

Original Research Article

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Unveiling the Bioactive Potential of Muttukadu Marine Bacterium for Antioxidant and Anticancer Applications

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ABSTRACT

Keywords

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Marine backwater sediments contain diverse cultivable microorganisms that may produce metabolites with useful biological activities. This study evaluated bacteria isolated from Muttukadu backwaters, Tamil Nadu, India, for free-radical-scavenging activity and preliminary cytotoxicity. Three sediment samples yielded twelve bacterial isolates, and ethyl acetate extracts of culture broths were examined using the DPPH assay. Four isolates were selected for secondary screening, of which isolate B4 showed the highest activity ($49.52 \pm 0.71\%$) and was taken forward for optimization and characterization. Variation in culture conditions altered the antioxidant response, and the optimized extract showed a concentration-related increase in DPPH scavenging, reaching $45.29 \pm 0.97\%$ at 0.5 mg/mL . TLC separation produced seven visible components, and the fraction at $R_f 0.71$ showed positive DPPH bioautography. Phenotypic and biochemical testing indicated a Gram-negative, rod-shaped, non-motile bacterium, while partial 16S rRNA analysis showed the closest relationship to *Alcaligenes faecalis* with 99% sequence similarity. The crude extract also decreased HeLa cell viability in a concentration-dependent manner, with an IC_{50} of $65.14 \text{ } \mu\text{g/mL}$. The findings demonstrate antioxidant activity in a cultivable bacterium from Muttukadu backwaters and identify an activity-positive extract that warrants further purification and chemical characterization.

Introduction

Reactive oxygen species are generated continuously during cellular metabolism and can become damaging when their production exceeds the capacity of endogenous protective systems. Persistent oxidative imbalance can affect lipids, proteins and nucleic acids, which has encouraged the search for antioxidant compounds from natural sources. (Almeida *et al.*, 2005; Fang *et al.*, 2002)

Marine and estuarine microorganisms are attractive candidates for this purpose because they inhabit environments with variable salinity, nutrient availability and other physical and chemical pressures. Bacteria from sediments and associated marine matrices are known to produce enzymes, pigments, peptides and small molecules with biological activities, making them useful targets for activity-guided bioprospecting. (Baharum *et al.*, 2010; Dhivya Balakrishnan *et al.*, 2014; Fenical and Jensen, 1993; Takamatsu *et al.*, 2003).

Microbial natural-product research also offers practical advantages. Selected organisms can be cultivated under controlled conditions, their metabolite production can be modified through medium composition and environmental parameters, and active fractions can be followed through chromatographic separation and bioassay-guided screening. (Fenical and Jensen, 1993; Newman and Cragg, 2012; Nickavar *et al.*, 2014; Takamatsu *et al.*, 2003). Muttukadu backwaters on the Tamil Nadu coast provide a locally relevant sedimentary environment in which cultivable bacterial diversity can be explored for useful metabolites. The present investigation was undertaken to isolate bacteria from Muttukadu backwater sediments, screen their crude extracts for DPPH radical-scavenging activity, select a high-performing isolate, optimize selected culture variables, locate antioxidant activity by TLC bioautography, characterize the organism phenotypically and by 16S rRNA analysis, and assess the effect of its crude extract on HeLa cell viability. (Dholakiya *et al.*, 2017; Velho-Pereira *et al.*, 2015)

Materials and Methods

Study material and sampling

Three marine sediment samples were collected from the backwater of Muttukadu Lake, Tamil Nadu, India, in sterile polypropylene containers. Samples were transported on the day of collection and processed for bacterial isolation.

Isolation of cultivable marine bacteria

One gram of each sediment sample was diluted in 100 mL sterile seawater and serially diluted. An aliquot (100 μ L) from the 10^{-6} dilution was spread on marine nutrient agar. Plates were incubated for three days, and morphologically distinct colonies were purified and maintained for subsequent screening. Twelve isolates were recovered from the three sampling sets.

Preparation of crude bacterial metabolites

Isolates were cultivated in marine nutrient broth. Following incubation, cultures were centrifuged and the recovered broth was extracted with ethyl acetate by liquid-liquid extraction. The solvent fraction was concentrated under reduced pressure and used as the crude metabolite preparation.

DPPH radical-scavenging assay

Antioxidant activity was determined by the DPPH radical-scavenging method. The reaction mixture contained 2 mL of 6×10^{-5} M methanolic DPPH solution and 50 μ L of methanolic sample solution. Absorbance was recorded at 515 nm for 16 min at room temperature. Assays were performed in triplicate.

Radical-scavenging activity was expressed as inhibition percentage using the control and sample absorbance values. (Yen *et al.*, 1993, 1994)

Primary and secondary screening

All isolates were screened initially for DPPH activity. Isolates A4, A5, B4 and C2 were selected for secondary screening on the basis of their preliminary performance. The isolate with the highest secondary activity was used for subsequent optimization and characterization.

Optimization of culture conditions

The selected isolate was evaluated with galactose, glucose, maltose and starch as carbon sources and ammonium chloride, beef extract, peptone and yeast extract as nitrogen sources. The effect of pH 6, 7, 8 and 9 and incubation temperatures of 25, 30, 35 and 40 °C was also assessed. Crude extracts from the optimization experiments were evaluated for DPPH activity. An optimized condition was subsequently used for a one-litre fermentation followed by ethyl acetate extraction and concentration.

Thin-layer chromatography and DPPH bioautography

The crude extract was applied to silica gel F254 TLC plates. Developed plates were examined under UV light at 254 and 365 nm and after exposure to iodine vapour. For DPPH bioautography, the extract was developed in hexane: ethyl acetate (1: 9), dried and sprayed with 0.2% methanolic DPPH. Yellow zones against the violet DPPH background were recorded as antioxidant-active regions. (Nickavar *et al.*, 2014)

Phenotypic and biochemical characterization

The selected isolate was examined by Gram staining and biochemical tests including catalase, oxidase, citrate

utilization, methyl red, Voges–Proskauer, urease, indole, starch hydrolysis, gelatin and triple sugar iron reactions.

Genomic DNA extraction and 16S rRNA analysis

Genomic DNA was prepared from the selected isolate. A 16S rRNA gene fragment was amplified in a 50 µL PCR reaction using 27F and 1522R primers. The amplified product was examined by agarose gel electrophoresis, purified and sequenced. The sequence was compared with GenBank records using BLASTN. Multiple sequence alignment and phylogenetic analysis were performed using CLUSTAL W and MEGA X; Kimura two-parameter distances and a neighbour-joining tree with 1,000 bootstrap replicates were used. (Thiel and Imhoff, 2003)

HeLa cell cytotoxicity assay

The crude extract was evaluated against HeLa cells using an MTT-based viability assay. Cells were maintained in DMEM supplemented with 10% fetal calf serum and exposed to the extract concentration series for 48 h. MTT was then added, the resulting formazan was solubilized, and absorbance was measured at 540 nm. The IC₅₀ value was obtained from the concentration–response relationship.

Results and Discussion

Recovery and screening of marine bacteria

The three sediment samples yielded twelve cultivable bacterial isolates. Primary screening showed differences in DPPH-scavenging activity, with A5 (50.79%), C2 (49.21%), B4 (45.87%) and A4 (45.40%) selected for secondary screening. In the secondary assay, B4 gave the highest activity ($49.52 \pm 0.71\%$), followed by C2 ($43.54 \pm 1.03\%$), A5 ($36.02 \pm 0.98\%$) and A4 ($27.58 \pm 1.18\%$). (Balakrishnan *et al.*, 2015; Dholakiya *et al.*, 2017) (Fig. 7–9).

Optimization of metabolite production

Among the carbon sources tested, glucose produced the highest DPPH-scavenging activity ($39.02 \pm 0.57\%$), followed by galactose ($20.93 \pm 0.43\%$), starch ($20.61 \pm 0.48\%$) and maltose ($18.52 \pm 0.64\%$). Yeast extract was the most effective nitrogen source ($42.13 \pm 1.05\%$), whereas ammonium chloride, beef extract and peptone

produced lower activities. The temperature series showed the highest activity at 30 °C ($34.89 \pm 1.40\%$). Across the tested pH range, pH 7 produced the highest response ($39.78 \pm 0.81\%$).

The optimized crude extract showed a progressive increase in DPPH scavenging across the concentration range tested, from $26.28 \pm 0.61\%$ at 0.1 mg/mL to $45.29 \pm 0.97\%$ at 0.5 mg/mL.

TLC profiling and antioxidant bioautography

TLC profiling of the B4 crude extract produced seven visible components with R_f values of 0.14, 0.29, 0.43, 0.71, 0.79, 0.89 and 0.99. The C4 band at R_f 0.71 produced the positive DPPH bioautography response. This result localized antioxidant activity to a discrete chromatographic region suitable for further purification.

Phenotypic and molecular characterization

Isolate B4 was Gram-negative, rod-shaped and non-motile. Catalase, oxidase, citrate utilization and TSI reactions were positive, whereas indole, methyl red, Voges–Proskauer, urease, starch hydrolysis and gelatin reactions were negative. A PCR product of approximately 1.5 kb was obtained for the 16S rRNA gene. Sequence comparison indicated the closest database relationship with *Alcaligenes faecalis* and approximately 99% sequence similarity. The sequence was further examined by phylogenetic analysis.

Cytotoxicity against HeLa cells

Exposure of HeLa cells to the crude extract for 48 h produced a concentration-dependent reduction in cell viability. Viability declined from 87.51% at 3.125 µg/mL to 9.96% at 200 µg/mL, compared with 100% in the untreated control.

The calculated IC₅₀ was 65.14 µg/mL. Because the tested material was a crude extract and only one cancer-derived cell line was examined, the result represents preliminary in-vitro cytotoxicity rather than evidence of selective anticancer activity.

The recovery of twelve cultivable bacterial isolates from three Muttukadu backwater sediment samples demonstrates that this habitat can support a range of metabolically distinct bacteria.

Table.1 DPPH radical-scavenging activity of bacterial isolates.

Isolate	Primary DPPH (%)	Secondary DPPH (%)
A1	24.60	—
A2	13.81	—
A3	16.51	—
A4	45.40	27.58 ± 1.18
A5	50.79	36.02 ± 0.98
B1	34.76	—
B2	43.81	—
B3	39.68	—
B4	45.87	49.52 ± 0.71
C1	20.00	—
C2	49.21	43.54 ± 1.03
C3	37.94	—

Table.2 Effect of culture variables on DPPH radical-scavenging activity.

Variable	Condition	DPPH scavenging (%)
Carbon	Glucose	39.02 ± 0.57
Carbon	Galactose	20.93 ± 0.43
Carbon	Maltose	18.52 ± 0.64
Carbon	Starch	20.61 ± 0.48
Nitrogen	Yeast extract	42.13 ± 1.05
Nitrogen	Ammonium chloride	16.99 ± 0.58
Nitrogen	Beef extract	16.77 ± 0.41
Nitrogen	Peptone	16.29 ± 1.47
Temperature	30 °C	34.89 ± 1.40
Temperature	35 °C	16.29 ± 1.05
Temperature	25 °C	15.59 ± 1.53
Temperature	40 °C	12.65 ± 1.30
pH	pH 6	15.27 ± 1.07
pH	pH 7	39.78 ± 0.81
pH	pH 8	17.53 ± 1.13
pH	pH 9	12.31 ± 1.50

Table.3 Concentration-dependent DPPH radical-scavenging activity of the optimized crude extract.

Extract concentration (mg/mL)	DPPH scavenging (%)
0.1	26.28 ± 0.61
0.2	31.07 ± 0.83
0.3	35.00 ± 1.21
0.4	37.48 ± 0.81
0.5	45.29 ± 0.97

Table.4 TLC profile and DPPH bioautography of the B4 crude extract.

Component	Rf	DPPH bioautography
C1	0.14	Not detected
C2	0.29	Not detected
C3	0.43	Not detected
C4	0.71	Antioxidant-active
C5	0.79	Not detected
C6	0.89	Not detected
C7	0.99	Not detected

Table.5 Phenotypic and biochemical characteristics of isolate B4.

Test	Result
Gram stain	Negative; rod-shaped; non-motile
Catalase	Positive
Oxidase	Positive
Citrate	Positive
Indole	Negative
Methyl red	Negative
Voges–Proskauer	Negative
Gelatin	Negative
TSI	Positive
Urease	Negative

Table.6 Effect of the crude extract on HeLa cell viability after 48 h.

Extract concentration (µg/mL)	HeLa cell viability (%)
0	100.00
3.125	87.51
6.25	78.18
12	70.32
25	59.93
50	43.79
100	22.53
200	9.96

Figure.1 Effect of carbon sources on DPPH radical-scavenging activity of isolate B4.

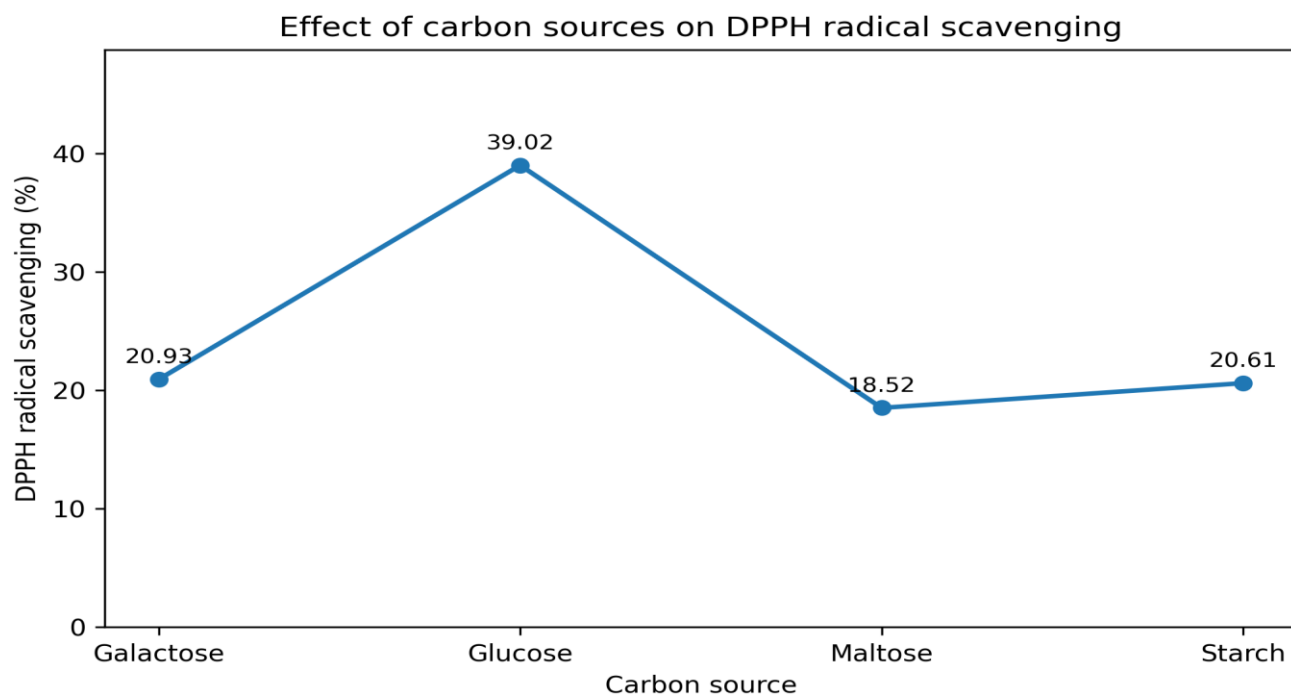


Figure.2 Effect of nitrogen sources on DPPH radical-scavenging activity of isolate B4.

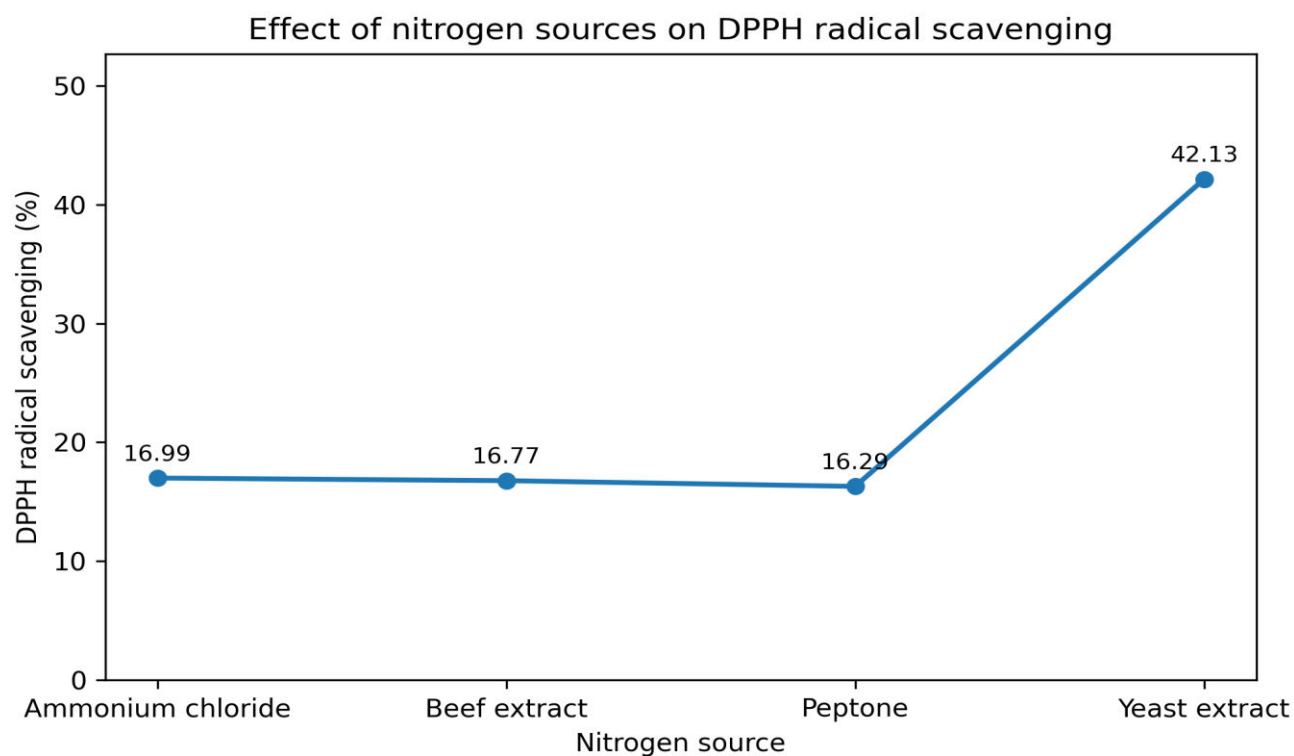


Figure.3 Effect of pH on DPPH radical-scavenging activity of isolate B4.

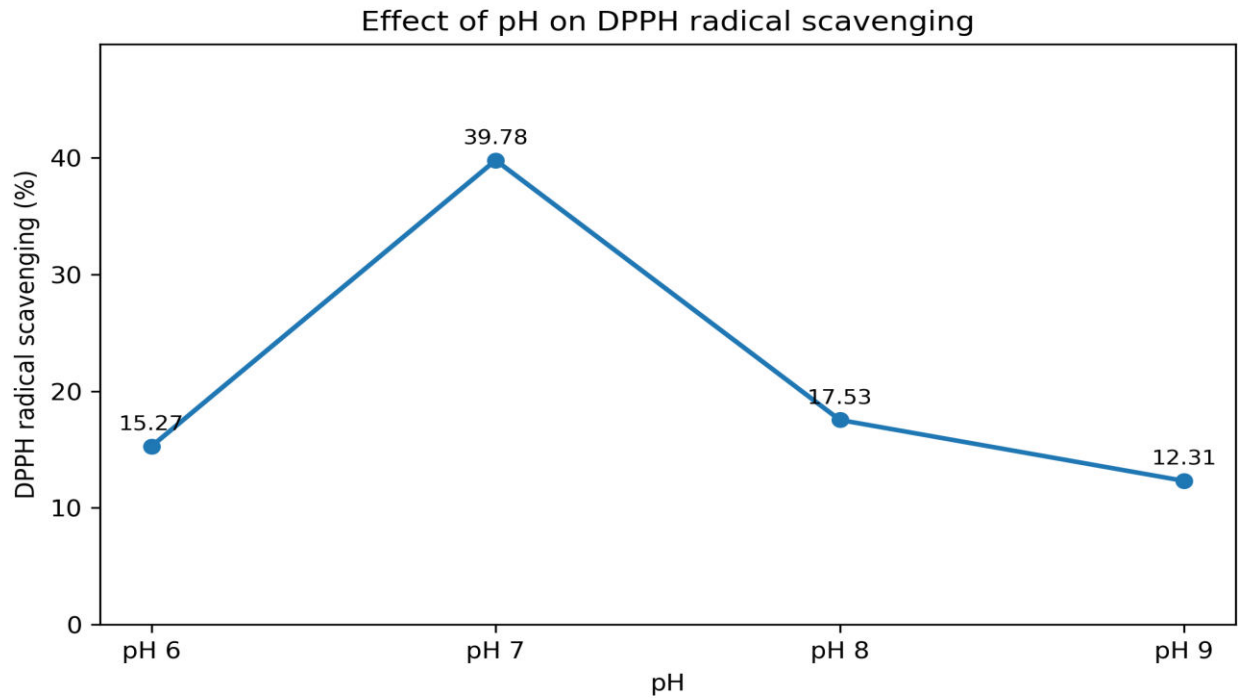


Figure.4 Effect of incubation temperature on DPPH radical-scavenging activity of isolate B4.

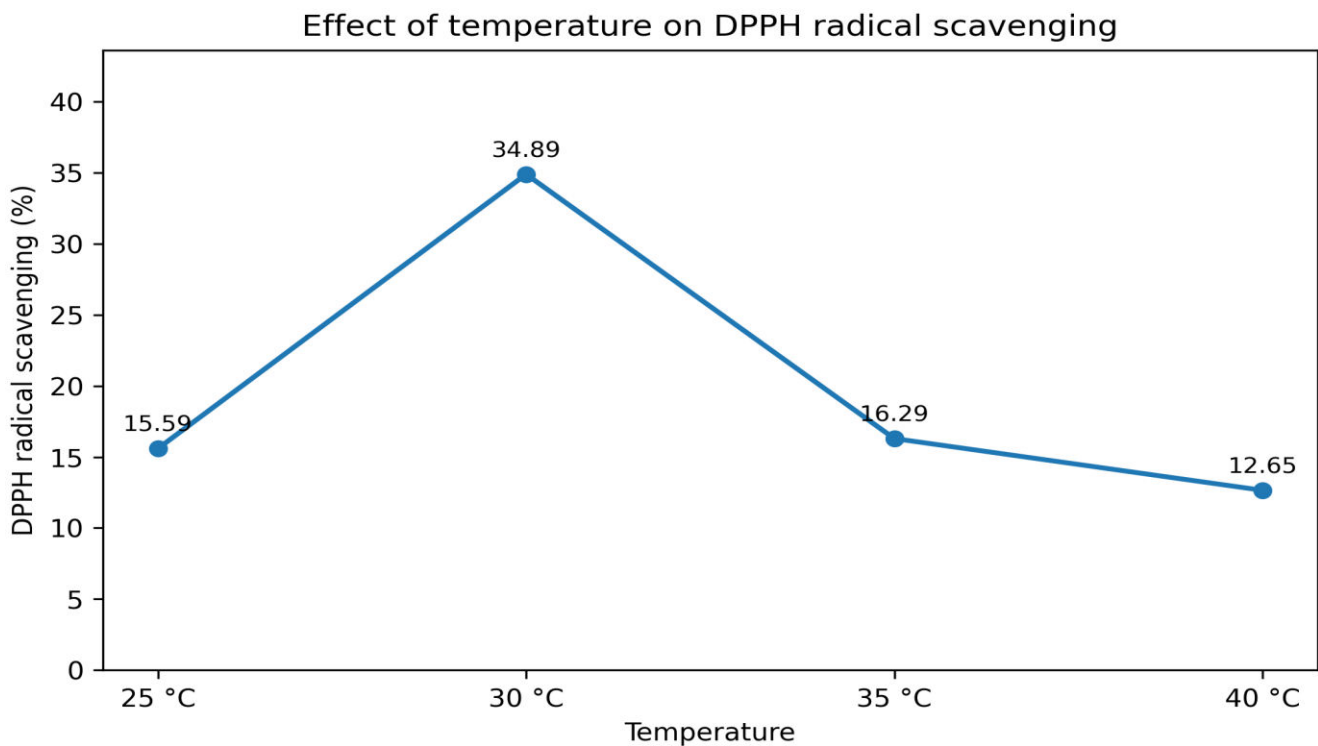


Figure.5 Concentration-dependent DPPH radical-scavenging activity of the optimized crude extract.

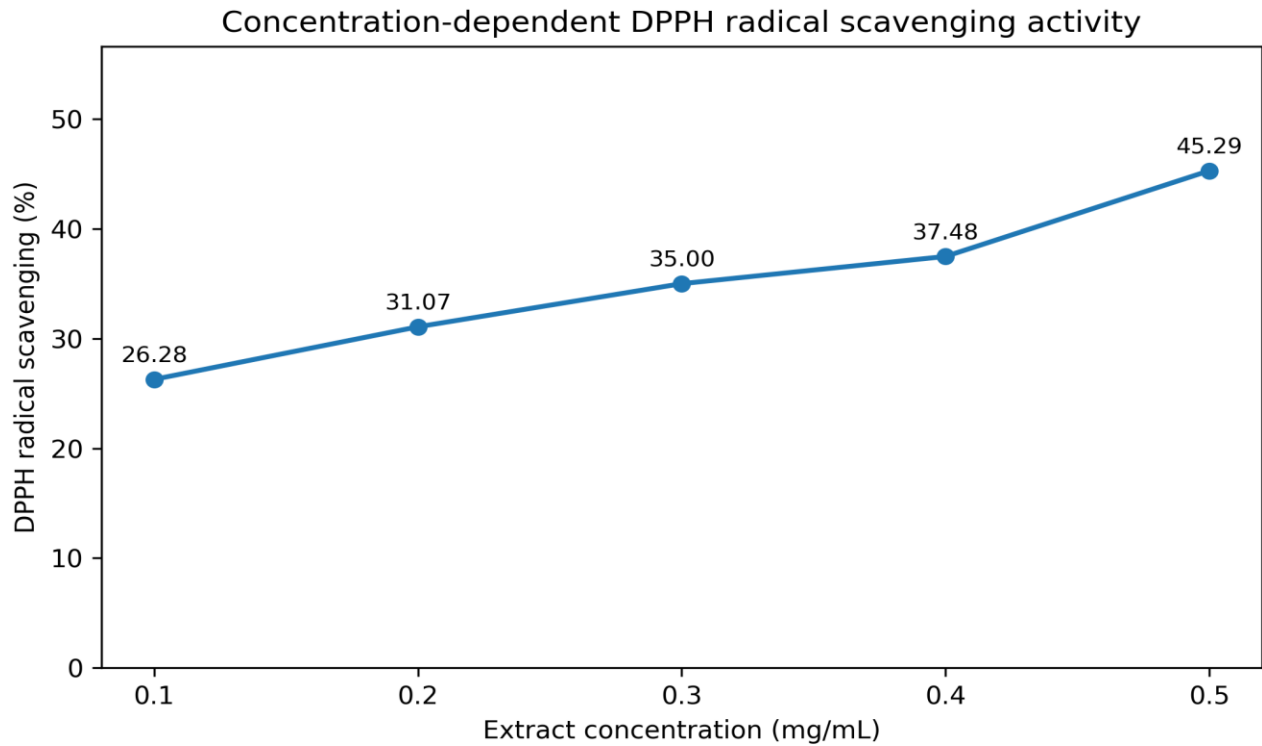


Figure.6 Concentration-dependent effect of the crude extract on HeLa cell viability after 48 h.

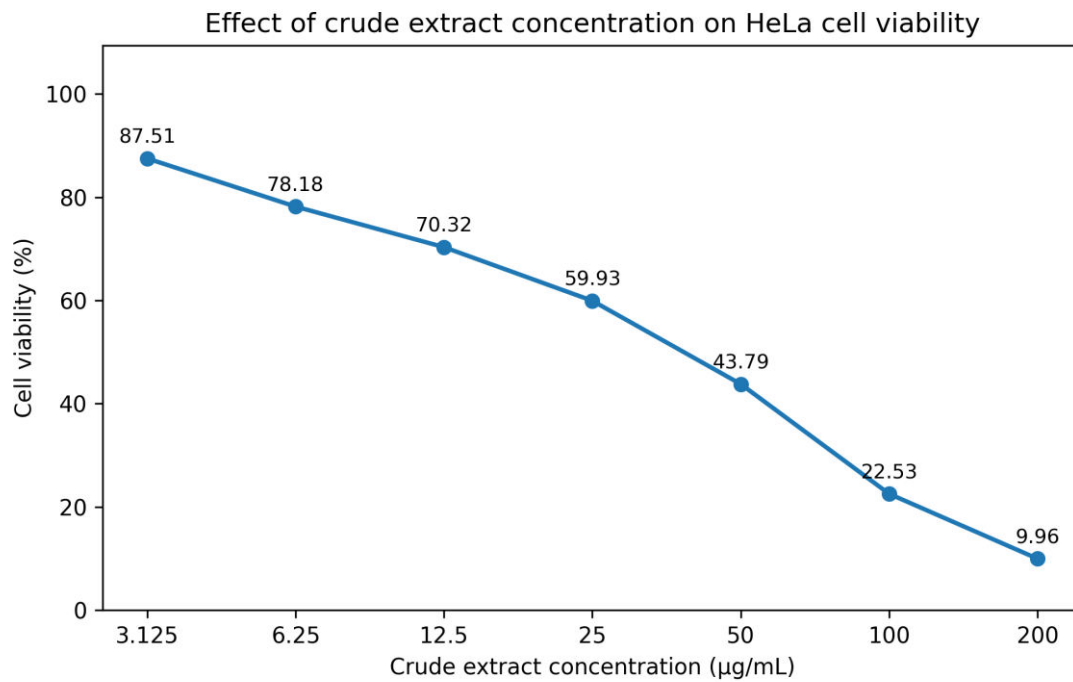


Figure.7 Muttukadu backwater sediment samples used for isolation of cultivable marine bacteria.



Figure.8 Representative colony morphology of bacterial isolates recovered from Muttukadu backwater sediment samples.



Figure.9 Representative DPPH screening assay images for bacterial isolates during primary and secondary screening.

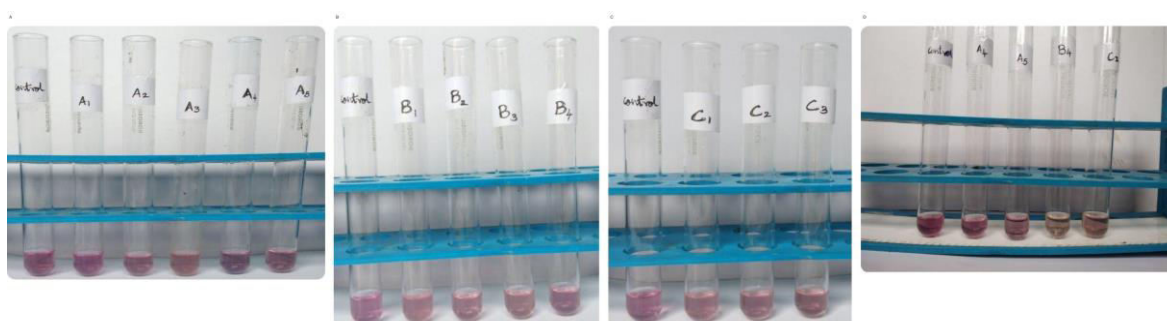


Figure.10 TLC profiling of the crude extract and visualization of chromatographic components associated with antioxidant screening.

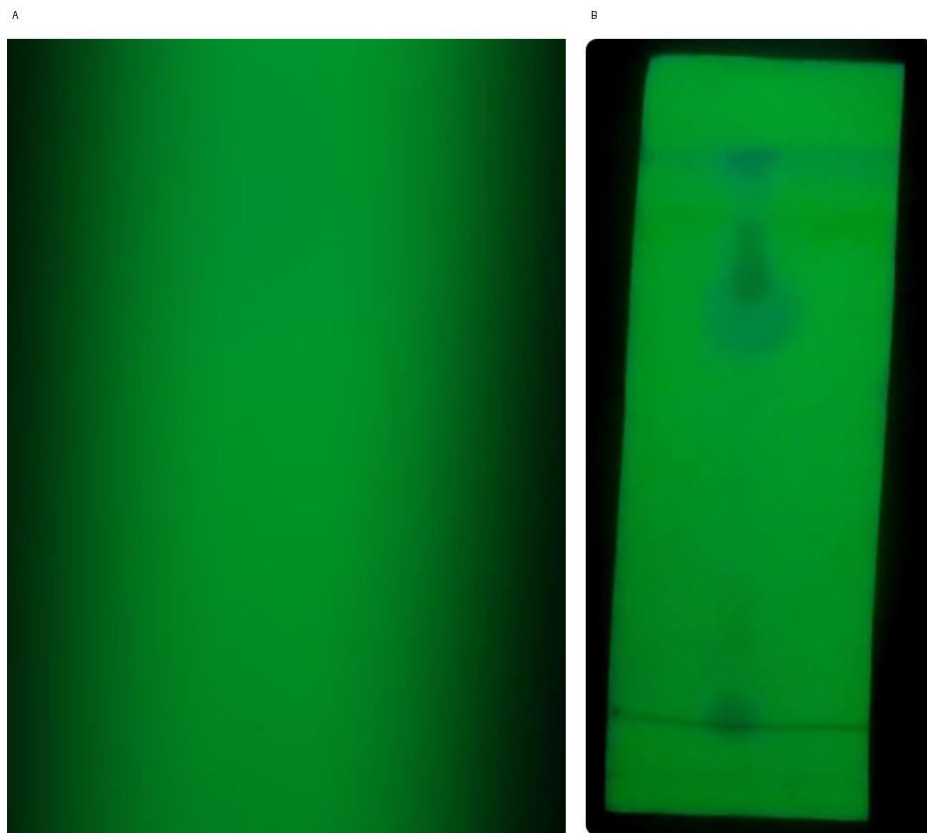


Figure.11 Representative biochemical characterization results for the selected isolate B4.



Figure.12 Molecular characterization of isolate B4: (A) 16S rRNA PCR product and (B) neighbour-joining phylogenetic tree showing its relationship to *Alcaligenes faecalis*.

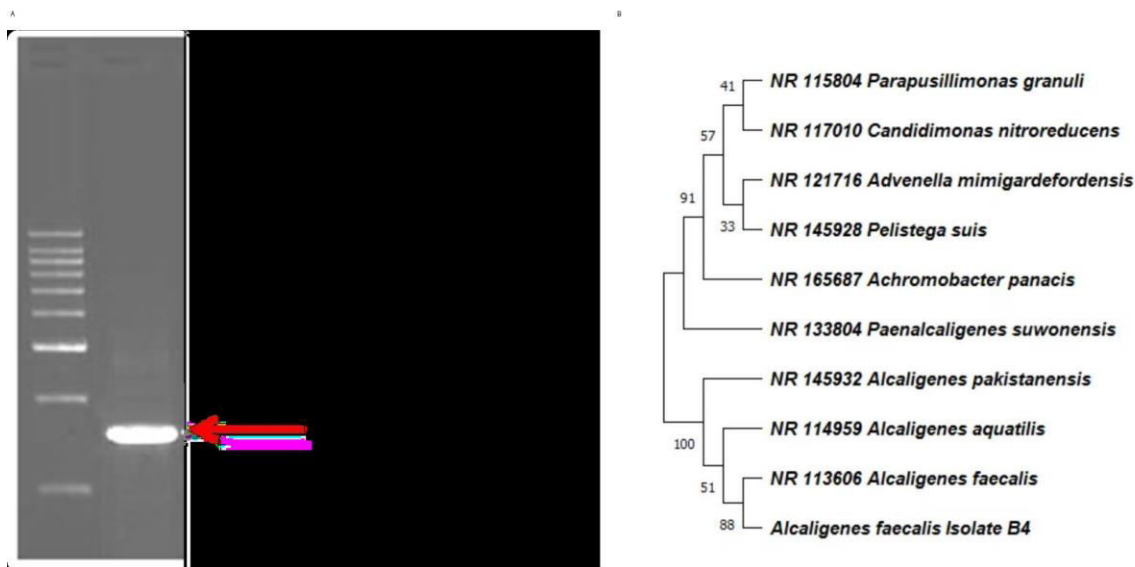
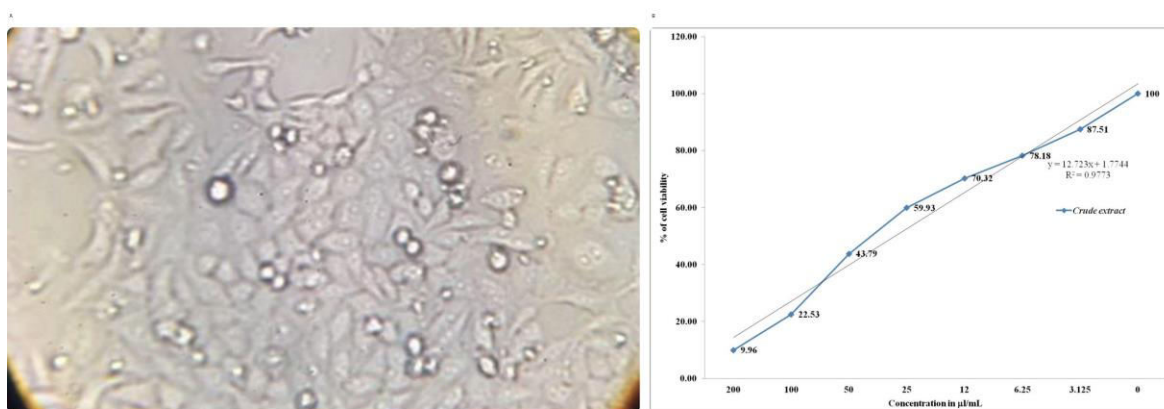


Figure.13 Representative bright-field microscopy images associated with the HeLa cell cytotoxicity assessment of the crude extract.



The marked variation in DPPH activity among the isolates justified the use of activity-guided selection, and B4 was retained for further work because it gave the strongest response in the secondary assay. (Balakrishnan *et al.*, 2015; Dholakiya *et al.*, 2017)

The antioxidant response changed with culture composition. Glucose and yeast extract produced the highest activities among the carbon and nitrogen sources examined, respectively. Changes of this type are expected in microbial secondary metabolism because nutrient availability can influence growth, precursor

supply and the expression of biosynthetic pathways. The present results establish an empirical optimization profile but do not identify the biochemical mechanism responsible for the altered activity. (Fenical and Jensen, 1993; Hamidi *et al.*, 2020; Takamatsu *et al.*, 2003)

The response to temperature and pH further indicates that culture conditions influenced metabolite-associated antioxidant activity. The strongest response occurred at 30 °C and pH 7 under the tested conditions. The progressive increase in DPPH scavenging with extract concentration provides additional evidence of

concentration-dependent activity, although DPPH scavenging alone cannot establish the identity or physiological role of the active molecule. (Fenical and Jensen, 1993; Hamidi *et al.*, 2020)

TLC-DPPH bioautography narrowed the antioxidant activity to the C4 region at Rf 0.71. Bioautography is useful because it links chromatographic separation directly to a measurable activity and can guide subsequent purification. The active band, however, should not be considered a purified compound; structural identification would require additional chromatographic and spectroscopic analysis. (Nickavar *et al.*, 2014; Takamatsu *et al.*, 2003) (Fig. 10).

Phenotypic testing together with partial 16S rRNA analysis placed B4 close to *Alcaligenes faecalis*. The sequence similarity supports a taxonomic relationship, but 16S rRNA alone may not provide sufficient resolution for definitive strain assignment. Whole-genome or multilocus analysis would strengthen the taxonomic conclusion. (Thiel and Imhoff, 2003) (Fig. 11–12).

The HeLa assay demonstrated a concentration-related decline in cell viability, with an IC₅₀ of 65.14 µg/mL. This finding indicates that the crude extract contains one or more components capable of affecting HeLa cell survival under the conditions tested. It does not establish selectivity, mechanism or the identity of the responsible compound. Comparative testing with non-cancer cells, purification of active fractions and mechanistic assays would therefore be appropriate next steps. (Siddharth and Vittal, 2018) (Fig. 13).

In conclusion, Activity-guided screening of cultivable bacteria from Muttukadu backwater sediments identified isolate B4 as the strongest DPPH-active producer among the isolates examined. Culture optimization, TLC bioautography and preliminary taxonomic characterization together provided a practical route for tracking the biological activity of its crude metabolites. The extract also showed concentration-dependent effects on HeLa cell viability. Further work should focus on purification and structural identification of the active metabolite(s), validation with complementary antioxidant assays, improved taxonomic resolution and mechanistic evaluation of the cytotoxic response. (Balakrishnan *et al.*, 2014; Dholakiya *et al.*, 2017; Fenical and Jensen, 1993; Takamatsu *et al.*, 2003)

Limitations and Future Work

- Only cultivable bacteria from a limited number of sediment samples were investigated; culture-independent microbial diversity was not assessed.
- DPPH was the principal antioxidant assay. Additional radical-scavenging and cellular antioxidant assays would provide a broader assessment.
- The DPPH-active TLC fraction was localized but the responsible chemical constituent was not identified.
- The cytotoxicity study used a crude extract and a single cancer-derived cell line; selectivity and molecular mechanism were not established.
- Whole-genome or multilocus sequence analysis would provide stronger taxonomic resolution than partial 16S rRNA analysis alone.

Declarations

Ethics statement: No human or animal subjects were used. The HeLa cell-line experiment was conducted entirely in vitro.

Conflict of interest statement: No known competing financial interests or personal relationships.

Data availability: The data supporting the findings are presented within the article.

Funding: No external funding for this study.

Author contributions: ¹Aarthi Monika B: conceptualization, investigation, methodology, data analysis, visualization and manuscript preparation. ²Dr.P. Nirmala* Designed the model and the computational framework and analysed the data.

Statement of originality: The manuscript is presented as an original research article and has been independently structured and written for journal submission. The reported numerical results correspond to the experimental study dataset.

Author Contributions

B. Aarthi Monika: Conceived the original idea and designed the model and wrote the manuscript. P. Nirmala: Designed the model and the computational framework and analysed the data.

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