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Isolation and Characterization of Antioxidant-Producing Thermophilic Bacteria from Tatapani Hot Spring, Chhattisgarh, India

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Abstract

Background: Oxidative stress, caused by an imbalance between reactive oxygen species (ROS) and antioxidant defences, is implicated in numerous degenerative diseases. The search for natural antioxidants has led to the exploration of thermophilic bacteria, known for thriving in extreme environments and potentially producing potent antioxidant compounds. **Objective:** The purpose of this study was to isolate and characterize thermophilic bacteria collected at Tatapani Hot Spring and evaluate their antioxidant potential. **Methods:** Water samples were collected from Tatapani Hot Spring and subjected to bacterial isolation using the Spread Plate and Enrichment Culture Method. Isolated strains were characterized morphologically, biochemically, and molecularly via 16S rRNA gene sequencing. Carotenoids and quinones were extracted from the fermentation medium and analyzed for antioxidant activity using UV-Visible spectroscopy. The antioxidant potential of the extracts was assessed through various assays, including DPPH radical scavenging, hydroxyl radical scavenging, nitric oxide radical scavenging, and Ferric ion reducing antioxidant power (FRAP) assays. **Results:** Two thermophilic bacterial strains, referred to as TR1 and TR2, were successfully isolated. Both strains are Gram-positive, endospore-forming rods. Strain TR1 exhibited 98.70% sequence similarity to *Bacillus nakamurai*, while strain TR2 showed 99.65% similarity to *Bacillus licheniformis*. Extracts from these strains revealed significant antioxidant activities, with ethanol extracts demonstrating superior efficacy. Specifically, the ethanol-dissolved extracts exhibited DPPH radical scavenging activity of approximately 80%, hydroxyl radical scavenging activity up to 95.5%, and nitric oxide radical scavenging activity around 87.4%. The FRAP assay confirmed the strong reducing power of the extracts. **Conclusion:** The thermophiles collected at Tatapani Hot Spring produce potent antioxidant compounds, primarily carotenoids and quinones, with substantial scavenging activities. These findings highlight the potential of these bacteria as sources of natural antioxidants for applications in

pharmaceuticals, nutraceuticals, and cosmetics. Future research should focus on optimizing the extraction processes and further characterizing these bioactive compounds to fully exploit their therapeutic potential.

Keywords: Thermophilic bacteria; Antioxidant activity; *Bacillus* species; Bioactive compounds

1 Introduction

Antioxidants have a key role in neutralizing oxidative stress, which occurs when there is an imbalance between reactive oxygen species (ROS) and the body's ability to detoxify these reactive intermediates or repair the resulting damage. ROS, including free radicals like superoxide anion and hydroxyl radical, as well as non-radical molecules like hydrogen peroxide, are highly reactive and can damage cellular components such as DNA, proteins, and lipids. Oxidative damage plays a key role in the development of numerous degenerative diseases, including cancer, cardiovascular diseases, diabetes, and neurodegenerative disorders like Alzheimer's and Parkinson's disease.[1] Additionally, oxidative stress accelerates the aging process and contributes to chronic inflammation, further exacerbating health issues. Antioxidants counteract ROS by donating electrons or hydrogen atoms to neutralize these reactive species, thus preventing cellular damage and maintaining redox homeostasis. Natural bacterial sources of antioxidants have emerged as significant contributors to the body's antioxidant defense mechanisms, providing a promising alternative to synthetic antioxidants.[2]

Thermophilic bacteria, thriving in the extreme conditions of Tatapani Hot Spring in Chhattisgarh, India, have garnered significant scientific interest due to their unique metabolic processes and potential applications, particularly their antioxidant activity. These bacteria, adapted to survive at high temperatures, typically between 45°C and 80°C, exhibit remarkable stability and robustness. The extreme environment of Tatapani Hot Spring has led to the evolution of resilient bacterial species that produce powerful antioxidant compounds to survive and thrive. The high-temperature environment challenges these bacteria to maintain cellular integrity and function, prompting the production of highly effective antioxidant metabolites.

These secondary metabolites include an array of bioactive compounds, with carotenoids and quinones distinguished by their potent antioxidant properties. Carotenoids are known for their ability to quench singlet oxygen, a highly reactive form of oxygen. This ability to stabilize free radicals by accepting or donating electrons helps prevent lipid peroxidation and protects cellular membranes from oxidative damage. Carotenoids also enhance antioxidant defence by interacting synergistically with other antioxidants, such as vitamin E, regenerating their active forms and amplifying their protective effects. Additionally, carotenoids can modulate gene expression related to oxidative stress response, further contributing to cellular protection. The robust antioxidant activity of carotenoids is attributed to their conjugated double-bond structures, which allow them to effectively interact with and neutralize ROS.

Quinones, another significant group of antioxidant compounds produced by these thermophilic bacteria, play a crucial role by undergoing redox cycling. This process allows quinones to alternate between oxidized and reduced states, thereby neutralizing ROS and maintaining cellular redox homeostasis. Quinones also act as electron carriers in the electron transport chain, facilitating energy production while mitigating oxidative stress. Beyond their direct antioxidant effects, quinones can influence various cellular signaling pathways involved in stress

responses and apoptosis, thereby enhancing the cell's overall antioxidant capacity and resilience to oxidative damage.[3] The diverse structural configurations of quinones contribute to their ability to participate in a diverse array of redox reactions, providing versatile protection against oxidative stress.[4]

Investigating the antioxidant potential in thermophilic bacteria isolated from Tatapani Hot Spring not only enhances our understanding of their survival mechanisms but also opens avenues for discovering new therapeutic agents. These naturally occurring antioxidants from thermophilic bacteria offer a sustainable and effective alternative to synthetic antioxidants in the pharmaceutical, nutraceutical, and cosmetic industries. The extraction and characterization of these metabolites could lead to the development of novel antioxidant formulations, targeting specific oxidative stress-related conditions. Thus, studying the secondary metabolites of these bacteria is crucial for bioprospecting novel antioxidants, potentially leading to significant advancements in health sciences and biotechnological innovations.[5]

2 Material and Methods

2.1 Chemicals and Reagents

All solvents, chemicals, and media used in this research were of analytical grade, obtained from Sigma-Aldrich. Pure bacterial cultures were sub-cultured on nutrient agar slants to ensure fresh cultures were available the day before each experimental procedure.

2.2 Isolation of Thermophilic Bacteria from Collected Water Samples

Twelve water samples were collected from Tatapani hot spring in Chhattisgarh using 200 mL sterile thermal glass containers. Located in the small village of Tatapani, Sarguja district (23.8275° N, 83.2977° E), samples were taken in triplicate from the same spot at depths of 10, 20, 30, and 40 cm. They were refrigerated at 4°C to maintain their physico-chemical and biological properties. Temperature and conductivity were measured with a thermometer and conductivity meter, respectively. The samples exhibited temperatures ranging from 47–62°C, pH levels between 7.1–7.6, and electrical conductivity of 1240–1300 μS/cm.

The Spread Plate and Enrichment Culture Method was employed for bacterial isolation using Nutrient Agar medium (NAM) adjusted to pH 6.8, containing 10 g peptone, 5 g yeast extract, 5 g sodium chloride, and 15 g agar per litre. Plates were inoculated and then incubated at temperatures ranging from 50°C to 80°C for 18 hours to enable the growth and isolation of thermophilic bacteria. Post-incubation, distinct bacterial colonies were identified and subsequently sub-cultured onto fresh NAM plates to ensure pure cultures. These isolated strains were preserved and maintained under refrigeration in the laboratory.[6]

2.3 Comprehensive Characterization of Bacterial Isolates: Colony Morphology, Biochemical Properties, and Growth Conditions

The bacterial isolates were analysed using a Zeiss compound microscope (AXIO SCOPE.A1 HBO 50, Zeiss, Germany) for Gram staining, Endospore staining, and colony morphology assessment. After incubation for 48 hours on selective medium maintained at 55°C, colony characteristics including color, size, shape, elevation, margin appearance, and surface texture were evaluated**.** Biochemical characterization involved conducting starch hydrolysis, Voges-Proskauer reaction, catalase activity, carbohydrate utilization (glucose, lactose, sucrose), indole production, methyl-red test, urease activity, citrate utilization, oxidase test, and nitrate reduction assays.[7] To assess the growth temperature range, the bacterial cultures were incubated at 0°C, 10°C, 37°C, 45°C, 55°C, 60°C, 65°C, 70°C, and 80°C. The pH tolerance was assessed by inoculating the strains in media adjusted to pH levels of 2, 4, 6, 7, 10, and 13. To evaluate salt tolerance, the isolates were cultured in media containing various concentrations of sodium chloride (0.1%, 0.2%, 0.4%, 0.5%, 1%, 2%, and 5%). These assays provided comprehensive insights into metabolic capabilities crucial for assessing therapeutic potential, while growth conditions were rigorously controlled to ensure accurate characterization.

2.4 Molecular Characterization of Bacterial Isolates using 16S rRNA Gene Sequencing

Chromosomal DNA was extracted using a spin column kit (e.g., HiMedia, India) following the manufacturer's specifications. The 16S rRNA gene was PCR-amplified using 704F (690 bp) and 907R (861bp) primer.[8] The PCR products were purified using Exonuclease I - Shrimp Alkaline Phosphatase (Exo-SAP) enzymatic treatment[9] and sequenced using the Sanger method on an ABI 3500xL genetic analyzer (Life Technologies, USA). The resulting .ab1 files were edited with CHROMASLITE (version 1.5) for accurate base calling. Sequence analysis was conducted using BLAST against the NCBI database to identify the closest matches, and the bacteria were assigned unique GenBank Accession numbers. A phylogenetic tree was constructed using Mega 6 software, comparing the bacterial sequences to 32 similar ones using the Maximum Likelihood approach and Kimura 2-parameter model to elucidate their evolutionary relationships.

2.5 Deposition of 16S rRNA Gene Sequences to the GenBank Database

The bacterial strains' 16S rRNA gene sequences were deposited in the GenBank database using the Blankit program provided by NCBI. Accession numbers for the strains were obtained through the submission process facilitated by NCBI.

2.6 Multi-Step Extraction and Characterization of Bioactive Compounds from Fermentation Medium

The extraction of bioactive compounds involves producing the active component in an optimized fermentation medium, followed by incubation and centrifugation to collect the supernatant.[10] Deproteinization is achieved using perchloric acid and subsequent neutralization with KOH.[11] The broth undergoes gel adsorption with activated silica gel and successive solvent extraction. The extracts are analyzed using UV-Vis spectrophotometry, providing detailed insights into their chemical structures and properties.[12] This multi-step process ensures thorough separation, identification, and characterization of bioactive compounds.[13]

2.7 Antioxidant Assays

Isolates from Nutrient Agar Medium (NAM) were first cultured in 15 ml of Nutrient Broth and incubated at 55°C for 24 hours.[14] This pre-culture was then used to inoculate 50 ml of the same culture medium in 100 ml conical flasks.[15] The flasks were incubated at 55°C for 36-48 hours with intermittent shaking to promote microbial growth and compound production. After incubation, the mixture was filtered to separate the precipitate from the supernatant, which was then analyzed for bioactive compounds.[16]

2.7.1 ***DPPH Assay***

The DPPH radical scavenging assay was performed with modifications.[17] DPPH solution was prepared by dissolving 2 mg of DPPH in 54 ml of methanol. Control solutions contained 3 ml of this DPPH solution, while the blank solution was made by adding 300 μl of distilled water to 2700 μl of 100% methanol. Sample solutions were formulated by mixing 300 μl of the supernatant with 2700 μl of the DPPH solution. Both control and sample solutions were incubated in the dark at room temperature for a duration of 30 minutes before measuring absorbance at 517 nm. The results were presented as ascorbic acid equivalents, calculated using the formula: Δ Sample = A0 of sample – A30 of sample and Δ Standard = A_0 of ascorbic acid – A_{30} of ascorbic acid. DPPH radical inhibition percentage was graphed against the sample concentration to assess antioxidant activity. The IC50 values were calculated, indicating the concentration required to inhibit 50% of DPPH radical activity.

2.7.2 ***Hydroxyl Radical Scavenging assay***

The hydroxyl radical antioxidant assay was conducted as follows. A 1 mM stock solution of salicylic acid, the probe molecule, was formulated in phosphate buffer (pH 7.4).[18] Hydroxyl radicals were generated by adding 1 mL of 1 mM $FeSO_4$ (iron (II) sulfate) with 1 mL of 1 mM hydrogen peroxide (H_2O_2).[19] Test samples and antioxidant standards were diluted to concentrations of 0.1, 0.5, 1.0, 5.0, and 10.0 mg/mL using methanol**.** In each reaction tube, 500 μL of the test sample was combined with 500 μL of the probe solution and 500 μL of the hydroxyl radical generation mixture. The tubes were incubated at 37°C for 30 minutes. After incubation, the absorbance was recorded at 517 nm using a spectrophotometer.[20] The percentage inhibition of hydroxyl radical generation was assessed using the formula:

% Inhibition = [(Absorbance of Control − Absorbance of Sample) / Absorbance of Control] × 100.

A standard curve was plotted to assess the antioxidant activity of the test samples, and IC50 values were determined as needed.

2.7.3 ***Nitric oxide radical scavenging assay***

The nitric oxide scavenging antioxidant assay was conducted with the following specifications. A 10 mM sodium nitroprusside solution was prepared by dissolving sodium nitroprusside in phosphate-buffered saline (PBS) at pH 7.4.[21] This solution was exposed to light to generate nitric oxide radicals. Test substances were diluted in PBS to various concentrations (e.g., 0.1, 0.5, 1.0, 5.0, and 10.0 mg/mL) and combined with an equal volume of the sodium nitroprusside solution. The mixtures were incubated at room temperature for 2 hours and 30 minutes. Following incubation, 500 μL of each reaction mixture was combined with 500 μL of Griess reagent, consisting of 1% sulfanilamide and 0.1% naphthyl ethylenediamine dihydrochloride in 2% phosphoric acid. The reaction was allowed to proceed for 10 minutes at room temperature, forming a pink-colored azo dye. Absorbance was recorded at 540 nm using a spectrophotometer.[22] The percentage inhibition of nitric oxide formation was calculated using the formula:

Percentage inhibition = [(A_control − A_sample) / A_control] × 100.

2.7.4 ***Ferric reducing antioxidant power) assay***

The Ferric Reducing Antioxidant Power (FRAP) assay was conducted to evaluate the antioxidant capacity of samples by measuring their capacity to reduce ferric ions. A 300 mM acetate buffer was made by dissolving 3.1 g of sodium acetate trihydrate in 900 mL of distilled water, adding 16 mL of glacial acetic acid, and adjusting the volume to 1 litre**.** A 10 mM TPTZ solution was made by dissolving TPTZ

in 40 mM HCl, and a 20 mM ferric chloride solution was prepared by dissolving ferric chloride in distilled water. The FRAP working reagent was freshly prepared by mixing the acetate buffer, TPTZ solution, and ferric chloride solution in a 10:1:1 ratio.[23] Standard solutions of Trolox were prepared with concentrations ranging from 3.90 μg/mL to 500 μg/mL. The assay was carried out by pre-warming the FRAP working solution to 37°C and adding 300 μL of each standard or sample solution to a 96-well microplate, followed by 2700 μL of FRAP reagent. The plate was incubated at 37°C for 30 minutes in the dark, after which the absorbance of the ferrous tripyridyltrazine complex was measured at 593 nm using a spectrophotometer. Absorbance values were plotted against standard concentrations to generate a standard curve, and the antioxidant capacity of the samples was assessed as Trolox Equivalent Antioxidant Capacity (TEAC)

2.7.5 ***Phosphomolybdenum assay for Total Antioxidant Capacity***

The phosphomolybdenum assay was employed to quantify antioxidant activity by measuring the reduction of molybdate (Mo (VI)) to molybdenum blue (Mo (V)) under acidic conditions.[24] Sulfuric acid, ammonium molybdate, sodium phosphate, and ascorbic acid were utilized. A standard solution of ascorbic acid (1 mg/mL) was prepared and serially diluted to 500, 250, 125, 62.5, 31.25, and 15.62 μg/mL. Sample solutions were dissolved in ethyl acetate or acetone to a concentration of 1 mg/mL and further diluted as necessary. The reagent solution consisted of 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. For the assay, 300 μL of each sample solution was mixed with 1 mL of the reagent solution, incubated at 95°C for 90 minutes, then cooled to room temperature. Absorbance was measured at 695 nm. A blank containing the reagent solution and solvent was used to zero the spectrophotometer and standard curve of ascorbic acid was utilized to determine the antioxidant capacity.[25]

2.8 Statistical analysis

All experiments were conducted in triplicate to ensure reliability, with the mean and standard deviation of the data calculated to quantify variability. One-way ANOVA was applied to determine whether the choice of solvent significantly affected the outcomes of the antioxidant assays. Statistical analyses, including these computations and the generation of relevant plots, were performed using Microsoft Excel, ensuring comprehensive data evaluation and interpretation.

3 Results

3.1 Isolation and Characterization of Thermophilic Bacteria: Morphology, Biochemistry, and Growth Conditions

Two thermophilic bacterial strains, termed as TR1 and TR2, were isolated from water samples of the Tatapani hot spring according to their thermostability. These isolates formed smooth, white, slimy, moist, rod-shaped colonies with undulate margins. Gram staining revealed that both strains are Gram-positive, non-motile rods. Both strains, TR1 and TR2, exhibited positive results for endospore staining, confirming their ability to form endospores, which are indicative of their resilience and survival capabilities under harsh environmental conditions. The dimensions of strain TR1 range from 2 to 6 μm in length and less than 1 μm in diameter, while strain TR2 measures 3 to 6 μm in length and 0.5 to 0.8 μm in diameter. Both strains are obligate thermophiles, unable to grow below 50°C. Strain TR1 can grow at temperatures up to 85°C, with optimal growth observed at 60°C, while strain TR2 can grow at temperatures up to 95°C, with optimal growth at 65°C.Strain TR1 exhibits growth within a pH range of 5 to 9, with optimal growth at pH 7. Conversely, strain TR2 demonstrates a broader pH tolerance, growing within a range of pH 3 to pH 12, with optimal growth at pH 6. Both strains can tolerate NaCl concentrations up to 0.5%. Strain TR1 shows optimal growth at 0.1% NaCl, whereas strain TR2 experiences inhibited growth at 0.2% NaCl. Growth of strain TR1 declines at 0.3% NaCl concentration. The results of various morphological and biochemical tests performed on the strains are detailed in Tables 1 and 2. These tests include assessments of carbohydrate fermentation, enzyme activity, and other metabolic capabilities, providing a comprehensive profile of the biochemical and physiological characteristics of TR1 and TR2.

3.2 Genomic DNA Isolation, 16S rDNA Sequencing, and Phylogenetic Analysis of the Isolates

Genomic DNA and 16S rDNA amplicons appeared as sharp bands on agarose gel. Sequencing revealed 690 and 862 nucleotide-long contigs of the 16S rRNA genes for strains TR1 and TR2, respectively. Strain TR1 exhibited 98.70% sequence similarity to *Bacillus nakamurai*, and strain TR2 showed 99.65% similarity to *Bacillus licheniformis*. The sequences were submitted to GenBank, NCBI, with *Bacillus nakamurai* strain RABN1 and *Bacillus licheniformis* strain TWR2 assigned accession numbers PQ044631 and PQ056784, respectively. A phylogenetic tree was constructed using the Neighbor-Joining method with 1000 bootstrap replicates, and evolutionary distances were computed using

Table 1. Morphological and Cultural Characteristics of Bacterial Strains

Morphological characteristic	*Bacillus nakamurai* (TR1)	*Bacillus licheniformis* (TR2)	Interpretation
Gram Staining	Positive	Positive	Both are Gram-positive bacteria (retain crystal violet stain).
Cell Shape	Rod-shaped	Rod-shaped	Both are rod-shaped (bacilli).
Cell Arrangement	Single or in short chains	Single or in short chains	Both cells are arranged singly or in short chains.
Endospore Formation	Positive	Positive	Both form endospores.
Motility	Positive	Positive	Both are motile.
Colony Morphology	Circular, flat, with irregular edges	Circular, flat, rough, with irregular edges	Both have circular, flat colonies with irregular edges.
Colony Colour	White to cream-colored	White to cream-colored	Both have white to cream-colored colonies.
Optimum Temperature for Growth	50-60°C	50-60°C	Both are thermophiles, growing optimally at 50-60°C.
Colony Elevation	Flat to slightly raised	Flat to slightly raised	Both have flat to slightly raised colonies.
Colony Surface	Smooth to rough	Rough	*Bacillus nakamurai* has smooth to rough colonies; *Bacillus licheniformis* has rough colonies.
Colony Margin	Undulate	Undulate	Both have undulate colony margins.
Spore Position	Central to subterminal	Central to subterminal	Both have centrally to subterminal positioned spores.
Spore Shape	Ellipsoidal	Ellipsoidal	Both have ellipsoidal-shaped spores.
Growth in Anaerobic Conditions	Facultative anaerobe	Facultative anaerobe	Both can grow in the absence of oxygen, indicating facultative anaerobic capabilities.
Morphology in Broth Culture	Uniform turbidity	Uniform turbidity	Both show uniform turbidity in broth culture.
Oxygen Requirement	Facultative anaerobe	Facultative anaerobe	Both can grow in both the presence and absence of oxygen.

the Maximum Composite Likelihood method.

The analysis included 29 nucleotide sequences and 3050 positions, conducted in MEGA11 (Figure 1).

3.3 Extraction and Spectral Analysis of Carotenoids and Quinones

Carotenoids and quinones from the strains were efficiently extracted using ethyl acetate, acetone, and ethanol. The resulting extracts were designated as TR1E7A3, TR1Et10, TR1E5A5, TR2E2A8, TR2E8A2 and TR2Et10, corresponding to the solvents used and the solubility of the compounds. Spectral analysis of the carotenoid extracts revealed absorption maxima for TR1E7A3, TR1E5A5 and TR2Et10 at 470 nm, 473 nm and 478 nm in the visible range. In contrast, the quinone extracts TR1Et10, TR2E8A2 and TR2E2A8 showed absorption maxima at 265 nm, 273 nm and 288 nm, respectively (Figure 2).

3.4 DPPH assay, hydroxyl radical assay, nitric oxide assay and FRAP assay

All of the extracts showed positive result in DPPH radical scavenging assay, hydroxyl radical scavenging assay, nitric oxide radical scavenging assay, Ferric reducing antioxidant power assay and the percentage inhibition property increased with increasing concentrations of extract. Amongst all the samples, carotenoids dissolved in ethyl acetate and acetone showed greater scavenging activity than ethanol. While in case of quinone extracts ethanol extracts activity was higher in comparison to ethyl acetate and acetone extracts. Scavenging activity of 1mg extracts of TR1E5A5 and TR1E7A3 showed similar DPPH scavenging activity of 90.38 $\pm$0.64, and 89.25$\pm$0.53% and TR1Et10 of 83.40$\pm$0.53% respectively, while that of TR2E8A2 and TR2E2A8 showed slightly lower activity of 82.44 $\pm$0.64, 80.33 $\pm$0.64 and TR2Et10 of 93.30$\pm$0.53%. The hydroxyl radical scavenging activity was 78.22$\pm$0.2% in case of TR1Et10, 79.66$\pm$1.2% in case of TR2Et10, 70.44$\pm$0.2% in case of TR1E7A3, 74.32$\pm$1.2% in case of TR1E5A5, 70.24$\pm$1.2% in case of TR2E8A2 and 68.54$\pm$1% in case of TR2E2A8. Ethanol-dissolved TR2Et10 and TR1Et10 extract showed a high amount of

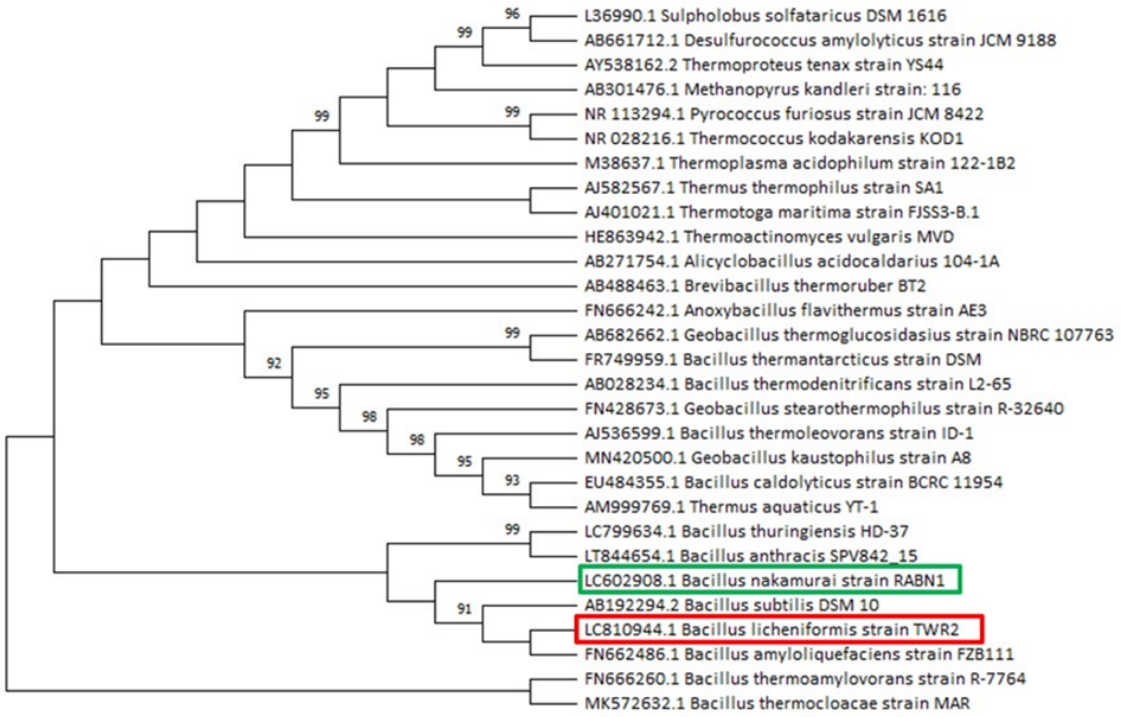

Fig 1. Phylogenetic Analysis

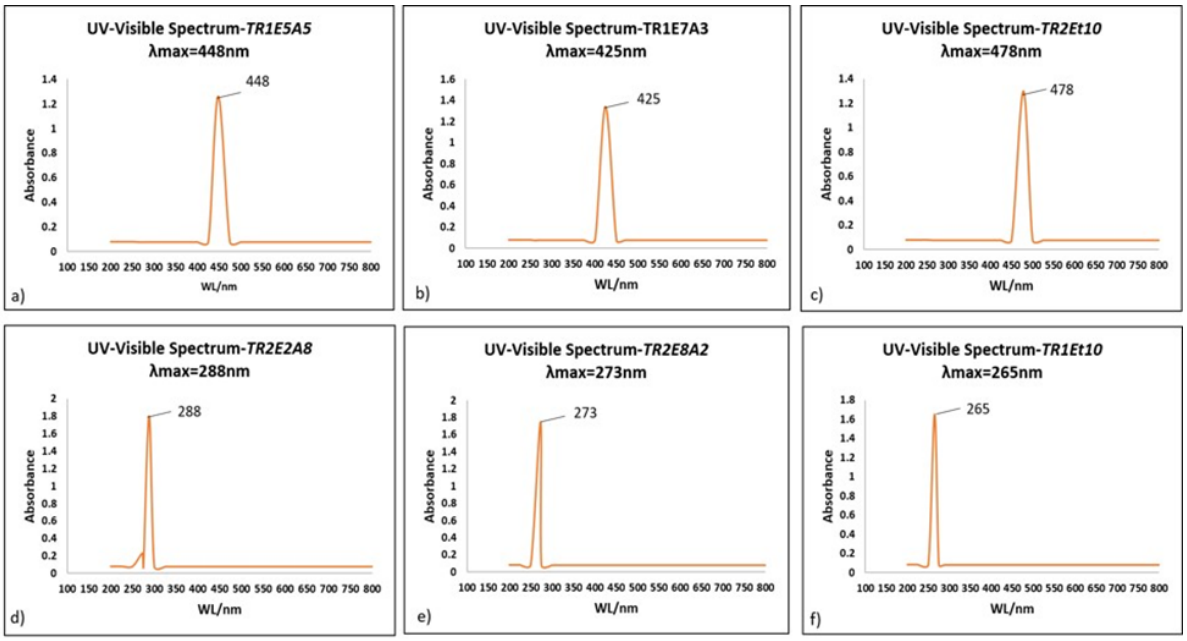

Fig 2. Spectral Analysis of Carotenoids and Quinones

Table 2. Biochemical Characterisation of Isolated Bacterial Strains

Biochemical Test	*Bacillus nakamurai* (TR1)	*Bacillus licheniformis* (TR2)	Interpretation
Urease Test	Negative	Positive	*Bacillus nakamurai* does not produce urease; *Bacillus licheniformis* does.
Catalase Test	Positive	Positive	Both produce catalase enzyme, which breaks down hydrogen peroxide.
Casein Hydrolysis Test	Positive	Negative	*Bacillus nakamurai* hydrolyzes casein (produces clear zone around growth); *Bacillus licheniformis* does not.
Citrate Utilization Test	Positive	Positive	Both utilize citrate as the sole carbon source, turning the medium blue.
Gelatin Hydrolysis Test	Positive	Negative	*Bacillus nakamurai* hydrolyzes gelatin (medium becomes liquid); *Bacillus licheniformis* does not.
Triple Sugar Iron (TSI)	A/A or K/A (H2S)	K/A	*Bacillus nakamurai*: Ferments glucose, may produce H_2S; *Bacillus licheniformis*: Ferments glucose, does not produce H_2S
Methyl Red Test	Positive	Positive	Both perform mixed acid fermentation (medium turns red)
Voges-Proskauer Test	Positive	Positive	Both produce acetoin
Starch Hydrolysis	Positive	Negative	*Bacillus nakamurai* hydrolyze starch (clear zone around growth on starch agar), while *Bacillus licheniformis* does not.
Indole Test	Positive	Positive	Both produce indole (medium turns red or pink)
Mannitol Salt Agar (MSA) Test	Negative	Positive	*Bacillus nakamurai* do not ferment mannitol on MSA while *Bacillus licheniformis* does
Xylose Fermentation Test	Positive	Negative	*Bacillus nakamurai* ferments xylose (medium turns yellow); *Bacillus licheniformis* does not.
Malonate Utilization Test	Positive	Negative	*Bacillus nakamurai* utilizes malonate (medium turns blue); *Bacillus licheniformis* does not.
Lactose Fermentation Test	Negative	Positive	*Bacillus nakamurai* does not ferment lactose; *Bacillus licheniformis* does.
Arabinose Fermentation Test	Positive	Negative	*Bacillus nakamurai* ferments arabinose (medium turns yellow); *Bacillus licheniformis* does not
Rhamnose Fermentation Test	Negative	Positive	*Bacillus nakamurai* does not ferment rhamnose; *Bacillus licheniformis* does.
Sorbitol Fermentation Test	Positive	Negative	*Bacillus nakamurai* ferments sorbitol (medium turns yellow); *Bacillus licheniformis* does not.
Sucrose Fermentation Test	Negative	Positive	*Bacillus nakamurai* does not ferment sucrose; *Bacillus licheniformis* does.

nitric oxide radical scavenging activity of 87.44±1.15% and 88.22±1.15% while that of TR2E2A8, TR1E5A5, TR2E8A2 and TR1E7A3 as 77.34±1.15%, 75.55±1.15%, 79.66±1.15% and 72.84±1.15%.

Ferric reducing antioxidant power activity was 84.38±1.15% of TR1Et10, 85.68±1.2% of TR2Et10, 70.44±1.2% of TR1E5A5, 75.05±1.2% of TR2E8A2, 74.23±1% of TR1E7A3 and 76.88±0.2% of TR2E2A8. Details of the result are given in Figure 3.

3.5 Phosphomolybdenum assay for Total Antioxidant Capacity

The total antioxidant capacity assay was seen to be much higher in all the extracts particularly that of TR1Et10 and TR2Et10, which increased with increasing concentrations of extract. 1mg of extracts of TR1E7A3, TR1Et10, TR1E5A5, TR2E2A8, TR2E8A2 and TR2Et10 showed total antioxidant capacities of 84.12±0.94, 92.38±1.2, 82.34±0.94, 72.84±1.2, 71.66±0.94 and 93.24±1.2, respectively (Figure 4).

4 Discussion

This study highlights the considerable antioxidant potential of thermophilic bacteria isolated from the Tatapani Hot Spring,

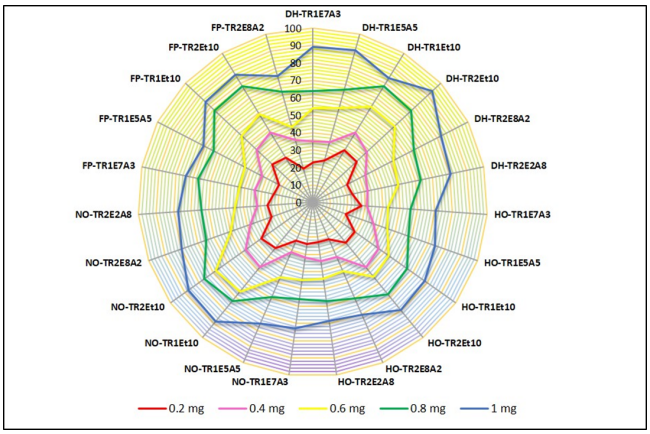

Fig 3. Antioxidant assay

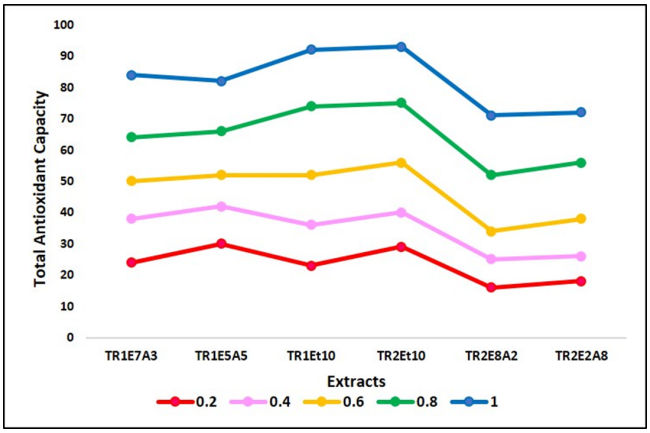

Fig 4. Total antioxidant capcity

emphasizing their significance as novel sources of natural antioxidants. The isolates, identified as *Bacillus nakamurai* (TR1) and *Bacillus licheniformis* (TR2), exhibited remarkable antioxidant activities, which can be attributed to their adaptation to extreme thermal conditions. The DPPH radical scavenging assay demonstrated exceptional activity, with TR2Et10 achieving a scavenging effect of 93.30%. This high activity suggests that the ethanol extract of TR2 possesses potent radical-neutralizing properties, likely owing to the presence of specific antioxidant compounds that effectively quench DPPH radicals. Both TR1Et10 and TR2Et10 showed significant hydroxyl radical scavenging activity, with TR1Et10 exhibiting 87.21% and TR2Et10 showing 87.68%, indicating their ability to mitigate oxidative damage caused by hydroxyl radicals. These findings align with previous studies reporting the high antioxidant potential of *Bacillus* species, often linked to their production of secondary metabolites with strong antioxidant properties. The nitric oxide scavenging activity was notably high in TR2Et10 (87.44%) and TR1Et10 (88.22%), underscoring their potential to counteract inflammation and associated oxidative stress. The high reducing power observed in the FRAP assay, particularly in TR1Et10 and TR2Et10, further corroborates their capacity to act as effective electron donors, neutralizing free radicals and reducing oxidative damage. The observed variation in antioxidant activities across different solvents highlights the critical role of solvent choice in extracting bioactive compounds. Ethanol emerged as the most effective solvent, likely due to its ability to dissolve a wide range of polar and non-polar compounds, thereby maximizing the extraction of antioxidant agents. This finding aligns with the understanding that solvent polarity is essential for extraction efficiency of bioactive compounds, with ethanol being particularly effective in extracting a broad spectrum of antioxidant compounds. The study underscores the importance of thermophilic bacteria from extreme environments as promising sources of novel antioxidants. These bacteria have adapted to survive in harsh conditions, leading to the production of robust antioxidant compounds. The high antioxidant activity observed in *Bacillus nakamurai* and *Bacillus licheniformis* extracts can be linked to their production of secondary metabolites including carotenoids and quinones. Carotenoids are known for their ability to quench singlet oxygen and stabilize free radicals, while quinones participate in redox cycling, neutralizing reactive oxygen species and maintaining cellular redox homeostasis. Future research should focus on the detailed biochemical characterization of these bioactive compounds, including the isolation and identification of active constituents, as well as exploring their stability and efficacy in various applications. Investigations should also examine the potential synergistic effects of these compounds with other known antioxidants, which could enhance their therapeutic effectiveness. Additionally, optimizing the extraction processes to maximize yield and activity, as well as evaluating the scalability of these processes for industrial applications, will be crucial steps toward the development of new antioxidant-rich products. In conclusion, the findings from this study validate the potential of thermophilic bacteria from extreme environments as sources of novel antioxidants. These insights pave the way for the development of new antioxidant-rich products with applications in pharmaceuticals, food preservation, and other industries, leveraging the unique properties of these extremophiles. Further research and development in this area could lead to significant advancements in health sciences and biotechnological innovations.

5 Conclusion

This study effectively highlights the significant antioxidant potential of thermophilic bacteria obtained from Tatapani Hot Spring, particularly *Bacillus nakamurai* (TR1) and *Bacillus licheniformis* (TR2). Both isolates demonstrated exceptional antioxidant activities across multiple assays, including DPPH radical scavenging, hydroxyl radical scaveng-

Figure Number	Legend
Figure 1	Evolutionary analyses were performed in MEGA11 using the Neighbour-Joining method. The bootstrap consensus tree, based on 1000 replicates, is used to depict the evolutionary history of the analyzed taxa analysed. The evolutionary distances were computed using the Maximum Composite Likelihood method.
Figure 2	The absorption spectra of carotenoid extracts of TR1E5A5, TR1E7A3 and TR2Et10 with absorption maxima of 448 nm,425 nm and 478 nm are given in a, b and c and that of quinone extracts of TR2E2A8, TR2E8A2 and TR1Et10 with absorption maxima of 288 nm, 273 nm and 265 nm are given in d, e and f, respectively.
Figure 3	Determination of percent scavenging activity of different concentrations of the carotenoid and quinone extracts of TR1 (*Bacillus nakamurai*) and TR2 (*Bacillus licheniformis*)- TR1E7A3, TR1Et10, TR1E5A5, TR2E2A8, TR2E8A2 and TR2Et10, dissolved in ethyl acetate (E), acetone (A) and ethanol (Et) of by DPPH assay (DH), Hydroxyl radical assay (HO), nitric oxide assay (NO) and FRAP assay (FP). Coloured lines indicate different concentrations: red—0.2 mg, pink—0.4 mg, yellow—0.6 mg, green-0.8 mg and blue—1.0 mg. The abbreviations are in the sequence of 'organism-solvent'.
Figure 4	Determination of percent scavenging activity of different concentrations of the carotenoid and quinone extracts of TR1 (*Bacillus nakamurai*) and TR2 (*Bacillus licheniformis*)- TR1E7A3, TR1Et10, TR1E5A5, TR2E2A8, TR2E8A2 and TR2Et10, dissolved in ethyl acetate (E), acetone (A) and ethanol (Et) of by DPPH assay (DH), Hydroxyl radical assay (HO), nitric oxide assay (NO) and FRAP assay (FP). Coloured lines indicate different concentrations: red—0.2 mg, pink—0.4 mg, yellow—0.6 mg, green-0.8 mg and blue—1.0 mg. The abbreviations are in the sequence of 'organism-solvent'.

ing, nitric oxide scavenging, and reducing power, with TR2 exhibiting the highest overall activity. These findings underscore the promising role of thermophilic bacteria as natural sources of potent antioxidants, driven by their adaptation to extreme thermal environments. The differential effectiveness of ethanol as a solvent for extracting bioactive compounds suggests that solvent choice plays a vital role in maximizing antioxidant recovery. This study not only establishes a foundation for the use of extremophilic microorganisms in developing antioxidant-rich products but also opens avenues for further research into the specific bioactive compounds responsible for these activities. Future studies should focus on elucidating the mechanisms behind these antioxidant properties, optimizing extraction processes, and exploring potential applications in diverse fields such as pharmaceuticals, food technology, and cosmetics. In summary, the thermophilic bacterial isolates sourced from Tatapani Hot Spring represent a valuable resource for identifying novel antioxidants and developing innovative solutions to combat oxidative stress and related diseases. Their unique properties and robust antioxidant activities present significant opportunities for advancing both scientific knowledge and practical applications in various industries.

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Appendices

Table Number	Legend
Table 1	This table presents the observed morphological and cultural features of various bacterial strains. The characteristics include colony morphology, Gram staining, motility, and growth requirements.
Table 2	This table presents the results of various biochemical tests performed on *Bacillus nakamurai* (TR1) and *Bacillus licheniformis* (TR2). The tests assess the ability of these strains to utilize different substrates, produce enzymes, and ferment various sugars. The results help differentiate the two strains based on their metabolic activities.

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