Production and characterization of antimicrobial peptides from *Bacillus subtilis* isolated from deep-sea core samples

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A new strain of *Bacillus subtilis* isolated from deep-sea core sediment sample (1400 m depth) produced antimicrobial peptides (AMPs) when cultured at 50 and 100 bar pressure conditions. The minimum inhibitory concentrations (MIC) showed that the AMPs had potent activity against *V. cholerae* and *K. pneumoniae*. AMPs extracted from cells grown at ambient and elevated pressure conditions exhibited distinct antifungal and antibacterial activities. Analysis of genes encoding AMPs revealed the presence of *srfAA*, *sbo* and *bmyB* biosynthetic genes. GC–MS analysis confirmed substantial accumulation of unsaturated fatty acids in membrane lipids of the cells in response to elevated pressure.

Keywords: Antimicrobial peptides, *Bacillus subtilis*, biosynthetic genes, deep-sea bacteria, piezotolerance.

Introduction

INCREASING microbial resistance to antibiotics has led to the search for new anti-microbial peptides (AMPs) from marine microorganisms. Antimicrobial compounds have been classified based on their biological functions, properties and chemical nature[1]. This includes a new antibacterial macrolide, macrolactin W, with potent antibacterial activity against both Gram-positive and Gram-negative pathogens[2]. Another such example is the antimicrobial linear lipopeptide from a marine *Bacillus subtilis* called gageostatin A-C[3]. The bacteria growing in the deep-sea ecosystem have several biochemical adaptations that enable them to survive in the harsh habitats. Pressure is a major challenge for deep-sea organisms and the pressure gradient (increase of 1 MPa for every 100 m) has been found to influence the metabolic pathways in microorganisms[4]. In recent years, researchers have isolated new piezophilic isolates or communities from habitats such as coal bearing deep-sea sediments[5], hydrothermal vents[6], whale fall associated sediments[7] and deep-sea methane cold seeps[8]. Deep-sea piezophiles acclimatize and respond to vast changes in an ecosystem immediately. Microorganisms surviving in deep-sea environment are known to produce unique compounds with broad biotechnological applications[9]. Many kinds of microorganisms, in particular *Bacillus* have been isolated from a wide range of deep-sea ecosystems. *Bacillus* group possesses distinct phenotypic characteristics, and it includes obligate aerobic and facultative anaerobes, halophiles and halotolerants, thermophiles and psychrophiles, piezophiles and piezotolerants. Several species of bacilli have been isolated from octacorals[10], deep-sea methane cold seep[11], shallow hydrothermal vents[12] and deep-sea hydrothermal sediments[13]. In the deep-sea oligotrophic environment, high hydrostatic pressure and low temperature are the key parameters that influence the metabolic activity of microorganisms. The microbial secondary metabolites that are produced in response to physical stress induced by pressure changes have shown to possess unique biotechnological values[14]. In contrast, there has been very little research probing the growth of deep-sea microbes under *in-situ* conditions of high pressure and low temperature. The objectives of the present study include (i) isolation and characterization of AMPs producing deep-sea strain, (ii) elucidating the biosynthetic genes, (iii) studying the growth of deep-sea strain under high pressure conditions, and (iv) evaluating the antimicrobial efficiency of AMPs.

Materials and methods

Sample collection

The deep-sea sediment core was collected from a depth of 1400 m (11°45.681′N, 80°03.718′E) of the Bay of Bengal, onboard Oceanographic Research Vessel (ORV) *Sagar Manjusha*. The sediment sample was enriched in marine broth (Difco, USA) at 10 bar pressure and 20°C for 3 weeks.

Biochemical, phenotypic and molecular characterization

The biochemical testing was done using test kits KB002, KB009A, KB009B and KB009C (HiMedia, India). Bacterial growth at different temperatures (10°–50°C); NaCl concentrations (0–15%); pH (4–10) was studied using a medium containing (w/v): 0.2% peptone, 0.2% yeast extract, 0.1% glucose, 0.02% KH_2PO_4, 0.005%

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$MgSO_4 \cdot 7H_2O$ prepared with 1 : 1 ratio of seawater distilled water. The purified 16S rRNA gene PCR product was sequenced and compared with gene sequences available on the NCBI GenBank database.

Piezotolerance analysis

In order to elucidate the piezotolerance capability and to assess changes at elevated pressures, the bacteria were cultured in customized high pressure and low temperature vessels in 1/4 concentration of marine broth (Difco, USA) at 10 bar pressure and 20°C for 2 days, after which pressure was increased up to 100 bar for 7 days. The growth and viability of the bacteria were determined by plate count in marine agar (Difco, USA) and optical density at 600 nm in UV spectrophotometer (Unicam UV 300, Thermo spectronic). AMPs were extracted from the stationary phase of cultures grown at atmospheric pressure and cultures grown at 50 and 100 bar pressure.

Partial purification of AMPs

Culture was grown in 100 ml nutrient broth medium at 28°C for 48 h. Cells were removed by centrifugation at 5000 *g* for 10 min and the supernatant was precipitated with 80% ammonium sulphate. Then the pellet was centrifuged, lyophilized and dissolved in 10 ml of 50 mM Tris–HCl (pH 7.5). The suspension was injected into a Sephadex G-50 column and major fraction was purified, using DEAE-cellulose column (5 cm × 25 cm) previously equilibrated with 50 mM Tris–HCl (pH 7.5) at a flow rate of 1 ml/min in GE-AKTA purifier GPC system. After washing the column with equilibration buffer, a linear 0–1.0 M NaCl gradient in 50 mM Tris–HCl (pH 7.5) was applied. The molecular weight of fraction was analysed, using 16.5% tricine SDS-PAGE. Fractions of 2.0 ml were collected manually and assayed for antagonistic activity using Microbial Type Culture Collection and Gene Bank (MTCC) type strains. The purified peak was analysed in MALDI-TOF-MS/MS (Applied Biosystems, USA). The purified AMPs were incubated with 10% NaOH in methanol for 16–24 h and lyophilized. This sample was re-extracted with methanol and tested.

Antimicrobial activity

The zone of inhibition and minimum inhibitory concentrations (MIC) against *Penicillium chrysogenum* MTCC 2725, *Aspergillus fumigates* MTCC 2550, *Aspergillus flavus* MTCC 277, *Aspergillus spinulosus* MTCC 377, *Candida albicans* 227, *Saccharomyces cerevisiae* MTCC 307, *Staphylococcus aureus* MTCC 7405, *Enterococcus faecalis* MTCC 2729, *Micrococcus luteus* MTCC 1809, *Pseudomonas aeroginosa* MTCC 4673, *Salmonella enterica* ser. *typhi* MTCC 734, *Proteus mirabilis* MTCC 425, *Escherichia coli* MTCC 730, *Vibrio cholerae-classical 01* MTCC 3904 and *Klebsiella pneumoniae* MTCC 4030 were determined by well diffusion method. All the analyses were performed in triplicates.

Identification of genes

The genes encoding AMPs in *B. subtilis* was identified using twelve biosynthetic genes chosen within the coding regions of bacyllomycin, bacylisin, ericin A, fengycin, haloduracin A1, haloduracin A2, iturin, mersacidin, surfactin, subtilin, sublancin and subtilosin[15–17]. The genes coding for polyketide synthases (PKS) and spore protein were also tested.

FTIR and SEM analysis

Purified AMPs obtained from cultures grown in various pressure conditions were lyophilized and analysed in FTIR spectroscopy (Affinity-1 Shimadhzu spectrometer) using the transmission mode at 4000–400 cm^{-1}. Morphological differentiation of cells grown at atmospheric condition and high pressure conditions (50 and 100 bar) was studied through SEM (TESCAN, SBU Vega 3).

Fatty acid methyl ethers analysis

The analysis of fatty acid methyl ethers was performed by GC–MS (GC 7890 A, 240-MS/4000-Agilent, USA). To an aliquot of lipid extract (10–30 mg) in a screw-capped glass (Teflon-lined) tube; 1.0 ml of anhydrous methanolic HCl was added and the mixture was heated to 100°C for 1 h. The mixture was extracted with hexane (1 ml) and analysed in a GC–MS under external ionization mode using HP-5 MS column (30 m × 0.320 mm × 0.25 μm), with helium as carrier gas at a flow rate of 1.0 ml/min.

Results

Characterization of deep-sea B. subtilis

The bioactive deep-sea isolate was aerobic, Gram-positive, spore forming and motile. In particular, sub-terminal ellipsoidal endospores were formed in non-swollen sporangia. Colonies grown on marine agar were round, creamy white, non-transparent and approximately 3–6 mm in diameter after two days of growth at 37°C. The optimum pH ranged between 5 and 8, NaCl concentration 2–4% and temperature 10–40°C. Isolate was catalase positive, reduces nitrate to nitrite, citrate is not utilized and acid is produced from cellobiose, D-arabinose, fructose, glucose, glycerol, L-arabinose, L-sorbose, maltose, mannose, mellibiose, sucrose, inulin, dulcitol, mannitol and

sorbitol. The phylogenetic analysis of the 16S rRNA gene sequence showed that the isolated strain belongs to *B. subtilis*, which showed 99% homology similarity. The deduced 16S DNA sequence (LN831186) was deposited at European Molecular Biology Laboratory (EMBL) database.

Piezotolerance and AMPs production

B. subtilis NIOT isolate was found to grow well and produce AMPs at atmospheric (1 bar) and elevated pressure (50 and 100 bar) conditions. The cell viability and antibacterial analysis revealed high production of AMPs at stationary growth phase. Cells grown at 50 and 100 bar pressure presented a different growth pattern and AMPs production when compared to the cells grown at 1 bar pressure. At 50 bar pressure conditions, a lag phase of 72 h was required for adaptation and the cells enter log phase (94–134 h). In 100 bar pressure conditions the cells required a prolonged lag phase of 96 h, but entered log and stationary phases at a faster growth rate. These findings elucidate the relationship between growth rate and pressure.

Partial purification and molecular characterization of AMPs

The AMPs were purified by gel filtration chromatography on Sephadex G-50 and ion-exchange chromatography on DEAE-cellulose column. The purification was about 98.9% with a yield of 0.3%. The purified major fraction was run on Tricine SDS-PAGE gels and a single band with 1.5 kDa was obtained. The major m/z ions were present at 1088 and 666.

Bioactivity of AMPs and marker genes

The AMPs were extracted after culture growth and antimicrobial activities were determined based on the degree of inhibition on agar plates. The test strains *A. fumigatus* and *A. spinulosus* were found to be more sensitive than other species and the MIC value was found to be 7.5 µg/ml. AMPs were also found to have high activity against *C. albicans* with MIC value of 17.5 µg/ml (Table 1). High activity was observed against *V. cholerae* and *K. pneumoniae* with MIC value of 7.5 µg/ml (Table 1). The AMPs of atmospheric pressure grown cells demonstrated strong bactericidal activity and AMPs of high pressure grown cells exerted high bacteriostatic rather than bactericidal activity. This change suggested that pressure induces intra-molecular alteration in structural and functional properties of AMPs. Studies using molecular markers confirmed the presence of three biosynthetic genes *srfAA*, *sbo* and *bmyB*. Interestingly, the *PKS* gene responsible for antimicrobial secondary metabolites production was also present. The presence of these genes in the isolate confirmed the active role of surfactin, subtilosin and bacyllomicin in providing advanced defence mechanism to survive in competitive marine environment.

FTIR analysis of AMPs – effect of pressure

AMPs were extracted from the cultures grown at different pressure conditions. The AMPs extracted from cultures grown in atmospheric conditions showed major peaks at 3394, 1645 and 1408 cm^{-1} (Figure 1 *a*) which may be attributed to α-helices patterns and some turns. In particular, many sharp bands observed around 1654 cm^{-1} were shifted towards 1638 cm^{-1} (β-helical proteins). Both the 50 and 100 bar spectra (Figure 1 *b* and *c*) were much similar. However, there is some oscillations in absorbance at 1740 cm^{-1} which is attributed for C=O stretches and this indicates changes in lipophilic moieties at elevated pressure. The pressure induces conformational changes in the peptide which causes shifts in its activity. The molecular changes in the peptide may be correlated with the shift in bactericidal to bacteriostatic activity.

Morphological alterations at elevated pressure – SEM

Bacteria exhibited typical rod-shaped morphology and the number of vegetative cells were more when compared to the presence of spores in cells when grown at atmospheric conditions (Figure 2 *a*). Sub-terminal endospores were observed with mild swelling of vegetative cells. The average cellular size of *B. subtilis* NIOT was 0.5–2.0 µm in length. Mild morphological changes were observed in the cells grown at 50 bar pressure (Figure 2 *b*). However, the elongated cells were observed under 100 bar pressure conditions (Figure 2 *c*). The elongated morphology showed an average cellular size of 4.0–6.0 µm in length.

Table 1. *In vitro* antimicrobial analysis and MIC of AMPs

	Zone of inhibition (mm)	MIC (µg/ml)
MTCC fungi tested		
P. chrysogenum	17.0 ± 5.5	12.5
A. fumigates	19.3 ± 2.5	7.5
A. flavus	10.0 ± 1.5	15.0
A. spinulosus	18.3 ± 2.2	7.5
C. albicans	13.6 ± 1.7	17.5
S. cerevisiae	20.0 ± 2.7	7.5
MTCC bacteria tested		
E. coli	14.5 ± 3.5	10.0
E. faecalis	10.2 ± 3.7	37.5
K. pneumoniae	20.0 ± 2.5	7.5
P. aeruginosa	12.5 ± 3.3	35.0
S. typhi	15.0 ± 1.5	12.5
S. aureus	15.0 ± 1.5	12.5
V. cholerae	17.5 ± 3.5	7.5

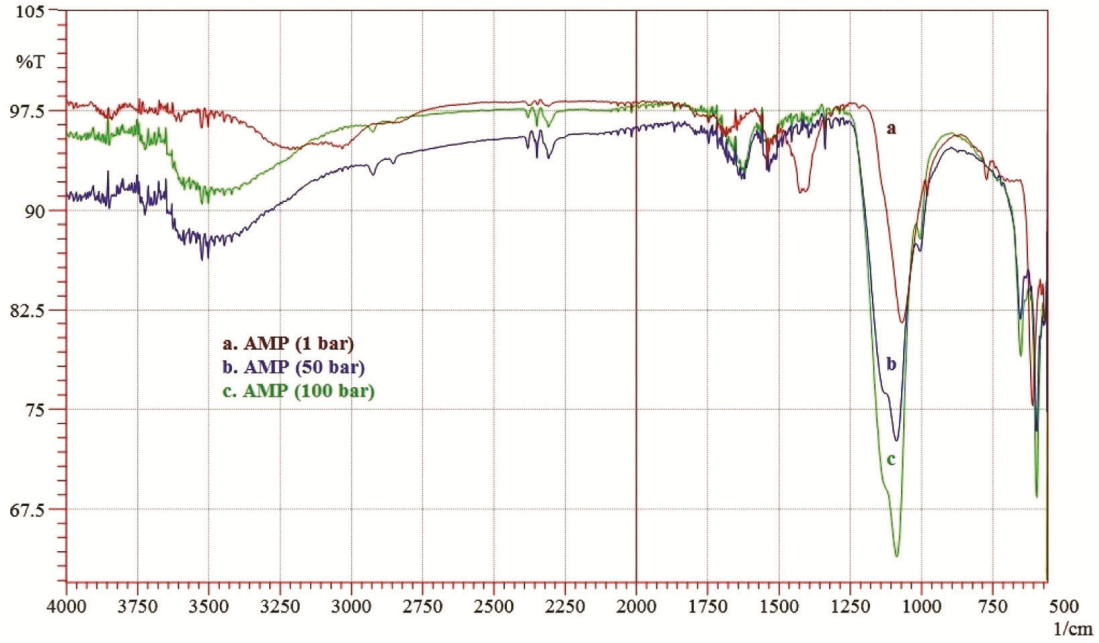

Figure 1. FTIR analysis of AMPs extracted from *B. subtilis* NIOT grown at different pressure conditions.

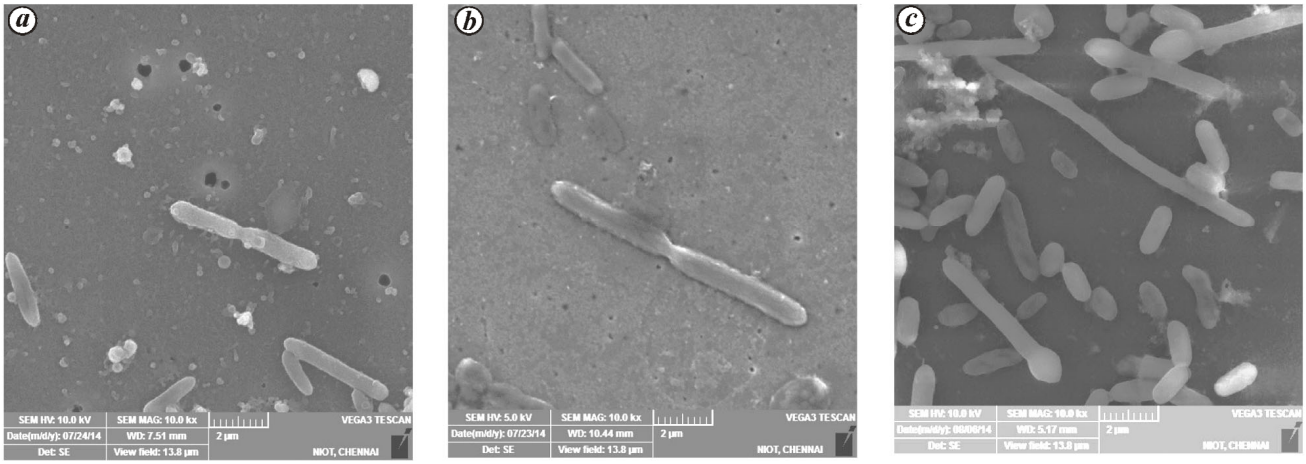

Figure 2. SEM analysis of *B. subtilis* NIOT grown at (***a***) 1 bar, (***b***) 50 bar and (***c***) 100 bar pressure conditions.

Table 2. GC–MS analysis of fatty acid composition of *B. subtilis* NIOT strain grown at 1, 50 and 100 bar pressure conditions

Fatty acid	1 bar	50 bar	100 bar
11 : 0	ND	ND	1.1
iso-13 : 0	ND	ND	1.2
13 : 0	12.5	ND	ND
14 : 0	18.7	6.5	10.1
15 : 0	1.85	ND	ND
16 : 0	13.5	29.3	18.8
16 : 1n-9	0.36	7.13	5.00
17 : 0	7.09	4.89	ND
18 : 0	11.8	1.12	5.23
18 : 1n-9	10.2	11.2	ND

ND, Not detected.

Elevated pressure effects on the fatty-acid composition of B. subtilis NIOT: GC–MS

Cells grown at 1, 50 and 100 bar pressure conditions were analysed for fatty acid compositions of total lipids and the results are shown in Table 2. In 1 bar grown culture, the major components were tridecanoic acid (13 : 0), tetradecanoic acid (14 : 0), hexadecanoic acid (16 : 0) and heptadecanoic acid (17 : 0). Minor levels of unsaturated fatty acid, palmitoleic acid (16 : 1n-9) were also observed. In the culture grown at 50 bar, the polyunsaturated fatty acid (18 : 1n-9) and monounsaturated fatty acid (16 : 1n-9) have been found to be increased. Interestingly,

at 50 bar condition the concentration of saturated fatty acid (16 : 0) increased by two-fold. In cells grown at 100 bar pressure, the 18 : 1n-9 level was decreased. The low levels of 18 : 1n-9 were on contrast to 18 : 0, which got reduced at 50 bar pressure and started to increase at 100 bar pressure.

Discussion

To our knowledge, this is the first report on exploring AMPs from deep-sea isolate *B. subtilis* and studying its functional alterations at elevated pressures. *Bacillus* sp. is considered as one of the best prokaryotes producing broad range of structurally diverse secondary metabolites[18]. The deep-sea isolate was aerobic, motile and formed sub-terminal ellipsoidal endospores. Colonies were approximately 3–6 mm in diameter, creamy white and non-transparent. The isolate grew well in 2–4% NaCl, pH 5–8 and temperature 10–40°C. Isolate was catalase positive, fermented cellobiose, D-arabinose, fructose, glucose, glycerol, L-arabinose, L-sorbose, maltose, mannose, mellibiose, sucrose, inulin, dulcitol, mannitol and sorbitol. The phylogenetic analysis of 16S DNA gene sequence confirmed the isolated strain as *Bacillus subtilis* with 99% homology similarity. Piezotolerance analysis studied the effect of high pressure on growth of *B. subtilis* NIOT using custom designed high pressure fermentor. There was an initial lag period during increase of pressure from 10 to 50 bar followed by extended exponential growth phase and in cells grown at 100 bar pressure conditions a prolonged lag phase was observed. This proved the requirement of phase shift time for intracellular and molecular changes to maintain the growth at different pressure gradients (50 and 100 bar). These results suggested that pressure induces significant alterations at cellular and molecular level for survival and maintenance of its activity[19]. AMPs were purified by a sequential ammonium sulphate precipitation, extraction and gel chromatography. The molecular weight of the purified peptide was about 1.5 kDa and the homogeneity of the fraction was confirmed by MALDI spectroscopy. The purified peptide exerted broad antifungal and antibacterial activity against various MTCC type strains *in vitro*. The MIC values were <15 μg/ml for all the filamentous fungi tested (*P. chrysogenum*, *A. fumigates*, *A. flavus*, *A. spinulosus*). Significantly, AMPs exerted inhibitory activity towards *C. albicans* with MIC value of 17.5 μg/ml (Table 1). The *C. albicans* is an important fungal pathogen of human causing life threatening disease under immunocompromised conditions[20]. The species of *Aspergillus* and *Penicillium* are considered as predominant phytopathogens causing considerable losses to agriculture[21]. Considering these medical and agricultural importances, the studied AMPs were considered to have significant applications. The characterization of antibacterial activity revealed differences in the activity of AMPs extracted from *B. subtilis* NIOT cells grown at ambient and elevated pressure conditions. Both variants displayed two distinct mechanisms of action, resulting in bacteriocidal and bacteriostatic activity. The bacteriostatic activity was found to be high in AMPs extracted from cells grown at elevated pressure whereas the potent bactericidal activity was observed in AMPs obtained from cells cultures at atmospheric pressure. These observations indicate the pressure-induced modification in activity of AMPs. The results suggest that high pressure can be applicable as a promising technology for enhancement of antimicrobial activity. The gene-specific antibiotic biosynthesis mechanism in *Bacillus* produces a wide range of antimicrobial metabolites with unique structural and functional properties[22]. The AMPs structural gene analysis confirmed the presence of *srfAA*, *sbo* and *bmyB*; and their possible association with *PKS* responsible for antimicrobial properties. Since the strain has three productive antimicrobial genes, it could have wider *in situ* application in the control of fungal pathogens. The FTIR analysis of AMPs purified from atmospheric pressure grown cells presented minor bands around 1600 cm^{-1} attributable to major amino acid chain vibrations (Figure 1). The multiple bands around 1650 cm^{-1} constitute for the presence of α-helices peptide components. The AMPs from cells grown under high pressure showed unaffected β-helices and sheets whereas minor shifts were observed in α-helices (Figure 1). The piezophysiology of *B. subtilis* NIOT may be the possible reason for conferring high stability in AMPs. Phenotypic variations with respect to change in pressure are shown in Figure 2. SEM analysis revealed the response of *B. subtilis* NIOT at 100 bar condition pressure by altering its length. Growth and morphological changes in *E. coli* are well elucidated when cells were exposed to different pressures[23]. These results confirmed that elevated pressure can bring about changes in the microbial growth patterns. Many researchers proved that piezophiles and piezotolerant bacteria in the deep-sea contain high percentage of unsaturated fatty acids[24]. The characterization membrane response to high pressure in *B. subtilis* NIOT clearly elucidates its acclimatization towards changes to the external environment. In particular, at 50 and 100 bar we found the occurrence of polyunsaturated fatty acid (18 : 1n-9) as a dominant fatty acid. In addition, substantial increase in monounsaturated fatty acid (16 : 1n-9) was found. The ratio of the unsaturated fatty acids also increased when the cells were grown at high pressure (Table 2). This phenomenon contributed to maintain the fluidity of the membranes at altered piezo-conditions.

Conclusion

The present study elucidated the production of AMPs at ambient and elevated pressure conditions with altered antimicrobial properties. AMPs produced by *B. subtilis*

NIOT exhibited both antifungal and antibacterial activity. The response of fatty acid biosynthetic machinery suggests the capability of deep-sea piezotolerant strain to adapt to the external piezo-changes. The study also highlighted that high pressure and low temperature conditions could be more suitable for deep-sea microorganisms to produce novel bioactive molecules.

Conflict of interest: The authors declare no conflict of interest.

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