered. The pie chart indicates the distribution of the subsystems for the *Pseudomonas sp.* strain ASTU00105 genome, as provided in Fig. 4B. The obtained data indicated a higher number of genes are involved in the metabolic functions which is followed by genes involved in protein processing.

The Consensus FASTA sequence of the strain was uploaded to the NCBI Nucleotide BLAST. The sequence showed the highest similarity (95.97%) to the sequence of *Pseudomonas sp.*, specifically strain *Pseudomonas stutzeri,* designated as *Pseudomonas sp*. strain ASTU00105. Phylogenetic analysis revealed that it formed a single clade with *Pseudomonas stutzeri* ATCC 17588 (Fig. 5).

Further analysis using the average nucleotide identity (ANI) value showed that the ANI between the isolated *Pseudomonas sp*. strain ASTU00105 and the closest strains of *Pseudomonas stutzeri* ATCC 17588 was 87.81%. These ANI values are below the typical cutoff range of 95–96% that is used to delineate different bacterial species. This result suggests that the newly isolated strain ASTU00105 represents an unexplored and distinct strain within the Pseudomonas genus (Fig. 6).

The biosynthetic gene clusters

The genome of *Pseudomonas sp*. strain ASTU00105 strain contains six biosynthetic gene clusters (BCGs) responsible for producing secondary metabolites with predicted bactericidal properties (Table 6). The identified BCGs were annotated as Betalactone, RRE-containing, Betalactone, NI-siderophore, Terpene, and Ectoine genes. Three BCGs identified in *Pseudomonas sp*. strain ASTU00105 shared homology with known BCGs, including lankacidin C (non-ribosomal peptide (NRP) + Polyketide, 13% similarity), fengycin (NRP, 20% similarity), and carotenoid (Terpene, 60% similarity). On the other hand, legonoxamine A/desferrioxamine B/legonoxamine B and ectoine did not match with previously reported BCGs.

BCG involving lankacidin C biosynthesis

Based on the in-silico analysis, the *Pseudomonas sp*. strain ASTU00105 genome revealed lankacidin gene clusters. The gene clusters proposed to produce lankacidin, whose similarity reached 13% with lankacidin C biosynthetic gene cluster from *Streptomyces rochei* based on KnownClusterBlast. This BGC was located in region 1.2 and spanned 30.96 kb in size. It contained

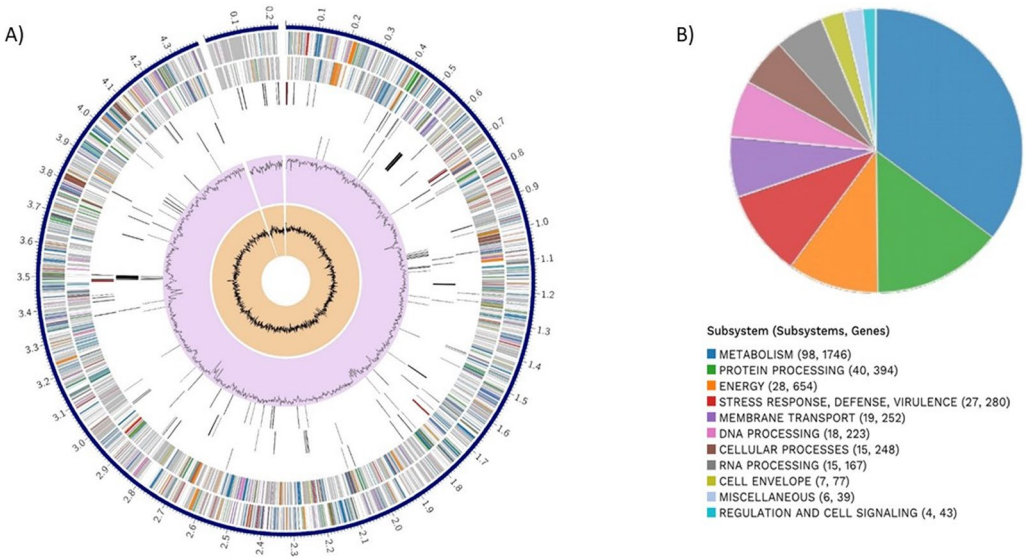

Fig. 4 **A** Circular genome of ASTU00105. From outer to inner concentric circle, Circle 1: the contigs, circle 2&3: CDS on the forward strand and CDS on the reverse strand; Circle 4: RNA genes; Circle 5&6: CDS with homology to known antimicrobial resistance genes and CDS with homology to know virulence factors; Circle 7&8: GC content and GC skew. The colors of the CDS on the forward and reverse strand indicate the subsystem that these genes belong to. **B** The pie chart showing a set of proteins with specific process or structural complex

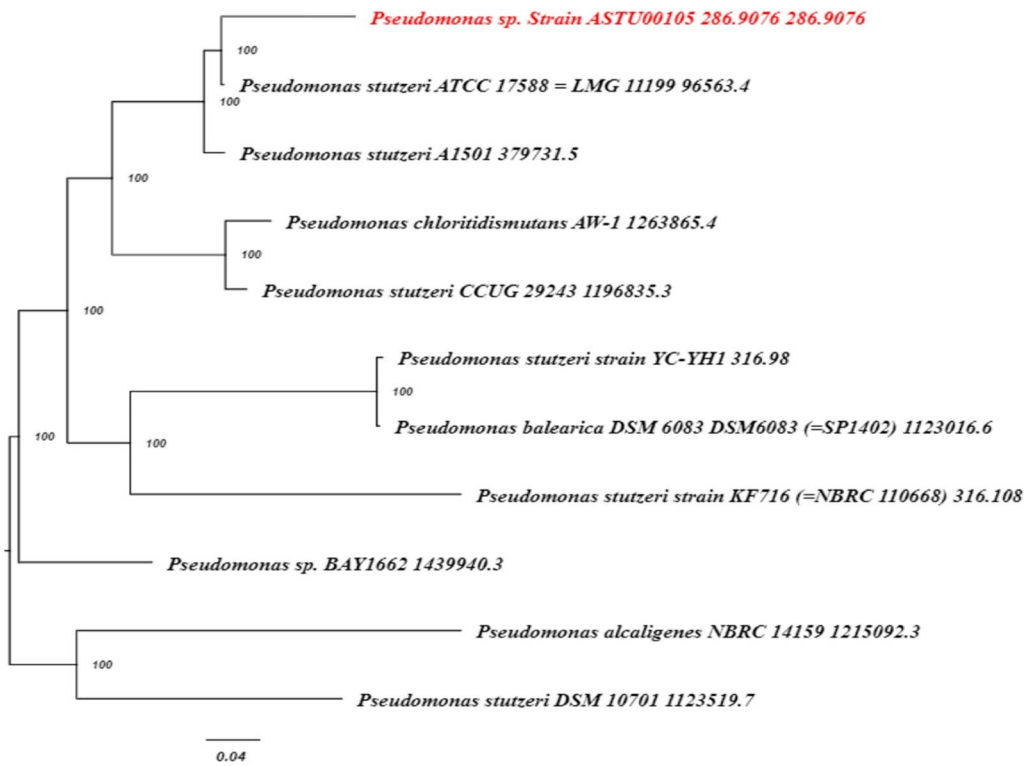

Fig. 5 Phylogenetic placement of *Pseudomonas sp.* strain ASTU00105

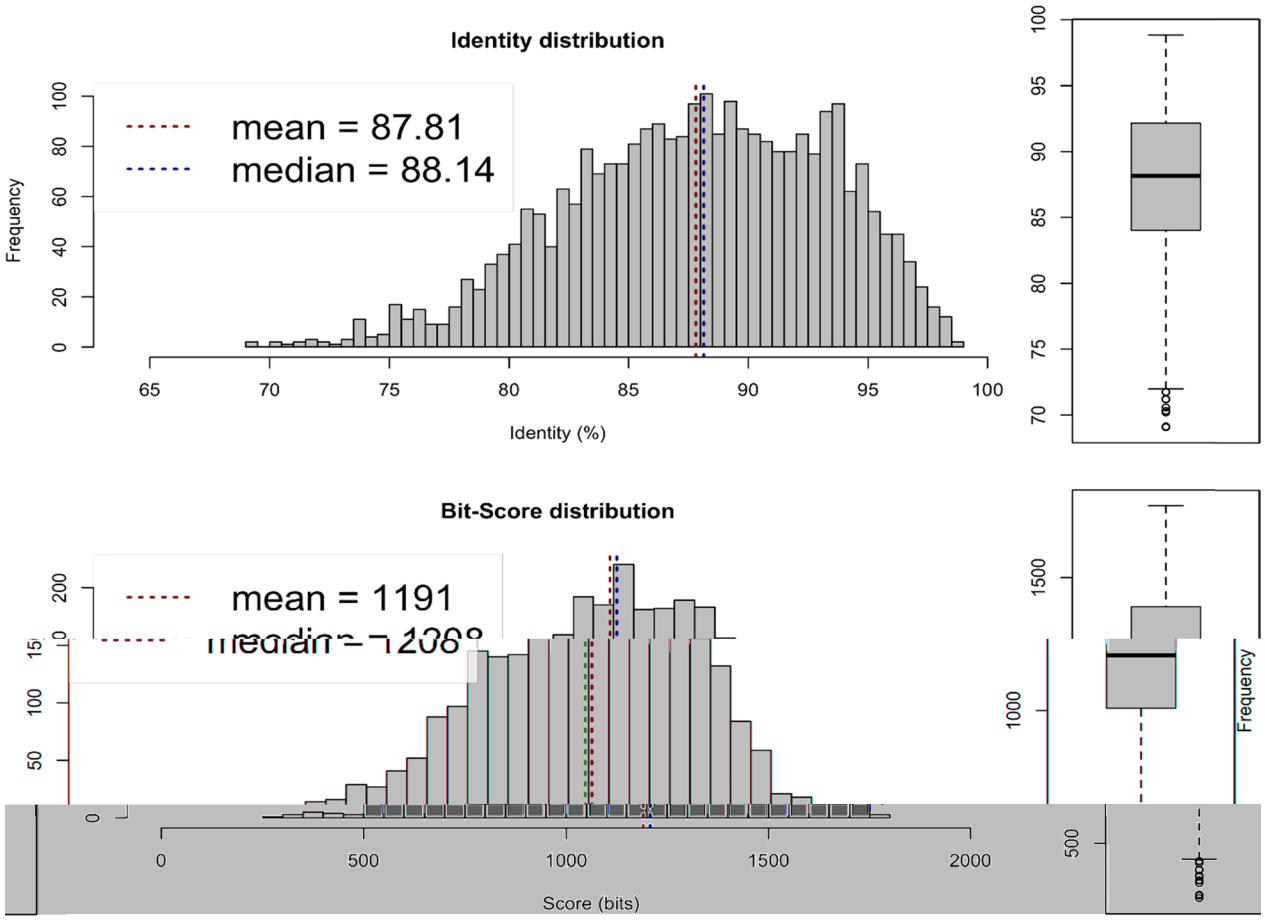

Fig. 6 Average nucleotide identity analysis between the newly identified strain in this study *Pseudomonas* sp. ASTU00105 and the closest strain *Pseudomonas stutzeri* ATCC 17588

Table 6 Identified biosynthetic gene cluster from the whole genome sequence of strain *Pseudomonas sp.* strain ASTU00105

Region	Gene type/Activity	Location (relative coordinate from (bp) to (bp)		Most similar known cluster	Type (% similarity)
1.1	Betalactone	26,226	54,102	–	–
1.2	RRE-containing	426,889	452,904	Lankacidin C	NRP + Polyketide (13)
1.3	Betalactone	1,651,529	1,680,875	Fengycin	NRP (20)
1.4	NI-siderophore	2,108,263	2,137,607	Legonoxamine A/desferrioxamine B/ legonoxamine B	Other (50)
1.5	Terpene	2,277,584	2,298,066	Carotenoid	Terpene (60)
1.6	Ectoine	2,930,026	2,940,583	Ectoine	Other (50)

genes including both non-ribosomal protein and Polyketide which were predicted to be involved lankacidin biosynthesis. Lankacidins are a class of polyketide/nonribosomal peptide natural products isolated from the soil bacterium *Streptomyces rochei* that feature a β-keto-δ-lactone core that is often contained within a 17-membered macrocycle (lankacidins 1–4 and 11).

BCG involving fengycin biosynthesis

The proposed gene clusters for fengycin production by *Pseudomonas sp.* strain ASTU00105 share a 20% similarity with the fengycin biosynthetic gene cluster found in *Bacillus velezensis* FZB42, as determined by KnownClusterBlast. This gene cluster, located at region 1.3 and spanning 29.347 kb, encompasses genes associated with non-ribosomal peptide synthesis. Fengycin synthesis is controlled by a biosynthetic gene cluster referred to as the fen cluster, which contains genes responsible for activating, loading, modifying, and cyclizing amino acids. NRPS genes like fenA, fenB, fenC, fenD, and fenE encode modules that sequentially activate and load amino acids onto the peptide chain. These modules operate in concert, with each incorporating specific amino acids into the developing fengycin peptide. The arrangement and interactions of these modules dictate the amino acid sequence and cyclization pattern in the resulting lipopeptide. Enzymes encoded by genes within the fen cluster modify amino acid residues and fatty acid chains, contributing to the structural diversity of fengycins. The interactions among these genes lead to distinct modifications that characterize different fengycin variants.

BCG involving carotenoid biosynthesis

The proposed gene clusters for carotenoid production by *Pseudomonas sp*. strain ASTU00105 exhibit a 60% similarity with the carotenoid biosynthetic gene cluster found in *Brevundimonas vesicularis*, as determined by KnownClusterBlast. This gene cluster is located at region 1.5 and spans 20.483 kilobases (kb). Within this gene cluster, there are genes associated with terpene production. Carotenoids, which are synthesized by these gene clusters, possess antioxidant properties that contribute to inhibiting the initiation of carcinogenesis, modifying biomembranes, strengthening the lipid bilayer, and reducing its fluidity. In the human body, natural carotenoids such as β-carotene aid in defending against reactive oxygen species (ROS) by scavenging them. Carotenoids also have additional effects, including up-regulating gap junction communication, inducing detoxifying enzymes, and inhibiting cell proliferation. These properties highlight the potential health benefits associated with carotenoid consumption.

GC–MS analysis of ethyl extract

The chemical composition of the EtaC extract from strain ASTU00105 was evaluated using GC–MS analysis. A total of 44 compounds were identified based on their retention time, molecular weight, and formula. As shown in Table 7, the dominant compound was the phenolic compound 2,4-bis(1,1-dimethylethyl) (36.64%), followed by 1,2-Benzenedicarboxylic acid, diethyl ester (12.22%), Eicosane (9.71%), Dibutyl phthalate (3.93%), and 1-Dodecanol (2.34%).

Discussion

The growing challenge of antibiotic-resistant pathogens necessitates the search for novel antimicrobials from natural resources. Microbes from untouched areas, such as stressful environments and plant rhizospheres, hold a potential for the identification of novel secondary microbial metabolites with antimicrobial activity. Hence, this study aimed to identify and characterize soil

Table 7 The compounds detected in the EtaC of strain ASTU00105 by GC–MS analysis

Formula	Compounds	Amount (%)	RT	Compound class	Biological activity	References
C14H22O	Phenol, 2,4-bis(1,1-dimethylethyl)	36.64	12.18	Phenol	Antimicrobial and antioxidant	[20]
C12H14O4	1,2-Benzenedicarboxylic acid, diethyl ester	12.22	12.73	Phthalate ester	Antimicrobial and Antioxidant Activities	[21]
C20H42	Eicosane	9.94	13.83	Alkane	Antibacterial, antioxidant and anti-inflammatory activity	[22, 23]
C16H22O4	Dibutyl phthalate	3.93	14.40	Ester	Antifungal and antimicrobial	[22]
C12H26	Dodecane	2.61	9.17	Straight-chain alkane	Antimicrobial	[24]
C12H26O	1-Dodecanol	2.34	11.88	Long-Chain Fatty Alcohols	Antimicrobial	[25]
C9H20O	1-Nonanol	1.47	7.36	Long-Chain Fatty Alcohols	Antimicrobial	[25]
C13H28	Tridecane	1.03	10.24	Straight-chain alkane	Antibacterial, antioxidant and anti-inflammatory activity	[26]
C11H24	Undecane	0.88	8.14	Straight-chain alkane	Antibacterial, antioxidant and anti-inflammatory activity, plant growth promotor	[26]
C13H28O	1-Tridecanol	0.75	12.65	Long-Chain Fatty Alcohols	Antimicrobial	[25]
C12H17NO2	Phenol, 3-methyl-5-(1-methylethyl)-, methylcarbamate	0.23	10.16	Phenol	Anticancer	[27]

bacteria using phenotypic, genotypic as well as chemical analytic approaches and evaluation of the antimicrobial potential of the secondary metabolite extracts from these microbes.

In this study, 22.37% of the isolates were found to be bioactive against most of the tested pathogens. The results of this study are comparable to the findings reported by Tawiah et al., [28] and Amankwah et al., [23] where 20.85% and 20.83% bacterial isolates tested were bioactive against bacterial pathogens, respectively. However, our results are significantly higher than the findings reported by Selvin et al., [29] and Prashanthi et al., [30] who reported 1.14% and 3.44% bioactive isolates, respectively. This difference could be attributed to variations in geographic location and the samples used for isolating the bioactive bacteria. Furthermore, differences in media, and methodology could also have a significant impact on the types of bacterial isolates and subsequent secondary bioactive metabolites elaborated.

Pseudomonas species are commonly identified using biochemical and molecular methods from various samples, including soil [23], plant rhizospheres [31], and sea sediments [32]. In this study, we identified the ASTU00105 using biochemical tests and confirmed using whole genome sequencing that it belongs to *Pseudomonas* species, specifically *Pseudomonas stutzeri*, with a 95.76% identity match. Previous studies have revealed that this species are known for producing secondary metabolites with antimicrobial activity. Gong et al., [32] isolated bioactive *Pseudomonas stutzeri* from sea sediment, which demonstrated effectiveness in controlling *Aspergillus flavus* through the production of antifungal volatiles. Uzair et al., [33] identified a compound called zafrin (4beta-methyl-5, 6, 7, 8 tetrahydro-1 (4beta-H)-phenanthrenone), which exhibited activity against several human pathogens, including *Staphylococcus aureus* and *Salmonella typhi*.

Pseudomonas species are well-known for their ability to synthesize secondary metabolites with a wide range of bioactivities, offering promising commercial applications in the pharmaceutical industry [34, 35]. These metabolites exhibit antitumor, antiviral, antioxidant, antihypertensive, immunosuppressant, and particularly antimicrobial properties. Serving as defensive compounds against microbial competition in their natural environments, notable examples include pyocyanin, which shows potential for drug development [36], and rhamnolipids, which are effective in bioremediation to mitigate environmental pollutants [37]. Additionally, these metabolites have applications in the cosmetic industry due to their antimicrobial and skin-conditioning effects. Overall, the diverse functionalities of *Pseudomonas* secondary metabolites position them as key contributors to advancing sustainable practices and innovative products across various markets.

In this study, strain ASTU00105 demonstrated antimicrobial activity against gram-positive and gram-negative bacteria as well as yeast. The extract exhibited a range of minimum inhibitory concentrations (MIC) from 125 to 31.25 µg/mL, with a lower MIC value observed against gram-positive bacteria in comparison to gram-negative bacteria. This result aligns with the findings of Uzair et al. [33], who also reported a lower MIC value for secondary metabolites derived from *Pseudomonas stutzeri* against gram-positive bacteria compared to gram-negative bacteria. Intriguingly, the MIC of the extract in our study was significantly lower than the MIC (3120 and 2500 µg/mL) extract from *Pseudomonas aeruginosa* Isolate reported by Amankwah et al., [23], indicating a greater inhibitory potential of the *Pseudomonas stutzeri* extract in our investigation. The antioxidant potential of the *Pseudomonas sp. strain* ASTU00105 extract was assessed using DPPH assay by measuring hydrogen atom transfer through single electron transfer. The extract demonstrated significant antioxidant activity, suggesting the presence of bioactive compound(s) with antioxidant properties.

The GC–MS analysis of the EtAc extract of ASTU00105 demonstrated the presence of several bioactive compounds with antibacterial and antifungal activities. The most abundant compound detected in the crude extract was Phenol, 2,4-bis(1,1-dimethylethyl) (36.64%). This finding is in line with the results reported by Mazumdar et al., [20] who found that Phenol, 2,4-bis(1,1-dimethylethyl) accounted for 30.03% of the extract from bioactive bacterial isolates. Mazumdar et al. further reported that these phenolic compounds exhibited also potent antioxidant and antibacterial properties, likely due to their lipophilic nature, which enhances their antimicrobial activity by facilitating interaction with the cell membrane. Additionally, other compounds, including 1,2-Benzenedicarboxylic acid, diethyl ester, Eicosane (9.71%), Dibutyl phthalate (3.93%), and 1-Dodecanol (2.34%), demonstrated significant antibacterial, antioxidant, and antifungal activities [21–23, 25–27].

In silico analysis has emerged as a key approach for the identification of Biosynthetic Gene Clusters (BGCs) within bacterial genomes[38]. The presence of BGCs indicates the potential of bacterial isolates to produce bioactive secondary metabolites, which can complement in vitro experimental studies[39, 40]. In this study, the whole genome scanning of *Pseudomonas sp.strain* ASTU00105 strain showed gene clusters closely resembling polyketide (PK), non-ribosomal pepetide (NRP), and terpene. Previous research has indicated that these

BGCs form the fundamental structural framework for the majority of secondary metabolites [41].

In conclusion, this study identified a novel *Pseudomonas sp*. strain ASTU00105 that produces secondary metabolites with the ability to inhibit pathogenic bacteria and fungi. The in-silico analysis of the WGS of *Pseudomonas sp*. strain ASTU00105 strain revealed six secondary metabolite BGCs containing potential compounds with antimicrobial activity. Furthermore, the GC–MS analysis of the extract identified several bioactive compounds with both antibacterial and antifungal activities.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-024-02589-6.

Supplementary material 1.

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Author contributions
SD: Conceptualization, methodology, validation, investigation, visualization; DO: editing and revising the draft; AA: Methodology, editing; GT: Methodology, editing; GDS: Methodology, editing; FF: Methodology, editing; AMS: methodology, editing; DW: editing and revising the draft; KT: Conceptualization, methodology, validation, investigation, writing original draft-writing-review and editing, visualization, supervision, project administration, funding acquisition.

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Data availability
The datasets generated during and/or analyzed during the this study are available from the corresponding author on reasonable request. The genomes of the *Pseudomonas sp*. strain ASTU00105 was submitted to the NCBI GenBank and was assigned the accession number SAMN44790702.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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