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Production and characterization of a new antibacterial peptide obtained from *Aeribacillus pallidus* SAT4

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ABSTRACT

A novel thermophilic bacterial strain of the genus *Aeribacillus* was isolated from Thar Dessert Pakistan. This strain showed significant antibacterial activity against *Micrococcus luteus*, *Staphylococcus aureus*, *and Pseudomonas aeruginosa*. The strain coded as 'SAT4' resembled with *Aeribacillus pallidus* in the morphological, biochemical and molecular tests. The production of antibacterial metabolites by SAT4 was optimized. These active metabolites were precipitated by 50% ammonium sulphate and purified through sephadex G-75 gel permeation chromatography and reverse phase HPLC. The molecular weight of 37 kDa was examined by SDS-PAGE. The structural elucidation of the purified product was studied by FTIR, ^{1}H and ^{13}C NMR. The X-ray diffractions study showed that the crystals belonged to the primitive orthorhombic lattice ($a = 12.137$, $b = 13.421$, $c = 14.097$ Å) and 3D structure (proposed name: Aeritracin) was determined. This new peptide antibacterial molecule can get a position in pharmaceutical and biotechnological industrial research.

1. Introduction

Microorganisms make up an everlasting reservoir of chemical compounds with pharmacological, physiological, medical or agricultural uses [45] and many bio active small molecules are synthesized by these naturally populated microbes [21,10]. The number of bioactive chemical compounds has been reported by bacilli, pseudomonas, actinomycetes and fungi [27]. Among them 800 peptide antibiotics have been described [33] and of this total, 66 various peptide antibiotics are particularized by strains of *Bacillus subtilis* and 23 are active metabolites of *Bacillus brevis* [18,31,20]. Some of these antibiotics including the polypeptide are being used against the common bacterial targets and thus having the broad spectrum activity. Bacilli producing peptide antibiotics (gramicidins, tyrocidines, and bacitracins) are mainly mesophilic [15]. Few of them are capable of growing at temperatures above 40 °C. They include *B. brevis* var. G-B, producing gramicidin C [14], and *Bacillus polymyxa*, producing gavaserin and saltavalin [38]. *Aeribacillus pallidus* belongs to class Bacilli and its growth occurs at 55–60 °C. Although the progress and speed of new antibiotic discovery has indeed slowed down [51] but in recent years the natural products are the most effective source of drugs and have gained valuable attention for their clinical practices and consistent therapeutic value. Therefore the importance and significance of antibiotics to medicine has led to much research into their discovery and production [29]. Here we describe the production and characterization of a new antibiotic molecule obtained from a newly isolated thermophilic *A. pallidus* strain SAT4.

2. Materials and methods

2.1. Isolation and screening

Thermophilic bacterial strains were isolated from soil sample of Thar Dessert, Pakistan by serial dilution method. Bacterial colonies were purified on nutrient agar medium at 50 °C by standard streaks plate technique. Antibacterial activity of these purified isolates were checked against *Micrococcus luteus* ATCC 10240, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 49189 and *Escherichia coli* ATCC 87064 by agar well diffusion assay.

2.2. Characterization

The purified bacterial isolate SAT4 was characterized on the basis of morphological, biochemical and genomic analysis.

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2.2.1. Morphological study

The morphological parameters were studied according to Buchanan and Gibbons [8]. For evaluation of colony morphology, under aseptic techniques, the purified and isolated colony was grown on nutrient agar media and was studied for colony morphology (size, pigmentation, form, margin, and elevation), gram's staining, spore staining and bacterial motility.

2.2.2. Biochemical study

The combination of API 50CHB V4.0 and API 20E kit (BioMerieux SA France; Lot No. 833022401) was used for biochemical analysis according to the manufacturer's protocol and test results were recorded after 24 h of incubation. The results were analyzed into a bio- Me'rieux identification software database (apiweb™; Bio-Merieux SA).

2.2.3. Molecular study

A 16S rRNA gene sequence to study bacterial phylotypes and taxonomy was used to identify bacterial isolate. DNA was extracted from bacterial cultures using Wizard genomic Kit (Promega, Madison, USA) according to the manufactures' specifications. DNA concentration in sample was determined using Nanodrop1000 (Thermo Scientific, Rockford, USA) as per standard procedure. PCR amplification and sequencing of 16S rRNA gene was carried out using a Takara 16S rDNA bacterial identification kit. 1 μl of the extracted DNA was amplified with universal primers F27 (5′-AGAGTTTGATCMTGGCTCAG-3′) and R907 (5′-CCGTCAATTCCTTTRAGTTT-3′), generating a PCR product. All reactions were carried out in 0.5 ml PCR tubes, containing 1 μl of each primer, 9.5 μl of sterile distilled water and 12.5 μl of Master Mix (PCR Master Mix 2X, Fermentas, #K0171). PCR was performed in a T-Personal combi PCR machine (Biometra, Germany, #2106284) with the following program: 3 min denaturation at 95 °C, followed by 30 cycles of 1 min denaturation at 94 °C, 1 min annealing at 58 °C, 1 min extension at 72 °C, and a final extension step of 3 min at 72 °C. PCR product of the correct size was purified by JET quick PCR products purification spin/250 kit (GENOMED, Germany). Extracted DNA was visualized by gel electrophoresis on 0.8 % agarose gel and 5 μl of PCR products on 1.5% agarose gel stained with ethidium bromide (0.5 mg/ml) in 1× TAE buffer (50× TAE buffer: 242 g/l Tris, 18.61 g/l NaEDTA.$2H_2O$, 57 ml glacial acetic acid). About 1 μl loading dye (30% v/v glycerol, 0.25 % w/v bromophenol blue, 0.25 % w/v xylene cyanol FF) was added to 3 μl of sample and mixed before loading into wells. DNA ladder (Kbp) (Fermentas GeneRulerTM, #SM0313) was used for PCR products. 2 μl of ladder was mixed with 2 μl of loading dye before its loading in the first well of the gel. The gel was run at 80 V for 45 mins. The gel was then observed for bands under UV using gel-dock imaging system (BioRad, Milan Italy).

Sequencing of PCR products was performed and analyzed in both directions using an ABI Prism 310 automated DNA sequencer using BigDye Terminator cycle sequencing kit (PE Applied and Biosystem USA). This kit contained a BigDye Terminator tube, filled with 10 μl of pinkish solution containing 2 μl of primer and 8 μl of BigDye Terminator Reagent. 10 μl of purified PCR product was transferred to this Big Dye Terminator tube. Then samples were sequenced. Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) was used to align the obtained bacterial 16S rDNA sequence with thousands of known different available 16S rDNA sequences in this database, and percent homology scores were generated to identify bacteria. Phylogenetic tree was constructed by the neighbor-joining method [40] using the MEGA 4.0 software package. Bacteria with 16S rDNA sequences >99% similarity was considered to be of the same phylotype [19].

2.3. Production of antibacterial compound

Shake flask fermentation experiments were carried out for the production of antibacterial metabolites by bacterial isolate SAT4. 100 ml of production medium [9] (L-glutamic acid: 5 gm/l; KH_2PO_4:0.5 gm/l; K_2HPO_4:0.5 gm/l; $MgSO_4$:0.2 gm/l; $MnSO_4$:0.01 gm/l; NaCl:0.01 gm/l; $FeSO_4$:0.01 gm/l; $CuSO_4$:0.01 gm/l; $CaCl_2$:0.015; Glucose:1%) was inoculated with 10% of bacterial inoculum and it was incubated at 50 °C in shaking incubator for 24 h at 150 rpm. After 24 h of incubation, sample was centrifuged for 15 min at 10,000 rpm by table top centrifuge to get cell free supernatant and antibacterial activity in the supernatant was analyzed against test bacterial strains by agar well diffusion assay [24,42].

2.4. Optimization of the production of antibacterial compound

The production of antibacterial compound was optimized by analyzing the time of incubation, pH, temperature, agitation rate, nitrogen, and carbon concentrations. Incubation time was analyzed from 24 to 144 h; pH was studied from 4 (acidic) to 9 (basic); temperature ranged from 45 to 60 °C; the concentration of glutamic acid used as a source of nitrogen varied from 0.25 to 2%; and carbon concentration by taking the glucose from 0.25 to 3%. The optimum levels were confirmed by agar well diffusion assay.

2.5. Purification of antibacterial compound

Precipitation, a method of protein purification [25] was carried out by ammonium sulphate [48,11]. The culture supernatant was treated with powdered ammonium sulphate (20, 40, 50, and 60% saturation). After sufficient shaking, solution was placed in the cold for one hour and precipitates were collected by centrifugation at 14,000 rpm for 15 min at 4 °C. The precipitates were resuspended in 15 ml of 0.05 M potassium phosphate buffer at pH 6. Dialysis was carried out against the same buffer for 24 h in dialyzing bag and the pallets were freeze dried. Further purification was processed by gel permeation chromatography. The column was carefully loaded with 3% sephadex G-75 gel suspended in 0.05 M phosphate buffer. The dialyzed protein sample was eluted at a flow rate of 36 ml/h and fractions (3 ml each) were collected. The absorption of these fractions was measured at 280 nm by UV-spectrophotometer (Agilent USA). The fraction showing antibacterial activity were pooled and lyophilized. The activity of these fractions were tested

Table 1
Antibacterial activity of selected thermophilic bacterial isolates against indicator bacterial strains.

Indicator bacterial species	Zone of inhibitions shown by the thermophilic bacterial isolates (mm)				
	SAT1	SAT2	SAT3	SAT4	SAT5
Staphylococcus aureus ATCC 6538	2	2	4	25	6
Micrococcus luteus ATCC 10240	1	2	2	22	6
Pseudomonas aeruginosa ATCC 49189	0	0	0	16	1
E. coli ATCC 87064	0	0	0	5	0

against indicator bacterial strains by agar well diffusion method. Finally, the purified sample was analyzed by reverse phase HPLC (C_{18} column; 5 μm; 4.6 mm × 150 mm) using isocratic elution of 20 mM Na_2HPO_4/130 mM NaCl at a flow rate of 1.0 ml/min. Polymyxin B was taken as standard peptide antibiotic due to same class of metabolite sample.

2.6. SDS-PAGE analysis

In order to monitor purity, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12% of resolution gel and 5% of stacking gel [30]. Sample was prepared by dissolving it with equal proportion of sample buffer and loaded onto several wells along with Prestained Protein Ladder (Fermentas, PageRuler, #SM 0671). One-half of the gel was stained with coomassie brilliant blue according to the method of Lee et al. [30] while the other half was assayed directly for antibacterial activity by the modified test [7].

2.7. FTIR and NMR analysis

FTIR spectra of sample were recorded in 3600–650 cm^{-1} region by Bruker Germany Alpha FT-IR model. The samples for NMR analysis were prepared by dissolving 15 mg of sample in 0.5 ml D_2O. NMR spectra were analyzed using Bruker 300 MHz spectrometer equipped with 5 mm of probehead for ^1H and ^{13}C analysis.

2.8. X-ray crystallography

Crystals of the lyophilized sample were developed at room temperature. The method involved mixing 2 ml of solvent solution (70% CH_3OH) with lyophilized sample (10 mg/ml) in a test tube for 30 h. Diffraction data related to X-ray crystal structure were collected, processed, and calculated from a single crystal source using a STOE-IPDS II equipped with an Oxford Cryostream low-temperature unit, Germany. Structure solution and refinement was done using SIR97, SHELXL97 and WinGX. During data collection, the crystal was maintained at cryogenic temperatures so as to reduce radiation damage.

2.9. Thermostability analysis

Thermostability was evaluated by taking 0.1% aqueous solution of pure compound in four different test tubes, kept them for one hour in water bath at 45, 50, 55, and 60 °C. Agar well diffusion method was used to measure the activity against *S. aureus* and *M. luteus*. The antibacterial activity of untreated sample measured at 37 °C was taken as control. Percentage loss of antibacterial activity was calculated using the following formula:

$$A_0 - \frac{A_f}{A_0} \times 100$$

where A_0: activity of control, A_f: activity after treatment.

3. Results

3.1. Screening of antibacterial producing bacterial species

Five isolates were purified (coded as SAT1 to SAT5) and antibacterial activity of strain "SAT4" showed maximum activity against *S. aureus* giving zone of inhibition of 25 mm followed by the 22 mm inhibitory zone against *M. luteus* (Table 1).

3.2. Characterization

Morphologically bacterial isolate SAT4 was a motile, spore forming and Gram positive rod arranged in pairs and colonies were white to off white in color, circular, flat, with entire margins. 98.4% significant taxon was identified as *Geobacillus pallidus* strain SAT4 (now known as *A. pallidus)* [34] when tested through biochemical kits (Table 2). 16S rDNA sequence was amplified using universal primers F27 and R907 generating a PCR product. Analysis of the 16S-rRNA gene sequences using NCBI server, this thermophilic bacterium showed 100% similarity with *A. pallidus* (accession number: JN986827). The maximum-likelihood phylogenetic tree and evolutionary relationship was analyzed (Fig. 1).

Table 2
Taxonomic characterization of bacterial isolates SAT4.

Test analysis	Types	Features	Result
Morphological characteristics	Microscopic examination	Gram's staining	+
		Shape	Rods arranged in pairs
		Spore formation	+
		Motility	+
	Macroscopic appearance: colony morphology	Color	White to off
		Elevation	Flat
		Colony shape	Circular
		Margins	Entire
Biochemical characteristics[c]	API 50CHB Tests that were positive for[a]		
	GLY GLU FRU MNE MAN SOR MDG NAG AMY ARB ESC SAL CEL SAC TRE LFUC		
	+ + + + + + + + + + + + + + + + + +		
	API 20E Tests that were positive for[b]		
	ONPG CIT GEL INO RHA ARA		
	+ + + + + +		
	Significant Taxon 98.4 % *Geobacillus pallidus*		
Molecular Characteristics	Universal primers F27 and R907		
	100% homology with *Aeribacillus pallidus* at NCBI server (accession number: JN986827) previously known as *Geobacillus pallidus* [21]		

Indications: + sign indicates the positive reaction (color change), GLY: glycerol; GLU: D-glucose; FRU: D-fructose; MNE: D-mannose; MAN: D-mannitol; SOR, MDG, NAG: *N*-acetylglucosamine; AMY: amygdalin; ARB: arbutin; ESC: esculin ferric citrate; SAL: salicin; CEL: D-cellobiose; SAC: D-sucrose; TRE: trehalose; LFUC: L-fucose.

[a] Only the tests with a positive result are included here (remaining tests were negative).

[b] Only the tests with a positive result are included here (remaining tests were negative). ONPG: 2-nitrophenyl-ß-D-galactopyranoside; CIT: trisodium citrate; GEL: gelatin; INO: inositol; RHA: L-rhamnose; ARA: L-arabinose.

[c] Biochemical tests: API kit system with percentage of positive tests after 24 h of incubation.

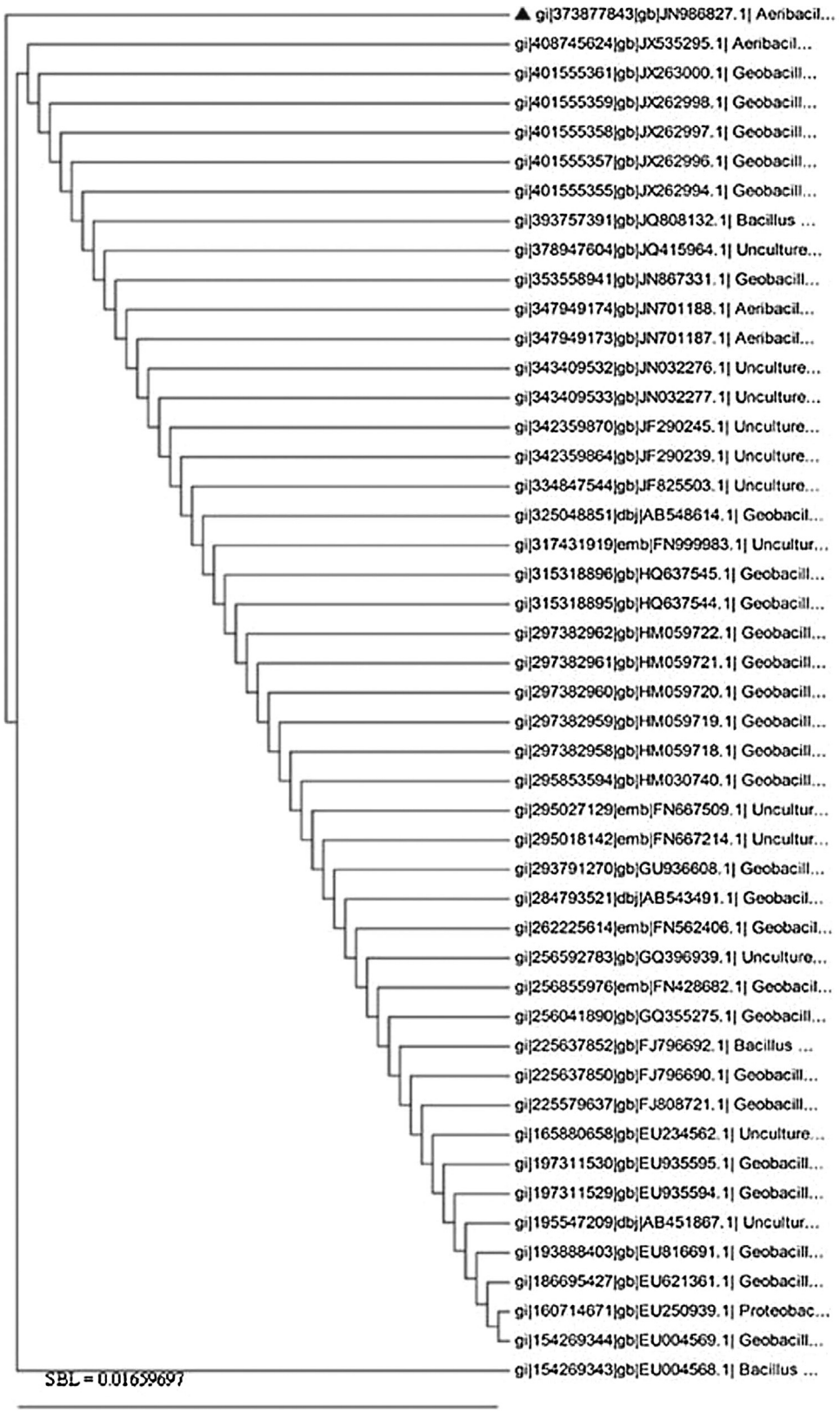

Fig. 1. Evolutionary and phylogenetic relationship of *Aeribacillus pallidus* SAT4 with 47 taxa (linearized).

3.3. Optimization analysis

The maximum production of antibacterial metabolites was observed at following parameters: 48 h of incubation, pH 5, 2% glucose, 1.5% glutamic acid, 100 rpm, and 55 °C temperature (Fig. 2).

3.4. Purification analysis

Antibacterial compound was purified using 50% saturated ammonium sulphate followed by the fractionation through sephadex G-75 gel permeation chromatography and protein concentration were estimated at 280 nm (Supplementary Fig. 1). The retention time of protein sample was 13.109 min when eluted through HPLC at 254 nm as compared to polymyxin as standard protein antibiotic at 11.952 min. These spectral peaks distinctively characterize the peptide antibiotic and differentiate them from other polypeptide antibiotics (Supplementary Fig. 2).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.btre.2015.09.003.

3.5. SDS-PAGE analysis

SDS-PAGE analysis of purified sample revealed single band at 37 kDa (Fig. 3). Zone of inhibition against *S. aureus* was observed when gel overlay test was performed at 37 °C corresponding to the protein band at the same position.

3.6. FTIR analysis

The spectrum of this ligand exhibited weak bands in the 3400–3100 cm^{-1} region; while strong bands appeared in the region of 3600–3500 cm^{-1} may be due to presence of H—O—H (asymmetric vibration). Similarly a band at 2900 cm^{-1} was observed which belongs to (=C—H) stretching. The strong bands belong to (—C=N), and (C=O) were found at 2359.86 cm^{-1}. In the spectrum of peptide ligand, other bands at 1700 cm^{-1}, 1540.81 cm^{-1} and 1257.49 cm^{-1} were appeared which attributed to asymmetric interaction (O—N—O), (N—H) and (C—O), respectively. The peptide bond formation could also be confirmed due to N—H stretching and C—O in frequency regions of 1540.81 and 1257.49 cm^{-1}, respectively with small contribution of C—N group (Table 3).

3.7. NMR analysis

The ^{1}H NMR spectrum of sample showed the signals about aliphatic groups. A —CH_3 resonance was observed at 1.195 ppm, while —C—CH_2—C— (sp^3 hydrogen) were recorded at 1.571 ppm. At high dilution, hydroxyl protons (OH) showed absorption near 0.5–1.0 ppm (0.751 ppm), while in concentrated solution their absorbance was closer to 4.5 ppm and it was not observed in the spectrum. The resonance of alkyl amines (RNH_2) was observed at 3.996 ppm (Supplementary Fig. 3a). ^{13}C NMR spectra were used to determine the number of nonequivalent carbons and to identify the types of carbon atoms which may be present in the compound.

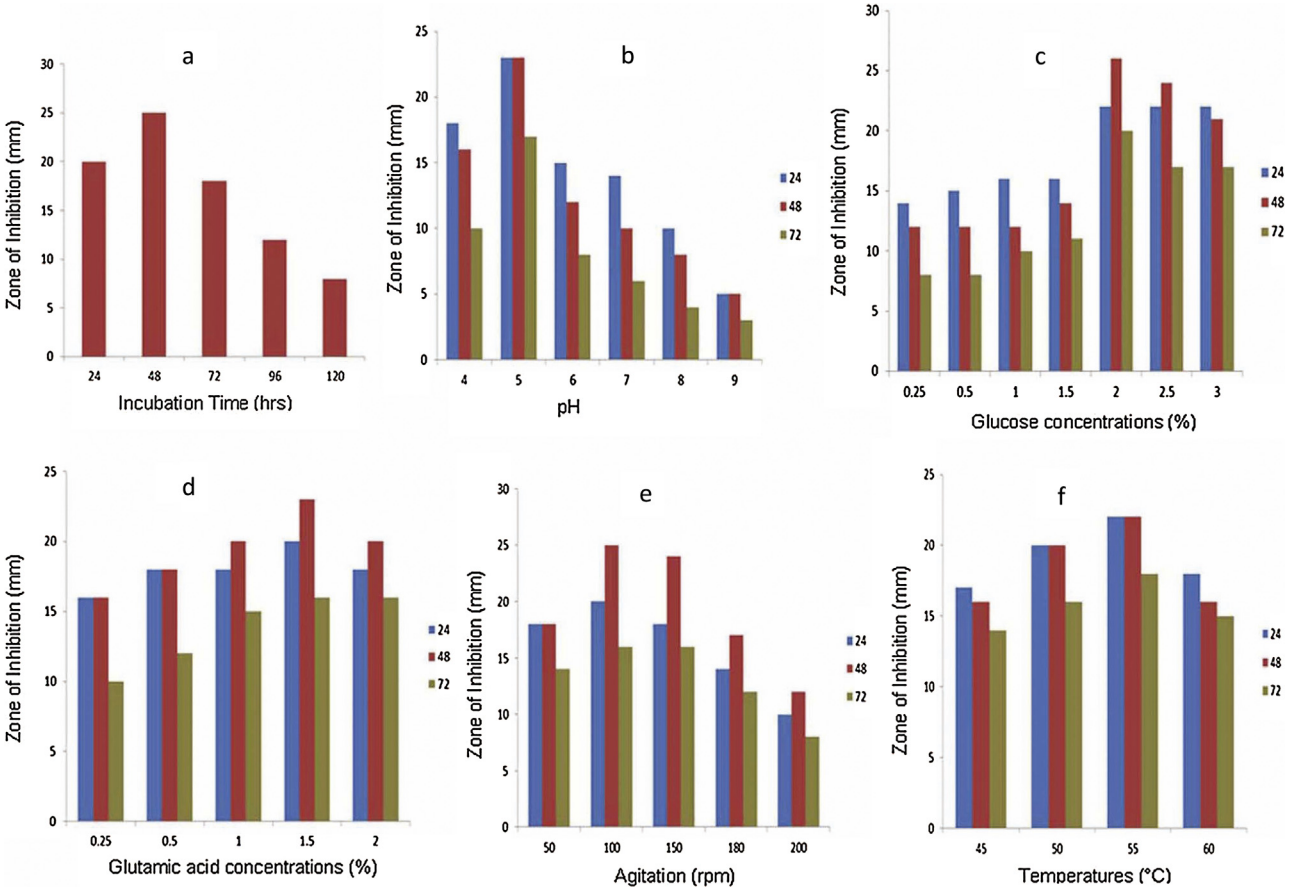

Fig. 2. Optimization of antibacterial compound production by *Aeribacillus pallidus* SAT4 (a) effect of Incubation time (hours) (b) various pH values (c) glucose concentrations, used as carbon source (d) glutamic acid concentrations, used as nitrogen source (e) Effect of agitation (rpm) rates (f) Effect of different temperatures.

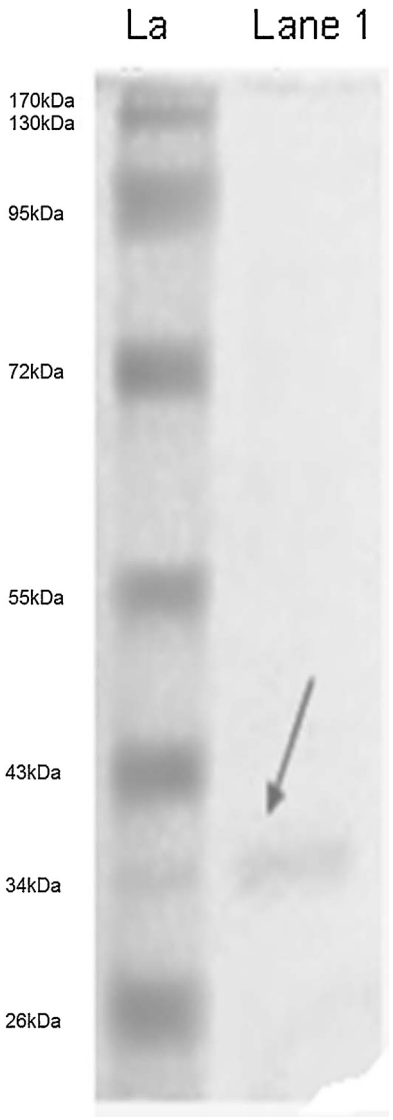

Fig. 3. SDS-PAGE analysis (La: Ladder; Lane 1: protein sample).

The ^{13}C NMR spectrum of sample demonstrated that saturated carbon CH_3, CH_2 appeared at 28.190 ppm and 28.738 ppm, respectively. The resonance at 69.69 ppm showed the effects of electronegative atom (—C—O) (Supplementary Fig. 3b).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.btre.2015.09.003.

3.8. Structure identification by X-ray crystallography

Antibacterial compound (proposed name: Aeritracin) isolated from *A. pallidus* SAT4 was subjected to X-ray diffraction study. The geometry of polypeptide contains various amino acids residues (Fig. 4a). The crystal system is triclinic with molecular formula of $C_{55}H_{82}N_{16}O_{17}$ showing the presence of histidine C27-N28 with bond lengths of (1.32909 Å (1.5)), N28-C29 (1.32989 Å (1.5)), C29-N30 (1.32887 Å (1.5)), N30-C31 (1.33197 Å (1.5)) and C31-C27 (1.38236 Å (1.5)). The selected bond lengths of (1.39026 Å (1.5)) and (1.39138 Å (1.5)) from C38-C43 and C71-C76, respectively showed the presence of two benzene rings. The bond lengths of (1.23148 Å (2)), (1.34144 Å (1)) and (1.55458 Å (1)) exhibited the existence of C=O (C9-O1), C—N (C9-N11) and CC (C12-C13), respectively. The crystals belong to the primitive orthorhombic lattice with the cell parameters $a = 12.137$, $b = 13.421$, $c = 14.097$ Å (Fig. 4b). The analytical data (Table 4) and the selected bond lengths are given in Supplementary Table S1.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.btre.2015.09.003.

3.9. Thermostability of the antibacterial compound

Thermostability of cell free supernatant and pure compound was tested at 45, 50, 55, and 60 °C and antibacterial activity was evaluated against *S. aureus* and *M. luteus* (indicator strains) by agar well diffusion assay. At 55 °C, 22 and 18 mm inhibitory zones were observed against *M. luteus* and *S. aureus,* respectively while activity was reduced at 60 °C (Table 5) while pure antibacterial compound using 0.1% concentration showed 25 and 22 mm inhibitory zones against these indicator strains, respectively at 37 °C. 18 and 20% loss of activity was observed against same strains at 45 °C, respectively.

4. Discussion

A. pallidus belong to genera Bacilli [51,6] producing antibacterial molecule against *S. aureus, M. luteus, and P. aeruginosa*. The bacterial metabolites possessing antimicrobial or antifungal activity including peptides [52] are getting worth now a day and due to number of clinical concerns their usage is important [41,47]. *A. pallidus* SAT4, a thermophilic bacterium, produced peptide antibacterial metabolites at 50 °C. In spite of the great attention to microorganisms living under extreme environmental conditions, including thermophiles [2] the secondary metabolism of thermophilic microorganisms is poorly understood and virtually nothing is known of thermophilic bacilli producing antibacterial substances, including peptides. Mostly peptide antibiotics are produced by mesophilic bacterial cultures [15]. Few of them are capable of growing at temperatures above 40 °C. They include *B. brevis* G-B, producing gramicidin C [14], *B. polymyxa* in gavaserin and saltavalin [38] and *Bacillus licheniformis* DSM-13 producing peptide lantibiotics [13]. Bacitracin is a mixture of related cyclic polypeptides with molecular formula of $C_{66}H_{103}N_{17}O_{16}S$ produced by mesophilic bacterial strain *B. subtilis*. It is broad spectrum antibiotic that disrupt both gram positive and gram negative bacteria by interfering with cell wall and peptidoglycan synthesis [44]. In 2002, Esikova et al. [17] reported the thermophilic Bacillus species strains VK2 and VK21 involved in the production of peptide antibacterial molecules. Streptomyces species SRDP-TK-07 has been reported to produce antibacterial metabolites at 45 °C [39]. *Geobacillus toebii* HBB-247, a thermophilic bacteria isolated from different thermal spring and soil has been reported for the production of bacteriocin like inhibitory substance which was found to be stable up to 60 °C [37].

Table 3
Prominent FT-IR bands for antibacterial peptide compound.

Compound	FT-IR (cm^{-1})
Thermostable peptide antibacterial compound produced by *Aeribacillus pallidus* SAT4	3600–3650 stretching (O—H), 2990–2800 stretching (—C—H sp^3), 2359 stretching (—N=C=O or N=C), 1860–1786 ($R_2C=CR_2$), 1820–1760 (C=O), 1540 bending (N—H), 1690 (O=C—NH_2), 1400–1220 week for (C—O—H), 1600–1475 stretching (C=C), 1257 stretching (—C—O—), 1015 (R-COR_2-R), 750 bending (—CH_2—), 794 (1,3-disubstituted ring), 668 bending (C=C, C—X)

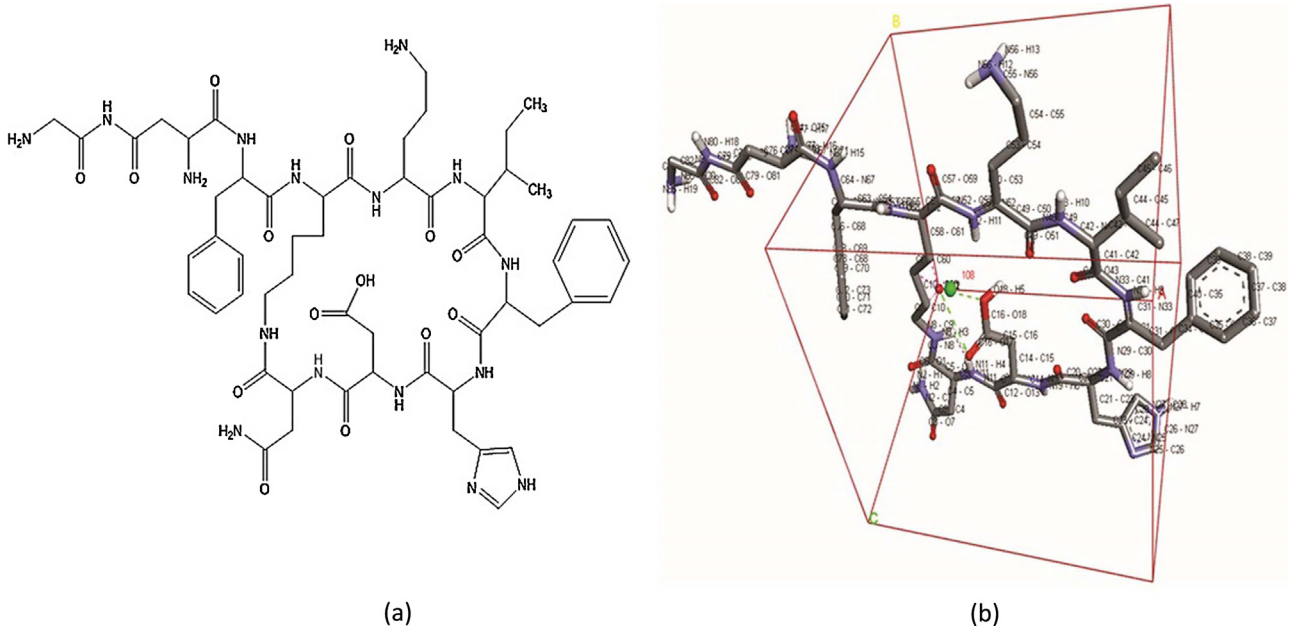

Fig. 4. (a) 2D structure [2-(9-((1H-imidazol-4-yl)methyl)-3-(2-amino-2-oxoethyl)-21-(2-(2-amino-4-(2-aminoacetamido)-4-oxobutanamido)-3-phenylpropanamido)-18-(3-aminopropyl)-12-benzyl-15-(sec-butyl)-2,5,8,11,14,17,20-heptaoxo-1,4,7,10,13,16,19-heptaazacyclopentacosan-6-yl) acetic acid] of antibacterial compound (proposed name: Aeritracin) obtained from thermophilic *Aeribacillus pallidus* SAT4 (b) The 3D crystal structure of antibacterial compound obtained from *Aeribacillus pallidus* SAT4 belong to the primitive orthorhombic lattice with the cell parameters $a = 12.137$, $b = 13.421$, $c = 14.097$ Å.

Antibiotic synthesis by using producing bacteria varies quantitatively and qualitatively based on the factors including the microbial strains and their growth fermentation conditions [49,3,12]. Considering the temperature and agitation rate as critical parameters, optimum production of antibacterial compound by SAT4 was observed at 55 °C and 100 rpm, respectively. During experimentation in the present study, incubation time was evaluated from 24 to 144 h with best activity found at 48 h of incubation and similar observation were recorded by Muaaz et al. [35], who reported that concentration of inhibitory compound was maximum at 48 h by *B. subtilis* MZ-7. pH was another important aspect of optimization study which was "5" from the experimental values of 4 (acidic) to 9 (basic) and Tang et al. in 1994 [46] reported that variations in fermentation broth pH affect several cellular developments like the regulation and controlling of the biosynthesis of microbial bioactive metabolites [46,5]. Addition of carbon and nitrogen sources in the production media optimally effect the production of antimicrobial compounds [22]. The optimum concentration of glutamic acid (as a source of nitrogen) and glucose (as source of carbon) was 1.5% and 2%, respectively. In 2006, Nasser discussed that galactose and glucose intensely improved the antibacterial activity of *Corynebacterium kutscheri* and *Corynebacterium xerosis*, respectively [9,36,23].

In 1991, Shimogki et al. reported that the first step in the purification of the antibacterial was separation of crude antibiotic from the microbial growth followed by precipitation of proteins by 70% ammonium sulfate [43,1,26]. The absorbance of protein fractions was observed at 280 nm [50,32] and electrophoresed [7,4]. The band on gel appeared at the position of 37 kDa while molecular weight of bacteriocin like substance produced by *G. toebii* strain HBB-247 was recorded about 38 kDa [37].

^{1}H and ^{13}C NMR spectra showed the presence of alkyl groups, amide linkage and carbonyl groups in the sample at 300 MHz. The resonance of alkyl amines (RNH_2) was observed at 3.996 ppm while Epperson and Ming recorded that ^{1}H NMR spectrum of the Co(II) complex of bacitracin mixture with amino acids appeared at 1–12 ppm and found the positions of the protons in the amino acid (α, β, etc.) at 11.7 ppm [16]. The resonance at 69.69 ppm showed

Table 4
Data processing statistics of antibacterial compound obtained by *Aeribacillus pallidus* SAT4 was identified and calculated from a single crystal source using a STOE-IPDS II equipped with an Oxford Cryostream low-temperature unit Germany.

Molecular formula	$C_{55}\ H_{82}\ N_{16}\ O_{17}$
Molecular weight	1,239.37
Molecular composition	C: 0.533, H: 0.067, N: 0.181, O: 0.219
Crystal system	Triclinic
Space group	P-1; Shoenflies: C1-1, $a = 12.137$, $b = 13.421$, $c = 14.097$
	Alpha = 84.269 (5) Å
	Beta = 81.71 (5) Å
	Gamma = 80.86 (5) Å
	$V = 2116.8$ (2) Å^3
Absorption coefficient	0.34 mm^{-1}
No. of reflections collected/unique	7955
No. of reflections used [$I > 2\sigma(I)$]	6045
Parameters	474
Final R indices	R1 = 0.0282, wR2 = 0.0677
R indices (all data)	R1 = 0.0394, wR2 = 0.0750
Structure determination	SIR97, SHELXL97 and WinGX
Equipment	STOE-IPDS II equipped with an Oxford Cryostream low-temperature unit

Table 5
Thermostability analysis of cell free supernatant and pure antibacterial compound at various temperatures against *Micrococcus luteus* and *Staphylococcus aureus* as indicator bacterial strains.

Sample	Zone of inhibition[a] (mm) (before treatment at 37 °C) (A_0)		Temperature (°C)	Activity against *M. luteus* (A_f)		Activity against *S. aureus* (A_f)	
	M. luteus	*S. aureus*		Zone of inhibition (mm)	% age loss of activity[b]	Zone of inhibition (mm)	% age loss of activity*
Cell free supernatant	22	25	45	14	36	12	52
			50	18	18	15	40
			55	22	0.00	18	28
			60	10	55	8	68
			45	18	18	20	20
Analysis after purification			50	15	31	16	36
			55	12	45	12	52
			60	8	63	10	60

[a] Formula: $A_0 - A_f/A_0 \times 100$, where A_0: activity control; A_f: activity after treatment.
[b] 0.1% concentration of purified antibacterial compound produced by *Aeribacillus pallidus* SAT4 was used before treatment.

the effects of electronegative atom (—C—O) while ^{13}C spectrum reported by Kim et al. showed carbon signal of C=O at 218.04 (C-3) and C=C at 150.96 (C-20), 109.47 (C-29) [28].

In 2001, Kim et al. reported that the structure of antibacterial compound was found to be orthorhombic which consisted of 3-different sides and three of 90° angle. The bond length of 1.2223 and 1.3639 Å exhibited the presence of C=O (C3-O1) and C=C (C20-C29), respectively [28].

The thermostability of both cell free supernatant and purified antibacterial compound showed that cell free supernatant was relatively stable at 55 °C showing 0 and 28% loss of activity against *M. luteus* and *S. aureus*, respectively. The activity of pure antibacterial compound was decreased 45% against *Micrococcus lutes* and 52% against *S. aureus* after treatment at 55 °C [13].

In conclusion, novel peptide antibacterial compound (proposed name: Aeritracin) produced by a thermophilic *A. pallidus* SAT4 is effective against *S. aureus*, *M. luteus*, and *P. aeruginosa*. This antibiotic would be a new addition in pharmaceutical research and drug development.

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