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Ultraviolet-radiation-resistant isolates revealed cellulose-degrading species of *Cellulosimicrobium cellulans* (UVP1) and *Bacillus pumilus* (UVP4)

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Abstract.

Among extremophiles, microorganisms resistant to ultraviolet radiation (UVR) have been known to produce a variety of metabolites (*i.e.*, extremolytes). We hypothesized that natural microbial flora on elevated land (hills) would reveal a variety of UVR-resistant extremophiles and polyextremophiles with modulated proteins and enzymes that had biotechnological implications. Microorganisms C*ellulosimicrobium cellulans* UVP1 and B*acillus pumilus* UVP4 were isolated and identified using 16S rRNA sequencing, and showed extreme UV resistance (1.03×10^6 and 1.71×10^5 J/m^2, respectively) from elevated land soil samples along with unique patterns of protein expression under UVR and non-UVR. A broad range of cellulolytic activity on carboxymethyl cellulose agar plates in *C. cellulans* UVP1 and *B. pumilus* UVP4 was revealed at varying pH, temperature, and inorganic salt concentration. Further, the microbial strain *B. pumilus* UVP4 showed the basic characteristics of a novel group: polyextremophiles with significance in bioenergy.

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1. Introduction

Extremophiles are microorganisms living under extreme environments such as hot springs, volcanic areas, the deep sea, extreme high and low temperatures (>45 or <15°C), pressure extremes, oxygen scarcity, and radiation [1],[2]. One type of radiation that is of particular concern to humans is ultraviolet radiation (UVR), which has been linked to many harmful effects, including immune suppression, dermatitis, premature aging, and skin cancer [3],[4]. Radiation energy in the form of particles or electromagnetic waves (gamma rays, X-rays, UVR, or radio waves) causes oxidative damage to various vital biomolecules, including proteins. However, several microorganisms are known to survive under these lethal conditions [5–7].

The ability of microorganisms to withstand extreme radiation is reported to be connected with their genome stability [8]. Several metabolites produced under extreme radiation are unique organic compounds that are not directly involved in normal growth, development, or reproduction of microorganisms [4],[9]. Unfortunately, these compounds have not yet been investigated for potential industrial or therapeutic significance. In addition to therapeutic applications, the green energy (biofuel) is of rapidly growing interest in the field of energy security, diversity, and sustainability as well as for greenhouse gas mitigation [10],[11]. Lignocellulosic biomass is a sustainable resource that can be converted to products of commercial interest such as bioethanol and lactic acid [11],[12]. Currently, the most widely studied cellulase, *Trichoderma* cellulase, has shown several disadvantages, including low enzyme yields, low specific activities, and end-product inhibition [13]. Thus, isolation and characterization of a stable microorganism able to thrive under extreme conditions and degrade cellulose are crucial, as this measure will produce enzymes that can also function under extreme conditions.

We hypothesized that UVR-resistant extremophiles from elevated land (hills) would reveal modulated proteins/enzymes and metabolites of biotechnological implications. Therefore, we aimed to isolate and characterize UVR-resistant microorganisms with cellulolytic activity from elevated land (hills) soil microbial flora. Our studies revealed specific UVR-resistant extremophiles and polyextremophilic strains of *Cellulosimicrobium cellulans* UVP1 and *Bacillus pumilus* UVP4, respectively, at elevated land (hills) with unique protein expression and stable cellulolytic activities at wider environmental conditions.

Abbreviations: CFU, colony forming unit; CMC, carboxymethyl cellulose; CPD, cyclobutane pyrimidine dimer; LESD, lag, exponential, stationary, and death; NA, nutrient agar; NB, nutrient broth; NER, nucleotide excision repair; OD, optical density; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; UVC, UV light subtype C; UVR, ultraviolet radiation.

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2. Materials and methods

2.1. Isolation of UVR-resistant extremophiles

The UVR-resistant microorganisms were isolated from soil samples collected at the Tracy Ridge recreation area, 2,245 ft. above sea level, in the Allegheny National Forest of northwestern Pennsylvania, in the month of August, as described by Copeland et al. [14]. Briefly, the microorganisms present in soil samples (1 g) were enriched aerobically in wide-mouthed glass bowls (105×40 mm^2) containing 50 mL nutrient broth (NB) medium, at 32°C, under germicidal UV light subtype C (UVC) at an intensity of 9.5 W/m^2. After UVR exposure, the microbial-enriched soil samples were subjected to serial dilutions in three replicates and spread onto nutrient agar (NA) plates for colony forming unit (CFU)/mL determination. The microbial growth and survival rate were determined at regular time intervals at 9.5 J/m^2/Sec from CFU counting, and they were grown at 32°C in the dark. The UVR-resistant microorganisms were obtained at UV exposure 1.03×10^6 and 1.71×10^5 J/m^2, and were denoted as UVP1 and UVP4. Further, the growth of the isolates under UV and non-UV was determined by measuring turbidity at optical density at 600 nm (OD_{600}). The isolated microorganisms were subjected to identification and characterization.

2.2. Morphological and physiological characterization

The morphological, physiological, and biochemical characteristics of the isolates were investigated by routine cultivation on NA and NB media. The cell morphology was examined under 40× and 100× objectives of the compound microscope (Leica CME, Wetzlar, Germany). Gram staining was performed using a BD Gram stain kit (BD Bioscience, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Growth of the isolate at different temperatures, NaCl concentrations, and pH levels was determined by measuring OD_{600} of the culture on a UV–vis spectrophotometer (DU-640; Beckman Coulter, CA, USA). The required temperature for optimal growth was determined in NB at varying temperatures from 10 to 55°C with an increment of 5°C. The effect of salt was determined using various NaCl concentrations (1.0%–10.0%, w/v, with an increment of 1.0%) in NB culture observing growth at OD_{600}. Similarly, the growth at different pHs was determined in NB that had been adjusted to various pH values before sterilization (pH 4.5–9.0 ± 0.05, in increments of 0.5 pH units) using 0.1 N HCl or 0.1 N Na_2CO_3. The starch hydrolysis was carried out by streak inoculation on NA plates supplemented with 0.4% soluble starch. After 72 H of incubation, the enzyme production was determined by flooding the plate with 3.0 mL of Gram's iodine (1 g KI and 2 g I per 100 mL). The catalase production of isolates was assayed on NA slants using 0.3% H_2O_2. The oxidase activity was detected using *N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine dihydrochloride. The cellulolytic activity was measured after 7 days' incubation on plates containing 0.5% carboxymethyl cellulose (CMC) as the carbon source, 0.3% yeast extract, and 1.5% agar. Plates were stained with a 1% congo-red water solution. Casein activity was detected on skimmed milk agar after 7 days' incubation. Other physiological and biochemical tests were done using BD™ BBL ™Enterotube-II (BD Bioscience, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Different carbon sources (D-glucose, sucrose, galactose, fructose, mannose, D-xylose, arabinose, D-mannitol, lactose, sorbitol, and inositol) were used to evaluate carbon utilization. All of the tests were performed at 32°C in the appropriate media and were conducted according to standard methods [15].

2.3. Sequence alignments and phylogenetic tree analyses

Total cellular DNA from single-cell purified colonies was extracted using PureLink™ Genomic DNA Mini Kit K1820-01 (Invitrogen Corp., Carlsbad, CA, USA). The 16S rRNA gene sequences from two most UVR-resistant isolates were amplified using universal primer (F-518: CCAGCAGCCGCGGTAATACG, R-800: TACCAGGGTATCTAATCC) and sequenced at Macrogen Service Center (Rockville, MD, USA). All the sequences were compared with closest relatives from GenBank and Ribosomal Database Project (RDP) release 10 (http://rdp.cme.msu.edu/index.jsp). Phylogenetic trees were constructed by the neighbor-joining method with pairwise deletion of gaps in the RDP database.

2.4. Nucleotide sequence accession numbers

The sequences of all pure cultures were deposited in the GenBank database under accession numbers JQ348903 and JQ348902 for UVP1 and UVP4, respectively.

2.5. UV radiation tolerance

To characterize UVR resistance, seed cultures of the isolated microorganisms UVP1 and UVP4 were prepared in NB medium at 120 rpm in a shaker orbital incubator at 32°C. The organisms were removed from the shaker at an OD_{600} of 1.25 microbial growth. To evaluate the UV tolerance of the isolated microorganisms, microbial seed cultures (1 mL) of the UVR-tolerant strains (UVP1 and UVP4) were inoculated in 50 mL NB medium and grown under a germicidal UVC lamp at radiation dosages of 1.03×10^6 and 1.71×10^5 J/m^2, respectively, in a glass bowl (105×40 mm^2) at 32°C. The UV tolerance was determined on the basis of the growth of microorganisms in liquid medium. At the end of incubation, the culture medium was transferred into a 250 mL Erlenmeyer flask recording OD_{600} as 0 H. The UV exposed microbial cultures were then incubated at 120 rpm at 32°C; 1 mL culture was withdrawn from flasks at regular time intervals and measured for growth at OD_{600}. The experiments were conducted under similar conditions for non-UV exposure, selecting UV-sensitive *Escherichia coli* as control.

2.6. UV-modulated protein expression in one-dimensional gel electrophoresis

To examine the protein modulation under UV exposure and non-UV conditions, total intracellular soluble protein was extracted and purified using a B-PER bacterial protein extraction reagent

kit from Pierce (Rockford, IL, USA) according to the manufacturer's instructions. Briefly, bacterial cells were collected in late log phase growth and pelleted by centrifugation at 5,000*g* for 10 Min. After enzymatic treatment with lysozyme and DNase I, 4 mL B-PER reagent per gram of cell pellet was added and incubated at room temperature for 10–15 Min. The total soluble proteins in lysate were separated from insoluble proteins by centrifugation at 15,000*g* for 5 Min. The supernatant was collected and estimated for total protein concentration using a RC DC protein assay kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Protein samples were aliquoted and stored at -80°C. Equal amounts (25 μg) of protein were denatured in a sample buffer containing 60 mM Tris (pH 6.8), 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, and 0.1% bromophenol blue and boiled for 5 Min, followed by centrifugation at 10,000*g* for 1 Min at 4°C. The samples were loaded on 10% Tris–glycine gel. A prestrained molecular weight marker was run along with the samples. Gel electrophoresis was performed using the Bio-Rad Mini Protein gel system (Bio-Rad) at a constant voltage of 60 V for 30 Min followed by 120 V for 120 Min or until the blue dye front reached the bottom of the gel. After electrophoresis, the gel was washed two times in distilled water and stained overnight in Coomassie Brilliant Blue dye (Bio-Rad). After three thorough washings in distilled water, the developed protein gel was scanned in a regular scanner for further analysis.

2.7. Determination of UVR-resistant cellulolytic activity and characterization

The UVR-resistant extremophiles were examined for cellulolytic activity on CMC agar plates (0.1% $NaNO_3$, 0.1% H_2PO_4, 0.05 KCl, 0.05 KCl, 0.05% $MgSO_4$, 0.5% CMC, and 1.7% agar) flooded with congo-red indicator followed by confirmation using 1 M NaCl and 1 M HCl to stop the enzymatic activity [16]. The microorganisms (UVP1 and UVP4) that revealed larger clear zones around the agar plugs due to cellulose degradation were selected and secured for further characterization. Agar plug assays were performed by first preparing seed cultures, incubating UVP1 and UVP4 in NB medium overnight at 120 rpm and 32°C. The grown culture (OD_{600} 2.2–2.5; 2 mL) was centrifuged at 10,000*g* for 5 Min and pelleted. The cell pellet was washed three times in sterilized distilled water and resuspended in 200 μL of sterilized distilled water. The washed cells (50 μL) were loaded in agar plugs formed using a sterile 5-mm cork borer on CMC plates. The plates were incubated at 32°C for 96 H. The cellulose degradation efficiency of these extremophiles was characterized at various temperatures (25, 32, and 45°C), pH levels (5, 7, and 9), and CMC concentrations (0.1%, 0.5%, 1%, and 2%), and using different inorganic sources, that is, $NaNO_3$ (0%, 0.05%, 01%, 0.5%, 1%, and 2%), $MgSO_4$ (0%, 0.01%, 0.05%, 0.1%, 0.5%, and 1.5%), K_2HPO_4 (0%, 0.05%, 0.1%, 0.5%, and 1%), and KCl (0%, 0.01%, 0.05%, 0.1%, 0.5%, and 1.5%). The cellulolytic activity of the UVR extremophiles (UVP1 and UVP4) was determined as described by Teather and Wood [16]. The cellulose-degrading zone index was calculated by measuring the diameter of the clear zone.

2.8. Statistical analysis

All experiments were carried out in triplicate, and the experimental results represent the mean of three identical sets of experiments. One-way analyses of variance followed by least significant difference (Tukey's honestly significant difference) were performed to evaluate the potential significant differences between the cellulose-degrading zone indexes.

3. Results

3.1. Isolation of UVR-resistant microorganisms

The UVR-resistant microorganisms were isolated at various dosages of UV (subtype C) irradiation by exposing the soil samples from a higher altitude to intense UV (9.5 W/m^2) for a prolonged time period (2.06×10^7 J/m^2). The initial dosages of UV (3.44×10^4 and 34.4×10^4 J/m^2) were observed to be fatal to the total microbial flora (3.27×10^5 and 3.43×10^{11} CFUs) compared with the non-UV-exposed batches (6.60×10^{14} and 5.80×10^{15} CFUs) (Fig. 1A). The number of microorganisms (CFUs)/ mL after 68.9×10^4 J/m^2 was observed to be consistent regardless (1.41×10^{14} CFUs), and there was a marginal decrease after further UV exposure up to 2.06×10^6 J/m^2 (Fig. 1A). The average survivability of total microbial flora over the course of UV exposure (2.06×10^6 J/m^2) was found to be 1.28%. The two most prominent and visible surviving colonies were selected and designated as UVP1 and UVP4, obtained at 3.44×10^5 and 1.03×10^6 J/m^2.

After purification by repeated streaking, both UVP1 and UVP4 were examined for their growth under the same intensity of UV irradiation compared with the non-UV-irradiated growth, along with the control microorganism *E. coli*, sensitive to UVR. A typical lag, exponential, stationary, and death (LESD) curve of microbial growth was observed in UVP1 and UVP4 under both UVR and non-UV conditions (Fig. 1B). The control microorganisms sensitive to UV did not grow under UVR (Fig. 1B), but showed a typical LESD curve under non-UV. During UV exposure, organisms UVP1 and UVP4 did not show any pigments in them, which suggests that pigmentation is not necessary for bacteria to survive in the presence of UVR.

3.2. Identification of UVR-resistant microorganisms

3.2.1. Morphology

The isolated microbes were translucent, whitish, convex, circular, smooth, and entirely edged. The older colonies had marginal edges. UVP1 and UVP4 were rod- and bacillus-shaped, respectively. The cells of both organisms were Gram positive. Microorganism UVP1 revealed motility in the liquid culture, whereas UVP4 did not depict motility (Table 1).

3.2.2. Physiological and biochemical characteristics of UVR-resistant UVP1 and UVP4

Both UVR-resistant isolates (UVP1 and UVP4) were tested for physiological and biochemical characteristics; a comparison is given in Table 1. UVP1 was aerobic but could also survive under oxygen-deficient conditions, whereas UVP1 was strictly aerobic.

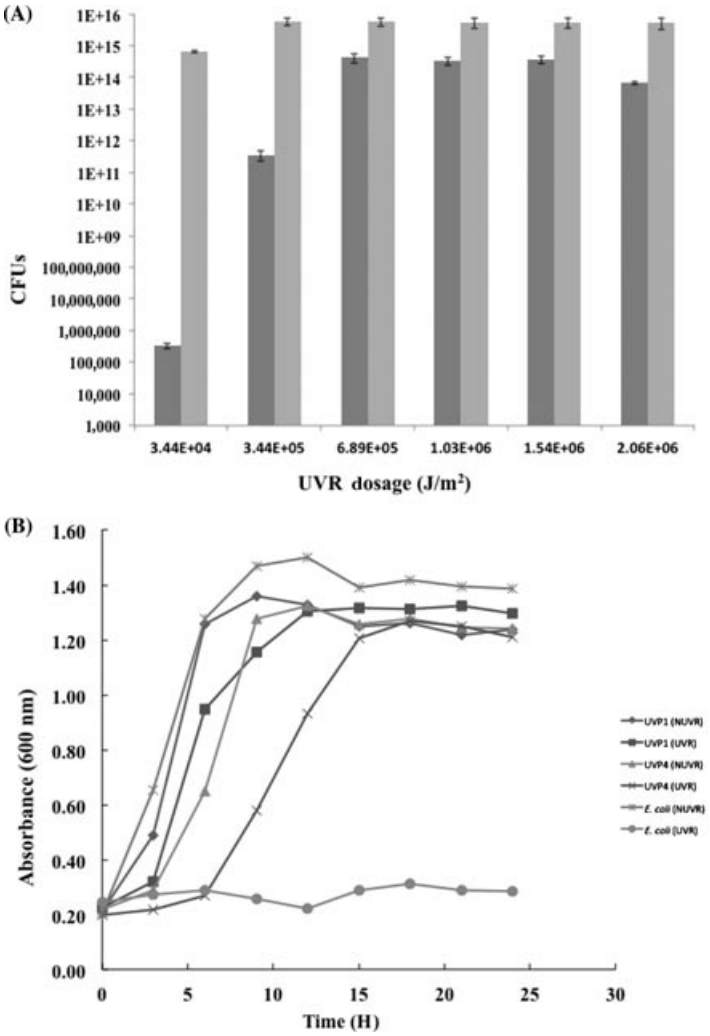

Fig. 1. Colony forming units (CFUs) and growth of UVR-resistant microorganisms under UV and non-UV irradiation. (A) CFUs of UVR-resistant microbial flora from higher elevation soil samples. The soil samples were enriched in nutrient broth in a wide mouth glass bowl as detailed in section *Materials and methods* under germicidal UV lamp (subtype C) at 9.5 W/m^2 at 32°C. At varying UVR dosages, the samples were plated on serially diluted nutrient agar plates. (Blue bars: UVR, green bars: non-UVR.) (B) Growth of UVR-resistant microorganisms under UV and non-UV irradiation. The UVR-resistant microorganisms were grown in nutrient broth medium at 32°C under UVC lamp at radiation dosage of 1.03×10^6 and 1.71×10^5 J/m^2, respectively, for UVP1 and UVP4. The microbial growth was determined at regular time intervals at OD_{600} and compared with UV-sensitive *E. coli* as positive and negative control. (UVR: grew under UVR; NUVR: grew in non-UVR.)

Table 1
Morphological and biochemical characterization of *Cellulosimicrobium cellulans* UVP1 and *Bacillus pumilus* UVP4 for identification

	Characterization results	
Test	*C. cellulans* UVP1	*B. pumilus* UVP4
Morphological		
Colony morphology	Rods	Bacilli
Grams staining	+	+
Motility	−	+
Spore formation	−	+
Biochemical		
Growth condition	Aerobic/facultative	Aerobic
Indole production	+	−
Methyl red	+	+
Voges–Proskauer	−	+
Nitrate reduction	−	−
Oxidase	+	+
Catalase	+	+
Starch hydrolysis	+	−
Cellulolytic	+	+
Casein hydrolysis	+	+
Lipase	−	+
DNase	−	−
Protease	+	+
Citrate utilization	+	−
Gas production (glucose)	−	−
Urea	+	+
Growth temperature	20–40°C	20–45°C
Growth pH	5–8	5–10
Substrate utilization		
D-Glucose	+	+
Sucrose	+	+
Galactose	+	+
Fructose	+	+
Mannose	+	+
D-Xylose	+	+
D-Mannitol	+	+
Arabinose	−	+
Lactose	+	+
Sorbitol	+	−
Inositol	−	−
Adonitol	+	−

Both strains produced catalase, showed cellulolytic activity as clear zones on CMC agar plates, were able to hydrolyze casein, showed protease activity, and showed positive urea tests. UVP1 was distinguished from UVP4 by indole production, starch hydrolysis, and citrate utilization, whereas UVP4 was observed to be distinct in Voges–Proskauer and lipase tests. Both isolates did not reduce nitrate into nitrite. The isolates grew over wide temperature and pH ranges (Table 1). The isolate UVP1 had a smaller temperature range for growth (20–40°C), whereas UVP4 showed optimum growth from 37 up to 45°C and could be designated as a thermophilic extremophile. The optimal pH range for growth was 6–7, but the organisms were also observed to grow at higher pH levels: 8 (UVP1) and 10 (UVP4). Both isolates showed a wide range of sugar substrate utilization, with the exceptions being sorbitol, adonitol for UVP4, and arabinose for UVP1 (Table 1).

3.2.3. 16S rRNA analysis

The two isolates, UVP1 and UVP4, obtained under UVR were further characterized for identification. Sequences for the 16S rRNA genes of UVP1 and UVP4, respectively, of 947 and 954 bp, were determined and compared with those of the other closely related taxa retrieved from the GenBank database. A comparison with 16S rRNA sequences held in GenBank indicated that the UVR-resistant isolates UVP1 and UVP4 are phylogenetically related to members of the families *Promicromonosporaceae* and *Bacillaceae*, respectively. The 16S rRNA homology and topology of the phylogenetic tree indicated that the isolates belonged to the genera *Cellulosimicrobium* and *Bacillus*. 16S rRNA sequence similarity revealed UVP1 and UVP4 showed 99.95% similarity to *C. cellulans* and *B. pumilus*, respectively (Figs. 2A and 2B). The sequences of *C. cellulans* UVP1 and *B. pumilus* UVP4 were deposited in the GenBank database under the accession numbers JQ348903 and JQ348902, respectively.

3.3. Unique proteome of UVR-resistant microorganisms

To investigate functional proteins under stress, the cellular survivability responsive unique radiation-sensitive proteins/enzymes were resolved on one dimensional (1D) SDS-PAGE. The total protein from *C. cellulans* UVP1 and *B. pumilus* UVP4 revealed 13 unique protein bands expressed at similar molecular weight ranges, 25–100 kDa, compared with those in the absence of UVR (Fig. 3). The species-specific protein expression is clearly visible in a controlled set of protein extracts where microorganisms grew under non-UVR conditions at 37°C. The distinct sets of protein expression in the absence of UVR appeared at different molecular weights from 25 to 50 kDa. Protein identification using liquid chromatography– mass spectrometry (LC-MS/MS) is under way and subject to further research into a protein- and enzyme-based defense against UVR in UVR-resistant extremophiles.

3.4. Measurement of cellulolytic activity in UV-resistant organisms

A variety of extremophiles have gained momentum for the production of sustainable and realistic alternatives to conventional fuels because of the darker consequences of fossil fuels in the bioenergy sector. The thermophiles and thermophilic enzymes have had the greatest impact in bioenergy. Microbial production of bioethanol from biomass has given impetus to the study of novel groups of microorganisms that can effectively degrade cellulosic materials. On the basis of the hypothesis that UVR-resistant microbes can potentially be used in biotechnological value-added products of commercial interest, the isolated UVR-resistant microorganisms *C. cellulans* UVP1 and *B. pumilus* UVP4 were studied and augmented for cellulolytic activity using agar-plug assays.

Studies were performed to determine the critical physical and chemical parameters for *C. cellulans* UVP1 and *B. pumilus* UVP4 to show maximum extracellular cellulolytic activity on CMC agar plates, as detailed in *Materials and methods*. The optimum temperature for cellulolytic activity was observed to be 32°C for both *C. cellulans* UVP1 and *B. pumilus* UVP4. However, *B. pumilus* UVP4 retained its cellulolytic activity with a marginal increase at 45°C with a larger zone index [33.75 mm ($P < 0.05$); Figs. 4A(i) and 4A(ii)], indicating that the enzyme produced by UVP4 is very thermostable and may hold thermostable cellulases.

The stability of cellulolytic activity in *C. cellulans* UVP1 and *B. pumilus* UVP4 was analyzed in acidic (pH 5), neutral (pH 7), and basic (pH 9) solutions [Figs. 4B(i) and 4B(ii)]. The zone indexes (27–30 mm, $P < 0.05$) revealed a broad range of enzymatic stability at pH 7 and 9 in both UVR-resistant isolates. Organism *B. pumilus* UVP4 was observed with slightly higher cellulolytic activity at pH 7 and 9, but both organisms showed enzymatic stability over a broader range [pH 5–9; Figs. 4B(i) and 4B(ii)]. The fact that optimal cellulase activity occurred at a neutral to basic pH (pH 7–9) in both UVP1 and UVP4 indicates their potential industrial applications in basic environmental conditions.

The concentration of CMC is one of the deciding factors that could regulate the substrate-based cost and yield of cellulase. Therefore, various concentrations (0.10%–2.0%) of CMC were tested to determine the effect on cellulase production. The larger cellulolytic zone indexes (32.8–34.5 mm) in both UVP1 and UVP4 showed that cellulose was easily degraded in the presence of low concentrations (0.1%) of CMC [Figs. 4C(i) and 4C(ii)]. The cellulolytic activity gradually decreased at 1% and 2% of CMC in agar plates. Thus, the organisms showed greater promise thriving on the smallest amount of cellulose.

The chemical parameters are among various detrimental factors to regulating microbial metabolism. Considering their roles in various regulatory pathways [17], it is unavoidable to optimize the required ionic balance of multiple inorganic salt types in growth medium. Various concentrations of different inorganic sources ($NaNO_3$, K_2HPO_4, KCl, and $MgSO_4$) were tested in individual sets of reactions with UVP1 and UVP4 (Figs. 5A–5D). The zone indexes (26.5–28.0 mm) of cellulolytic activity in *C. cellulans* UVP1 and *B. pumilus* UVP4 were largely unaffected at lower concentrations (0.01%, 0.05%, and 0.1%) of optimized inorganic salts [Figs. 5A(ii)–5D(ii)]. The organism *B. pumilus* UVP4 showed extreme stability in cellulolytic activity over a wide range of increasing concentrations (2.0%, 1.0%, and 1.5% of $NaNO_3$, K_2HPO_4, and KCl, respectively), compared with *C. cellulans* UVP1 (Figs. 5A–5C). The higher concentrations (0.5% and 1.5%) of $MgSO_4$ caused severe inactivation of cellulolytic activity in both UVP1 and UVP4, although cellulase activity in both UVP1 and UVP4 peaked in the presence of 0.01% $MgSO_4$ [Figs. 5D(i) and 5D(ii)].

4. Discussion

This report represents the first characterization of UVR-resistant extremophiles from higher-altitude microbial flora. Because UVR causes unsafe mutations that lead to skin cancer in humans [4],[18], it is vital to investigate organisms that thrive under extreme UVR to develop new therapies. Traditionally, random

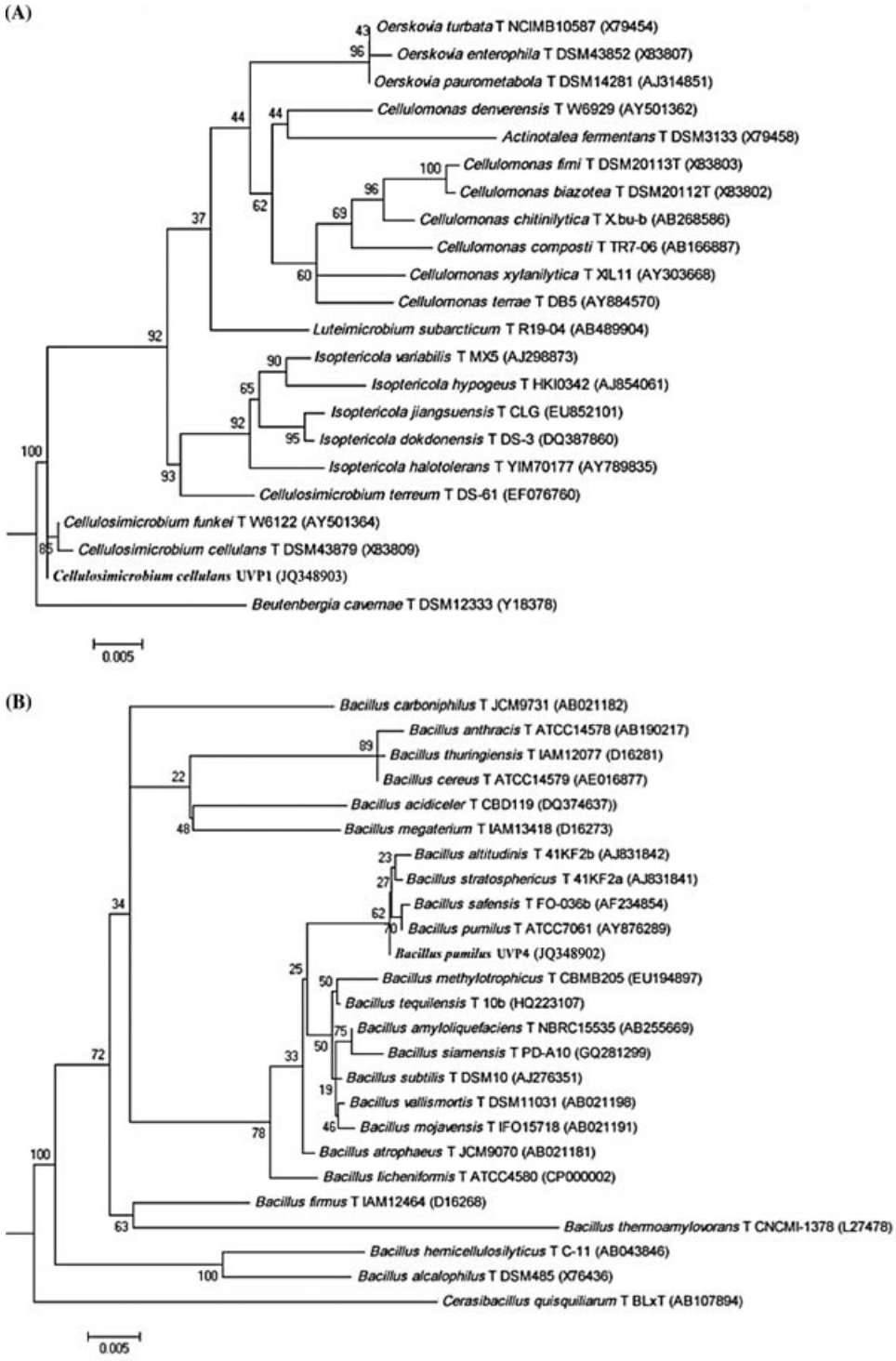

Fig. 2. 16S rRNA gene sequence-based neighbor-joining phylogenetic tree showing the relationship between UVR-resistant microorganisms UVP1 and UVP4 with related microbial strains. (A) The microorganism UVP1 revealed close proximity with genus *Cellulosimicrobium*. *Beutenbergia cavernae* T DSM12333 was used as the outgroup. Numbers at nodes indicate levels of bootstrap support based on neighbor-joining analysis of 1,000 resampled datasets. GenBank accession numbers are given in parentheses. Bar, 5 substitution for 1,000 nucleotide positions. (B) Isolate UVP4 showed close proximity with the genus *Bacillus*. *Cerasibacillus quisquiliarum* T BlxT was used as the outgroup. Numbers at nodes indicate levels of bootstrap support based on neighbor-joining analysis of 1,000 resampled datasets. GenBank accession numbers are given in parentheses. Bar, 5 substitution for 1,000 nucleotide positions.

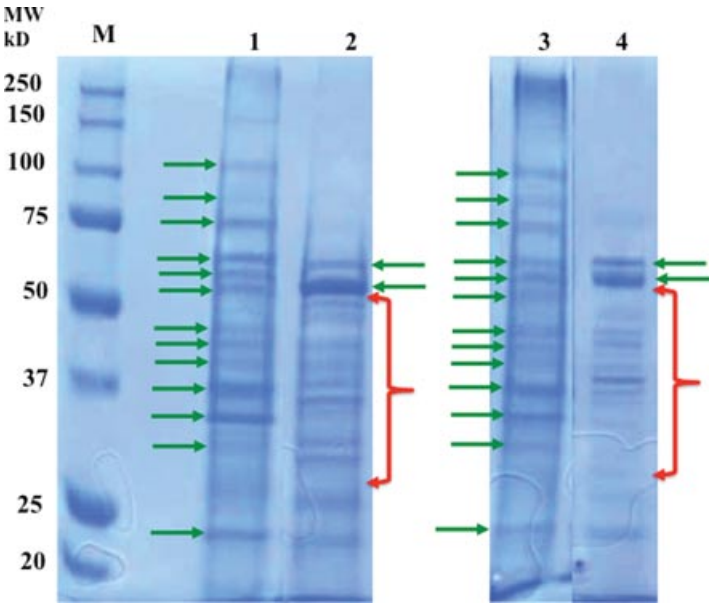

Fig. 3. Total protein profile of UV responsive protein profile from UVR extremophiles on 1D SDS-PAGE. The UVR-resistant microorganisms *C. cellulans* UVP1 and *B. pumilus* UVP4 were grown under UV irradiation 1.03×10^6 and 1.71×10^5 J/m^2, respectively. The total protein was extracted and resolved on 10% SDS-PAGE as detailed in *Materials and methods*. The total protein expression was visualized by Coomassie blue stain and imaged at higher resolution. The control sets of experiments were performed in parallel with *C. cellulans* UVP1 and *B. pumilus* UVP4 grown under non-UV condition at 37°C. (Lanes: 1, *C. cellulans* UVP1-UV irradiated; 2, *C. cellulans* UVP1-non-UV irradiated; 3, *B. pumilus* UVP4-UV irradiated; 4, *B. pumilus* UVP4-non-UV irradiated; green arrow indicates the protein bands expressed at similar molecular weights, red bracket shows the protein bands expressed at different molecular weights.)

mutagenesis has been performed using short exposure of UVR to alter the microbial properties. However, such alteration limits with potential consequences of microbial reversion after few generations. The prolonged exposure of UVR in certain microorganisms may develop stable change to their genome creating resistance by modifying their metabolic profile to thrive under a high-energy environment such as UVR. The UVR-resistant microorganisms have shown tremendous stable biotechnological implications [4, 14, 19]. Various extremophiles, especially thermophiles, have also gained momentum in the bioenergy sector for the production of thermostable cellulose-degrading enzymes [20]. These enzymes are being considered to reduce the cost of bioethanol production from biomass as a sustainable resource of biorefinery. Thus, the investigation of microbial flora thriving under extreme conditions such as UVR is necessary to move toward energy sustainability and novel therapeutic applications.

4.1. Isolation and identification of UVR-resistant extremophiles

Extreme environmental conditions such as high levels of UVR, low nutrients, and heavy metals are found at elevated land sites. UVR is one of the most limiting abiotic factors for microbial communities, and thus it is possible that microorganisms found at high altitudes will have UVR-resistant properties. Morphological and biochemical characterizations followed by 16S rRNA sequencing analysis revealed UVP1 and UVP4 to be of the species *C. cellulans* and *B. pumilus*, respectively [Table 1; Figs. 2(A) and 2(B)]. The growth observation of both isolates UVP1 and UVP4 revealed their tremendous ability to survive at higher dosages of UVR [Fig. 1(B)]. Several UVR-resistant microorganisms have been isolated in the past: Yuan et al. [21] isolated *Deinococcus radiodurans* that had the ability to withstand 1,000 J/m^2 UVR, and Link et al. [22] were able to isolate several strains of UV-resistant *B. pumilus* from a spacecraft assembly. However, the maximum dosage of UVR that the strains were able to withstand was 3,500 J/m^2 [22]. The microorganisms isolated in this report were able to thrive at 1.03×10^6 (*C. cellulans* UVP1) and 1.71×10^5 J/m^2 (*B. pumilus* UVP4) [Fig. 1(B)].

The genus *Cellulosimicrobium* was first characterized by reclassification of *Cellulomonas cellulans* to *Cellulosimicrobium cellulans* [23]. A characteristic feature of the members of this genus is cellulolytic activity. *C. cellulans* has been reported to be widely distributed in soil, clinical samples, and marine sponges [24, 25]. This organism has also been found to live in extreme environments of the Antarctic [26]. However, the current study is the first attempt at presenting and designating the strain *C. cellulans* UVP1 as a UVR extremophile.

B. pumilus is a Gram-positive, aerobic, spore-forming bacillus that is commonly found in soil. With the exception of the strain ATCC 7061, most *B. pumilus* strains have shown high resistance to UV radiation [22]. However, the resistance at 3.44×10^5 J/m^2 was not reported in earlier studies. Because of the presence of *B. pumilus* in soil, it has been known to encode for various types of xylanases, including β-1,4-endo- and exoglucanase [27]. This report presents the newly isolated *B. pumilus* UVP4 as capable of withstanding higher dosages of UVR; its ability to degrade cellulose was unaffected by physical and chemical environmental factors.

This study focuses on potential microorganisms that thrive under extreme UVR subtype C [Fig. 1(B)] and show stable cellulolytic activity across a wider range of environmental conditions (Figs. 4 and 5). It is known that UVC radiation (254 nm wavelength) is the most mutagenic part of the spectrum and leads to alterations of DNA bases, which result mostly in formation of dimeric photoproducts [18]. Our study suggests that the ability of these microorganisms to survive under high UVR dosages (1.03×10^6 and 1.71×10^5 J/m^2) may be due to the enzymatic mechanisms used for repairing DNA damage, as no pigmentation was observed during or after the UV exposure. Under UVR, the growth rates of both *C. cellulans* UVP1 and *B. pumilus* UVP4 were higher than that of the control *E. coli* [Fig. 1(B)].

The inability of *E. coli* to survive at high UVR levels is partly because of the fact that it lacks the metabolites,

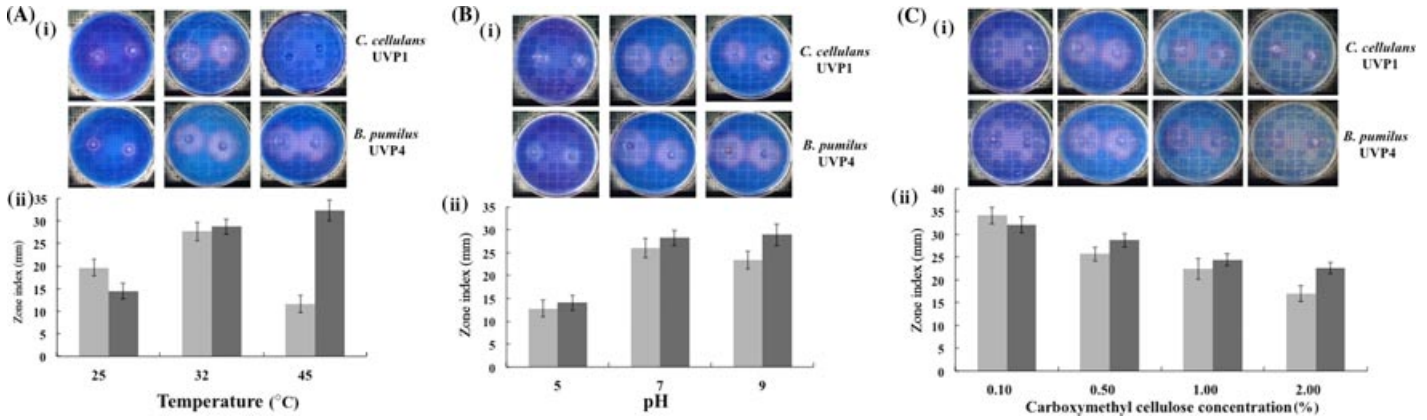

Fig. 4. The cellulolytic ability of UVR-resistant microorganisms *C. cellulans* UVP1 and *B. pumilus* UVP4. (A) The seed cultures of respective microorganism were prepared and loaded in agar plugs. After incubation at various temperatures (25, 32, and 45°C), the plates were flooded with congo-red indicator and clear zone was measured as detailed in *Materials and methods*. (B) UVR-resistant microorganisms *C. cellulans* UVP1 and *B. pumilus* UVP4 revealed cellulolytic activity at various pHs. (C) Carboxymethyl cellulose modulated cellulolytic activity in UVR-resistant microorganisms *C. cellulans* UVP1 and *B. pumilus* UVP4. (i) Congo-red flooded CMC agar plates followed by confirmation using 1 M HCl to stop the enzymatic activity. (ii) The calculated zone index at respective experimental values in *C. cellulans* UVP1 (grey bar) and *B. pumilus* UVP4 (dark bar).

proteins, and enzymes to repair DNA damage caused by UVR. The enzyme involved in *E. coli* DNA repair is photolyase, which is a class I cyclobutane pyrimidine dimer (CPD) and contains 471 of its amino acids, two cofactors, flea allergy dermatitis, and a folate derivative, 5,10-methenyltetrahydropterolypolyglutamate (MTHF) [28],[29]. The other mechanism by which *E. coli* repairs DNA damage is nucleotide excision repair (NER). It has been shown to repair 20 different types of chemically distinct DNA lesions [30]. The protein subunits in NER, UvrA, UvrB, and UvrC work in sequential steps to recognize a damage-containing DNA fragment. This fragment is released by UvrD helicase and the filling of the resultant gap is catalyzed by DNA polymerase I and DNA ligase. However, these mechanisms were not able to withstand the higher UVR (1.03×10^6 and 1.71×10^5 J/m^2) that *E. coli* was exposed to in this study.

4.2. Proteome of UVR extremophiles revealed by 1D gel electrophoresis

Because of the unavailability of a comprehensive report on the UVR-responsive global proteome, we attempted to investigate the global protein profile within the limits of 1D SDS-PAGE in the line of unique radiation-sensitive proteins that may have potential impact in radiation prone disease types, and in cellulose degradation responsive pathways. The current analysis relied upon the protein expression based on the respective molecular weights in the presence and absence of UVR. The total protein profiles of *C. cellulans* UVP1 and *B. pumilus* UVP4 under UVR revealed 13 unique protein bands expressed at similar molecular weight ranges (25–100 kDa) in Coomassie blue stained gel images, compared with those in the absence of UVR (Fig. 3). This suggests that the DNA repair genes and proteins unique to *B. pumilus* and *C. cellulans* allow the organisms to grow in the presence of high ionizing radiation. The *B. pumilus* genome is known to contain the *PhrB* gene, which encodes for a photolyase enzyme that repairs the CPD formed by high-energy UVR [31].

Several other species of the genus *Bacillus*, as well as *E. coli*, have homologs of the *PhrB* gene. However, *B. pumilus* only shares 32% of its amino acid identity with *E. coli PhrB* and 46% sequence identity with its closest homolog, *B. firmus* [31]. In addition to the *PhrB* gene, two other genes involved in DNA repair have been found in *B. pumilus*, including a gene sequence (BPUM_0608) similar to a Superfamily II helicase [32]. This mechanism has been implicated to function in repair pathways to correct the DNA damage induced by UVR. *B. pumilus* also encodes a C-5 cytosine-specific DNA methyltransferase [33]; no homolog of this gene is found in the other *Bacillus* species, and it may be involved in the UVR-induced DNA repair mechanism. In our studies, analysis of radiation-sensitive proteins from both *C. cellulans* UVP1 and *B. pumilus* UVP4 is under way to determine their protein- and enzyme-based defenses against UVR, which will eventually assist in our efforts to target the radiation-prone genes and proteins.

4.3. Implications of UVR extremophiles in bioenergy

The current challenge in biomass conversion by cellulases is finding an efficient and inexpensive process for the degradation of cellulose. To increase cellulase efficiency and lower the cost, cellulases need to be improved to produce higher catalytic efficiency on cellulose, greater stability at elevated temperatures, and nonphysiological pH including a higher tolerance to end-product inhibition [25],[34],[35]. UVR-resistant microorganisms may also have the potential to thrive in the environment near nuclear power plants [36]. Other radiation-resistant microorganisms in the genus *Deinococcus* have been well studied [21],[37], but their biotechnological importance has yet to be discovered. Both *C. cellulans*

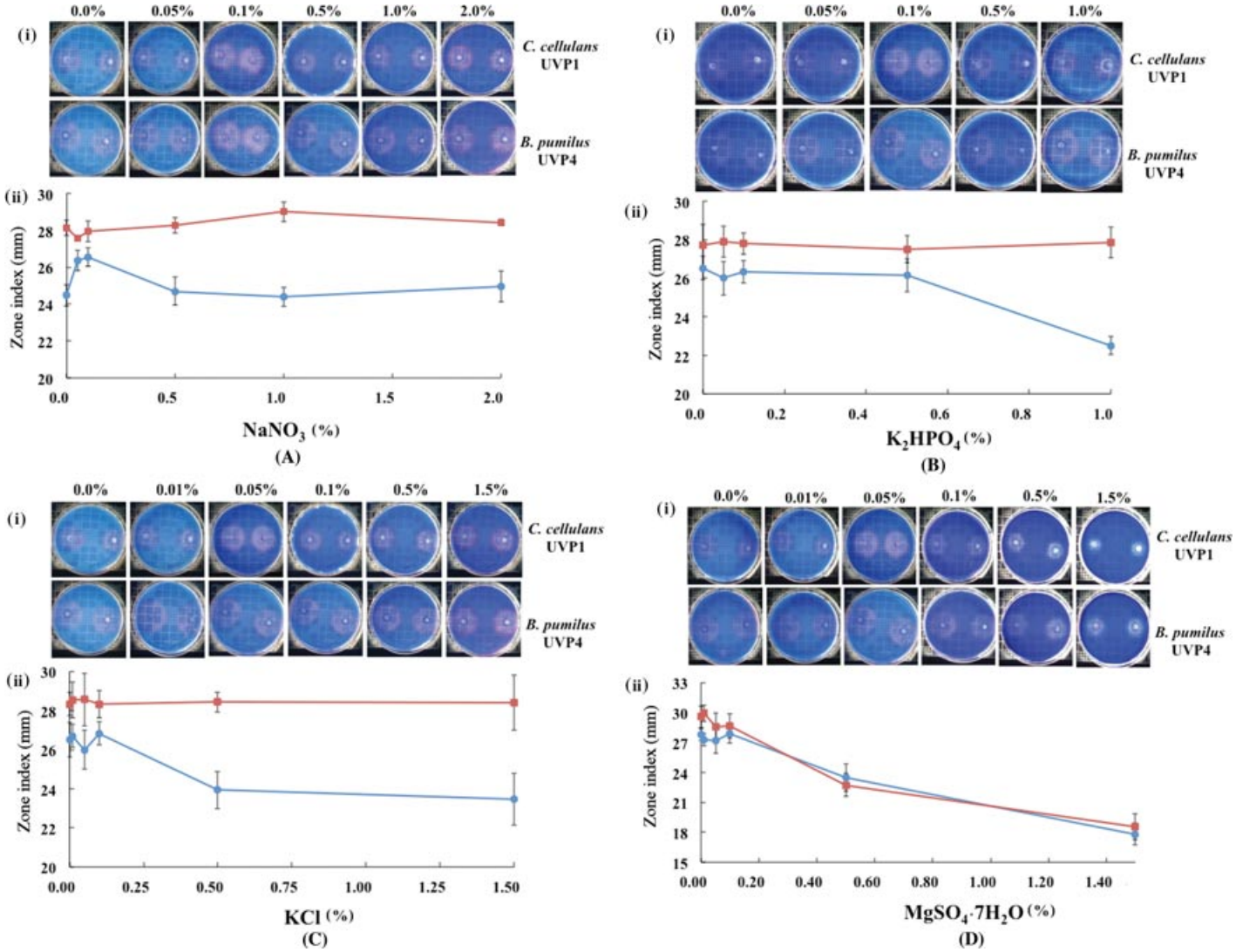

Fig. 5. (A–D) Stability of cellulolytic activity at various concentrations of inorganic salts in UVR-resistant microorganisms *C. cellulans* UVP1 and *B. pumilus* UVP4. The seed cultures of respective microorganisms were prepared and loaded in agar plugs on CMC medium supplemented with varying inorganic salts, (A) $NaNO_3$, (B) K_2HPO_4, (C) KCl, and (D) $MgSO_4 \cdot 7H_2O$. After incubation at 32°C, the plates were flooded with congo-red indicator and clear zone was measured as detailed in *Materials and methods*. (i) Congo-red flooded CMC agar plates followed by confirmation using 1 M HCl to stop the enzymatic activity. (ii) The calculated zone index at respective experimental values in *C. cellulans UVP*1 (circles) and *B. pumilus* UVP4 (squares).

UVP1 and *B. pumilus* UVP4 in the current study could thrive at higher UVR and revealed stable cellulolytic activity under broader physical and chemical conditions. CMC was used as a medium to reveal bioenergy and biotechnology implication, as it resembles cellulose and lignocelluloses, the most widely available material.

Strain *B. pumilus* UVP4 showed efficient cellulolytic activity at a higher temperature (45°C) with a greater zone index [Figs. 4A(i) and 4A(ii)] and thus is both radiation and temperature resistant. Thermophilic cellulases are the key for efficient biomass degradation. Their importance arises from the fact that cellulose swells at high temperatures, increasing the surface area and allowing the enzymes easier access for degradation.

Both *C. cellulans* UVP1 and *B. pumilus* UVP4 showed cellulolytic activity in acidic (pH 5) and basic (pH 9) media. However, the cellulolytic activity in *B. pumilus* UVP4 was most efficient at pH 9, revealing that the strain is most active under basic conditions [Figs. 4B(i) and 4B(ii)]. In agreement, Duarte et al. [38] showed that xylanases produced by four different strains of *B. pumilus* were most active at pH 9, and Kim et al. [39] characterized alkaline cellulase from alkalophilic *Bacillus* sp. HSH-810.

Further, UVP4 was unaffected even at higher concentrations of $NaNO_3$ (2.0%), K_2HPO_4 (1.0%), and KCl (1.5%), which were inhibitory to *C. cellulans* UVP1 (Figs. 5A–5C). In a study by Kachlishvili et al. [40], the effects of nitrogen sources on the production of cellulase varied depending on the

microorganism and the product to be tested. These results were in accordance with previous reports indicating optimal enzyme activity in the presence of inorganic nitrogen sources [26]. The purified thermophilic fungal cellulases have been characterized in terms of their optimal pH, temperature, thermostability, and glycosylation [41].

Purified thermophilic fungal cellulases have been characterized in terms of their optimal pH, temperature, thermostability, and glycosylation [41]. However, there is a lack of research into bacterial cellulases that are stable under broader environmental conditions. Here, we attempted to fill this gap by characterizing the thermostability of strains *B. pumilus* UVP1 and *C. cellulans* UVP4 and the pH ranges at which they can function. It is of crucial importance that we find thermostable cellulases from bacterial sources, as most of the currently used industrial enzymes are of fungal origin and only function below 50°C. Both *C. cellulans* UVP1 and *B. pumilus* UVP4 have potential to be utilized in the bioenergy sector toward efficient bioprocess development. In addition, the strain *B. pumilus* UVP4 can be categorized in the novel group of "polyextremophiles" [1]. Further studies are in progress to examine the UVR defense mechanisms in the *C. cellulans* UVP1 and *B. pumilus* UVP4 isolates, which will extend our understanding along new avenues, including space and defense science exploration.

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