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Bioprospecting of Cow Farmyard Manure for Bioactive Compound-Producing Microorganisms: Isolation, Characterization, Crude Extraction, and Antimicrobial Evaluation of a Plant-Growth-Promoting *Bacillus pumilus* BIOSHIELD-L Isolate

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Abstract

Cow farmyard manure represents a complex microbial habitat containing bacteria with potential applications in agriculture and biotechnology. In this study, a bacterial isolate designated BIOSHIELD-L was recovered from cow farmyard manure collected in the Latur region of Maharashtra, India. Morphological and biochemical characterization, together with 16S rRNA gene sequencing, supported its identification as Bacillus pumilus. The approximately 1,465-bp 16S rRNA gene sequence showed 99.32% similarity to B. pumilus. The sequence has not yet been deposited in GenBank and therefore has no accession number.

The antimicrobial activity of the cell-free culture supernatant and ethyl-acetate crude extract was evaluated against six bacterial pathogens relevant to aquaculture. Inhibition zones ranged from 9.8 to 13.5 mm for the supernatant and from 14.1 to 18.6 mm for the crude extract. The crude extract yield was 0.42 g/L. The extract showed DPPH and ABTS radical-scavenging activities of 78.6 ± 1.8% and 82.4 ± 1.6%, respectively, and a total antioxidant capacity of 146.3 ± 4.2 mg AAE/g extract. Anti-inflammatory screening showed 71.3 ± 2.1% protein-denaturation inhibition. HEK-293 cell viability remained at 86.7% or above at concentrations up to 200 µg/mL. GC-MS profiling identified hexadecanoic acid methyl ester, 9-octadecenoic acid methyl ester and phytol among the major constituents. In the pot experiment, BIOSHIELD-L treatment was associated with increases in germination, root length, shoot length, fresh weight and dry weight compared with the untreated control. The findings indicate that BIOSHIELD-L merits further investigation as a plant-growth-promoting bacterial candidate and as a source of metabolites for potential agricultural and aquaculture applications.

Keywords: Cow farmyard manure; Bacillus pumilus; BIOSHIELD-L; antimicrobial activity; antioxidant activity; GC-MS; plant growth promotion; biofertilizer

1. Introduction

Cow farmyard manure is an organic amendment and a habitat for diverse microbial communities involved in organic matter decomposition, nutrient cycling and mineralization. Manure-associated microorganisms may include spore-forming bacteria with extracellular enzymatic activities and plant-beneficial properties, making them relevant sources for agricultural inoculants and biotechnological applications [1–3].

Members of the genus Bacillus have attracted attention because of their metabolic versatility, ability to form resistant endospores and capacity to produce extracellular enzymes and secondary metabolites. Bacillus pumilus strains have been investigated for plant-growth-promoting and other biological properties; however, these characteristics are strain-dependent and require experimental evaluation for each isolate [3–5].

The present study examined BIOSHIELD-L, an isolate obtained from cow farmyard manure collected in Latur, Maharashtra, India. The work included morphological and biochemical characterization, 16S rRNA gene-based identification, antimicrobial screening against selected aquaculture pathogens, antioxidant and anti-inflammatory assays, cytotoxicity assessment using HEK-293 cells, GC-MS profiling of the crude extract and a plant-growth-promotion pot

experiment. The study aimed to assess the biological properties of BIOSHIELD-L and its potential relevance to agricultural and aquaculture applications.

2. Materials and Methods

2.1 Sample Collection and Bacterial Isolation

Cow farmyard manure samples were collected aseptically from a managed dairy farm in the Latur region, Maharashtra, India. Bacterial isolation and purification were performed using serial dilution and spread-plate techniques on nutrient agar. A distinct bacterial colony was selected, purified and designated BIOSHIELD-L. The purified isolate was maintained on nutrient agar slants at 4 °C until further analysis.

2.2 Morphological and Biochemical Characterization

Colony characteristics, including colour, shape, texture and opacity, were examined on nutrient agar. Cellular morphology and Gram reaction were determined by microscopic examination following Gram staining. Biochemical characterization included catalase, oxidase, indole, Voges–Proskauer, starch hydrolysis, gelatin liquefaction, citrate utilization, methyl-red and nitrate-reduction tests. The combined phenotypic profile was used for preliminary characterization of the isolate.

Table 1. Biochemical Characteristics of *Bacillus pumilus* BIOSHIELD-L

Biochemical test	**Observed result**
Gram staining	Gram-positive rods with endospores
Catalase	Positive
Oxidase	Negative
Indole	Negative
Voges–Proskauer	Negative
Starch hydrolysis	Positive
Gelatin liquefaction	Positive
Citrate utilization	Positive
Methyl-red (MR)	Positive
Nitrate reduction	Positive

2.3 Molecular Identification and Phylogenetic Analysis

Genomic DNA was extracted from the BIOSHIELD-L isolate, and the 16S rRNA gene was amplified using universal bacterial primers. The amplified product was sequenced, and the resulting consensus sequence was approximately 1,465 bp in length. Sequence similarity was assessed using BLAST against nucleotide sequences available in the NCBI database. The sequence showed 99.32% similarity to Bacillus pumilus. The isolate was therefore identified as B. pumilus BIOSHIELD-L on the basis of the sequence result in conjunction with its morphological and biochemical characteristics. Phylogenetic analysis was conducted using the neighbour-joining method. The sequence has not yet been deposited in GenBank; no accession number is available.

2.4 Antimicrobial Activity Assay

The antimicrobial activity of the cell-free culture supernatant and ethyl-acetate crude extract of B. pumilus BIOSHIELD-L was evaluated by the agar-well diffusion method [6]. The test organisms were Aeromonas hydrophila, Edwardsiella tarda, Pseudomonas fluorescens, Flavobacterium columnare, Streptococcus agalactiae and Vibrio anguillarum. Mueller–Hinton agar plates were seeded with standardized bacterial inocula. Wells of 6 mm diameter were prepared and filled

with the respective test preparations. The crude extract was reconstituted in dimethyl sulfoxide (DMSO). Plates were incubated at 37 °C for 24 h, after which inhibition-zone diameters were measured in millimetres. Standard antibiotic discs and DMSO were included as positive and negative controls, respectively. Assays were performed in triplicate, and results are expressed as mean ± SD.

Table 2. Antimicrobial Activity of Cell-Free Culture Supernatant and Ethyl-Acetate Crude Extract of *Bacillus pumilus* BIOSHIELD-L Against Selected Aquaculture Pathogens

Test organism	Cell-free supernatant (mm)	Ethyl-acetate crude extract (mm)
Aeromonas hydrophila	11.4 ± 0.8	16.2 ± 1.1
Edwardsiella tarda	10.2 ± 0.6	15.4 ± 0.9
Pseudomonas fluorescens	12.1 ± 0.7	17.8 ± 1.2
Flavobacterium columnare	9.8 ± 0.5	14.1 ± 0.8
Streptococcus agalactiae	13.5 ± 0.9	18.6 ± 1.3
Vibrio anguillarum	12.8 ± 0.7	17.2 ± 1.0

2.5 Crude Secondary-Metabolite Extraction

BIOSHIELD-L was cultivated in nutrient broth at 37 °C for 48 h under agitation. The culture broth was centrifuged at 10,000 rpm for 15 min to separate the bacterial biomass from the culture supernatant. The supernatant was extracted twice with an equal volume of analytical-grade ethyl acetate (1:1, v/v) using a separating funnel. The organic phase was collected, dehydrated over anhydrous sodium sulfate and concentrated by rotary evaporation under reduced pressure. The resulting dried crude extract was collected for subsequent analyses. The extraction yield was 0.42 g per litre of culture broth.

2.6 Antioxidant and Anti-Inflammatory Assays

The antioxidant potential of the crude extract was assessed using DPPH and ABTS radical-scavenging assays, total antioxidant capacity and reducing-power assay. DPPH activity was determined following the method of Brand-Williams et al. [7]. Total antioxidant capacity was expressed as milligrams of ascorbic acid equivalents per gram of extract (mg AAE/g extract), and reducing power was recorded as absorbance at 700 nm. Anti-inflammatory potential was evaluated using protein-denaturation inhibition, albumin-denaturation inhibition and heat-induced membrane-stabilization assays. Results were expressed as percentage activity or inhibition, as appropriate.

Table 3. Antioxidant and Anti-Inflammatory Activities of the Crude Extract of *Bacillus pumilus* BIOSHIELD-L

Assay parameter	Measured activity (mean ± SD)
DPPH radical scavenging (%)	78.6 ± 1.8
ABTS radical scavenging (%)	82.4 ± 1.6
Total antioxidant capacity (mg AAE/g extract)	146.3 ± 4.2
Reducing power (A700 nm)	0.86 ± 0.03
Protein-denaturation inhibition (%)	71.3 ± 2.1
Albumin-denaturation inhibition (%)	68.7 ± 1.9
Heat-induced membrane stabilization (%)	63.9 ± 2.3

2.7 Cytotoxicity and Cell Viability Assessment

The cytotoxicity of the crude extract was evaluated using the human embryonic kidney cell line HEK-293 by the MTT colorimetric assay [8]. Cells were exposed to extract concentrations of 25, 50, 100 and 200 µg/mL for 24 h. DMSO was maintained as the vehicle control. Cell viability was calculated relative to the control and expressed as a percentage.

Table 4. Effect of Different Concentrations of *Bacillus pumilus* BIOSHIELD-L Crude Extract on HEK-293 Cell Viability

Extract concentration (µg/mL)	HEK-293 cell viability (%)
25	96.1 ± 1.5
50	93.2 ± 1.7
100	90.4 ± 1.9
200	86.7 ± 1.8
DMSO control	100.0 ± 0.0

2.8 Gas Chromatography–Mass Spectrometry (GC-MS) Profiling

The ethyl-acetate crude extract was analysed by gas chromatography–mass spectrometry to characterize its chemical constituents. Compounds were assigned by comparison of their mass-spectral fragmentation patterns with entries in the NIST spectral library. Retention times and relative peak areas were recorded, with relative abundance expressed as a percentage of the total chromatographic peak area.

Table 5. GC-MS Profile of the Ethyl-Acetate Crude Extract of *Bacillus pumilus* BIOSHIELD-L

Compound	Retention time (min)	Relative peak area (%)
Hexadecanoic acid, methyl ester	14.72	18.45
9-Octadecenoic acid, methyl ester	17.21	18.32
Phenol, 2-methoxy-4-(2-propenyl)-	21.08	12.64
n-Hexadecanoic acid	22.38	10.73
Phytol	26.14	8.96
Other minor constituents	—	33.90

2.9 Plant-Growth-Promotion Pot Experiment

Plant-growth-promoting potential was evaluated in a pot experiment comparing untreated control plants with plants treated with B. pumilus BIOSHIELD-L. At the end of the trial, germination percentage, root length, shoot length, fresh weight and dry weight were recorded. Germination was expressed as a percentage, root and shoot lengths in centimetres, and fresh and dry weights in grams per plant. Percentage increase was calculated relative to the untreated control.

Table 6. Effect of *Bacillus pumilus* BIOSHIELD-L Treatment on Plant-Growth-Promoting Parameters

Parameter	Control (mean ± SD)	BIOSHIELD-L treatment (mean ± SD)	Increase (%)
Germination (%)	68.0 ± 3.1	92.0 ± 2.3	35.29
Root length (cm)	6.8 ± 0.6	10.9 ± 0.7	60.29
Shoot length (cm)	12.6 ± 0.8	18.6 ± 0.9	47.62
Fresh weight (g/plant)	0.85 ± 0.08	1.38 ± 0.06	62.38
Dry weight (g/plant)	0.18 ± 0.02	0.31 ± 0.02	72.22

2.10 Statistical Analysis

Quantitative data are presented as mean ± standard deviation (SD). The experimental assays were reported as having three independent biological and technical replicates (n = 3). Statistical evaluation was performed using analysis of variance (ANOVA), followed by an appropriate post-hoc test. The significance threshold was set at $p < 0.05$.

3. Results

3.1 Isolation and Characterization of BIOSHIELD-L

The selected BIOSHIELD-L isolate produced irregular, cream-coloured, rough and opaque colonies on nutrient agar. Microscopic examination showed Gram-positive, rod-shaped cells with endospores. The isolate was positive for catalase, starch hydrolysis, gelatin liquefaction, citrate utilization, methyl-red and nitrate-reduction tests, whereas oxidase, indole and Voges–Proskauer tests were negative (Table 1).

3.2 Molecular Identification

The 16S rRNA gene sequence of BIOSHIELD-L was approximately 1,465 bp and showed 99.32% similarity to B. pumilus in the NCBI nucleotide database. The phylogenetic analysis placed the isolate with closely related B. pumilus sequences. The sequence has not yet been deposited in GenBank.

3.3 Antimicrobial Activity

Both the cell-free culture supernatant and ethyl-acetate crude extract showed inhibitory activity against the six tested aquaculture pathogens (Table 2; Figure 1). The inhibition zones produced by the supernatant ranged from 9.8 mm against F. columnare to 13.5 mm against S. agalactiae. The crude extract produced zones ranging from 14.1 mm against F. columnare to 18.6 mm against S. agalactiae. For each test organism, the crude extract produced a larger inhibition zone than the supernatant.

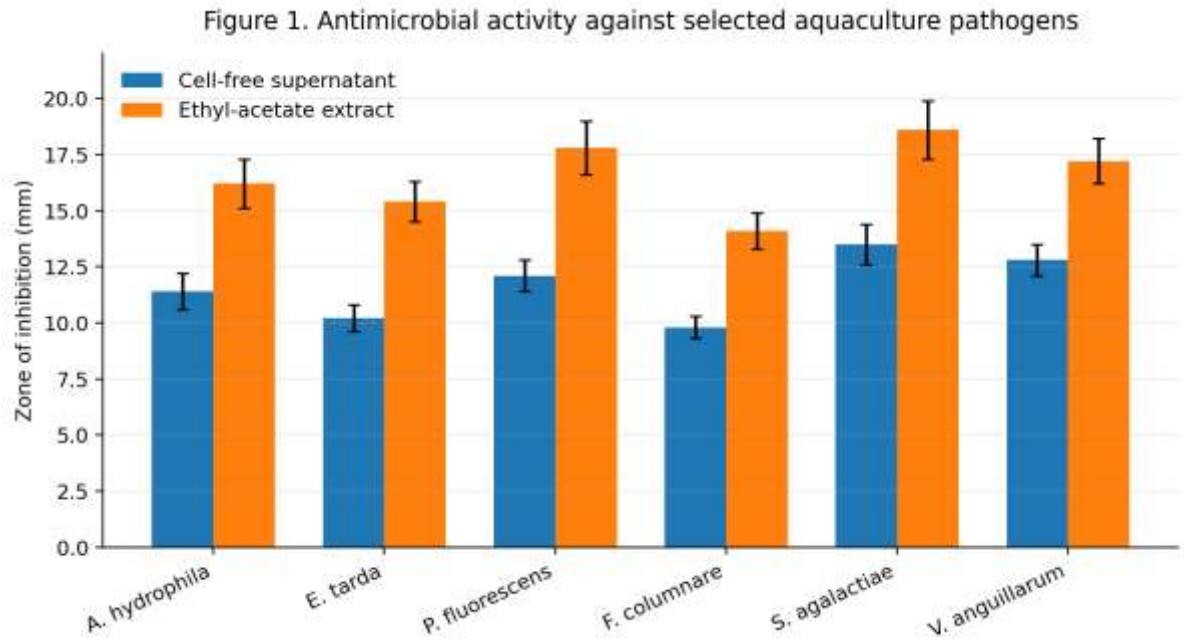


Figure 1. Inhibition-zone diameters produced by cell-free supernatant and ethyl-acetate crude extract against the tested aquaculture pathogens.

3.4 Crude-Extract Yield, Antioxidant and Anti-Inflammatory Activities

Ethyl-acetate extraction yielded 0.42 g of crude extract per litre of culture broth. The extract showed DPPH and ABTS radical-scavenging activities of 78.6 ± 1.8% and 82.4 ± 1.6%, respectively. Total antioxidant capacity was 146.3 ± 4.2 mg AAE/g extract, while reducing power was 0.86 ± 0.03 at 700 nm. Protein-denaturation inhibition was 71.3 ± 2.1%, albumin-denaturation inhibition was 68.7 ± 1.9%, and heat-induced membrane stabilization was 63.9 ± 2.3% (Table 3; Figure 2).

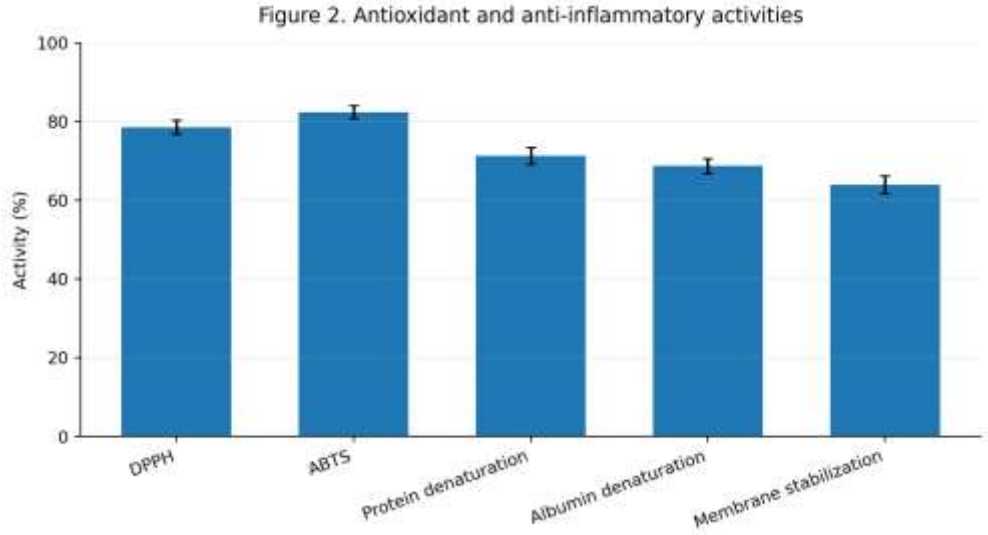


Figure 2. Percentage values for radical-scavenging and in-vitro anti-inflammatory assays. Total antioxidant capacity and reducing power are reported separately in Table 3.

3.5 Cytotoxicity Assessment

HEK-293 cell viability decreased gradually with increasing extract concentration. Viability values were 96.1 ± 1.5%, 93.2 ± 1.7%, 90.4 ± 1.9% and 86.7 ± 1.8% at 25, 50, 100 and 200 µg/mL, respectively, compared with 100.0 ± 0.0% in the DMSO control (Table 4; Figure 3).

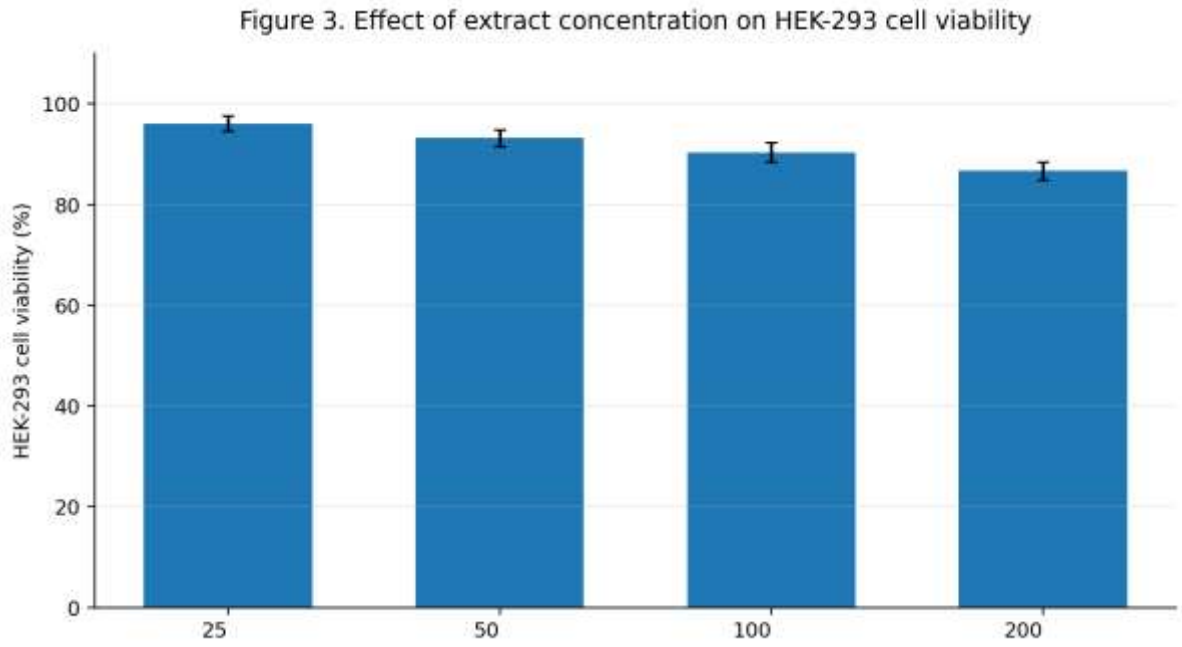


Figure 3. HEK-293 cell viability following 24-h exposure to different concentrations of BIOSHIELD-L crude extract.

3.6 GC-MS Profile

GC-MS analysis identified five major constituents with individual relative peak areas of 18.45%, 18.32%, 12.64%, 10.73% and 8.96%, respectively (Table 5; Figure 4). These corresponded to hexadecanoic acid methyl ester, 9-octadecenoic acid methyl ester, phenol, 2-methoxy-4-(2-propenyl)-, n-hexadecanoic acid and phytol. The five listed constituents accounted for 69.10% of the total peak area; other minor constituents represented 33.90% as reported in the chromatographic profile.

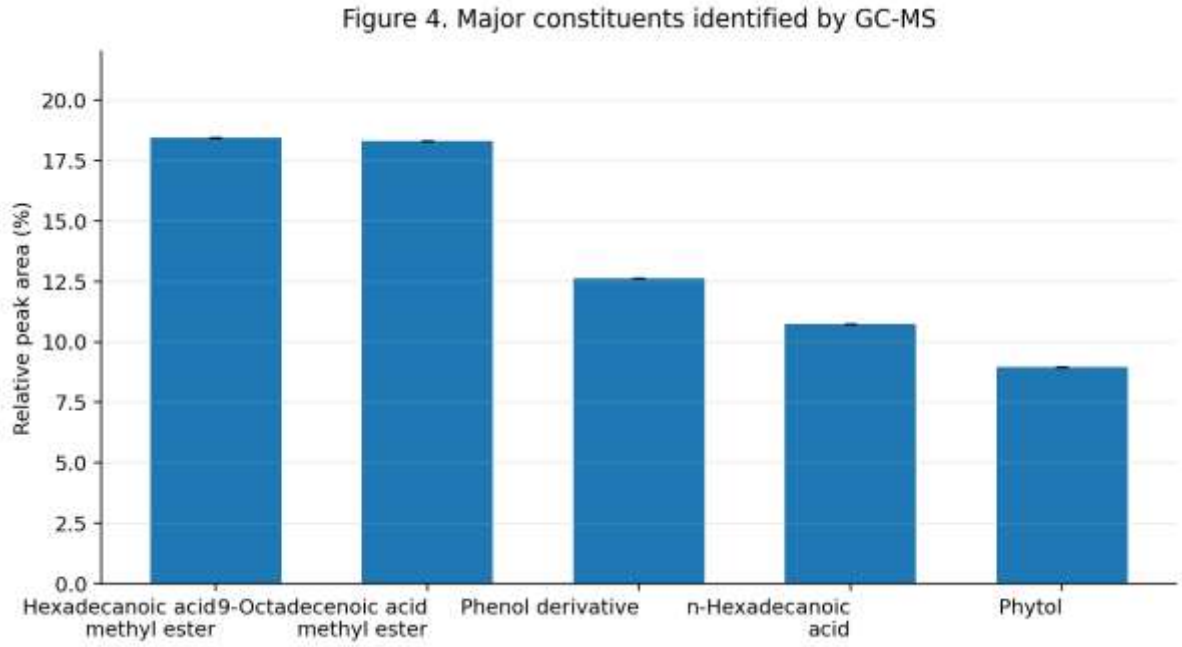


Figure 4. Relative peak areas of the five major compounds reported in the GC-MS profile.

3.7 Plant-Growth-Promotion Response

BIOSHIELD-L-treated plants showed higher mean values than the untreated control for all five measured growth parameters (Table 6; Figure 5). Germination increased from 68.0 ± 3.1% in the control to 92.0 ± 2.3% in the treated group, corresponding to a 35.29% increase. Root length increased by 60.29%, shoot length by 47.62%, fresh weight by 62.38% and dry weight by 72.22% relative to the control.

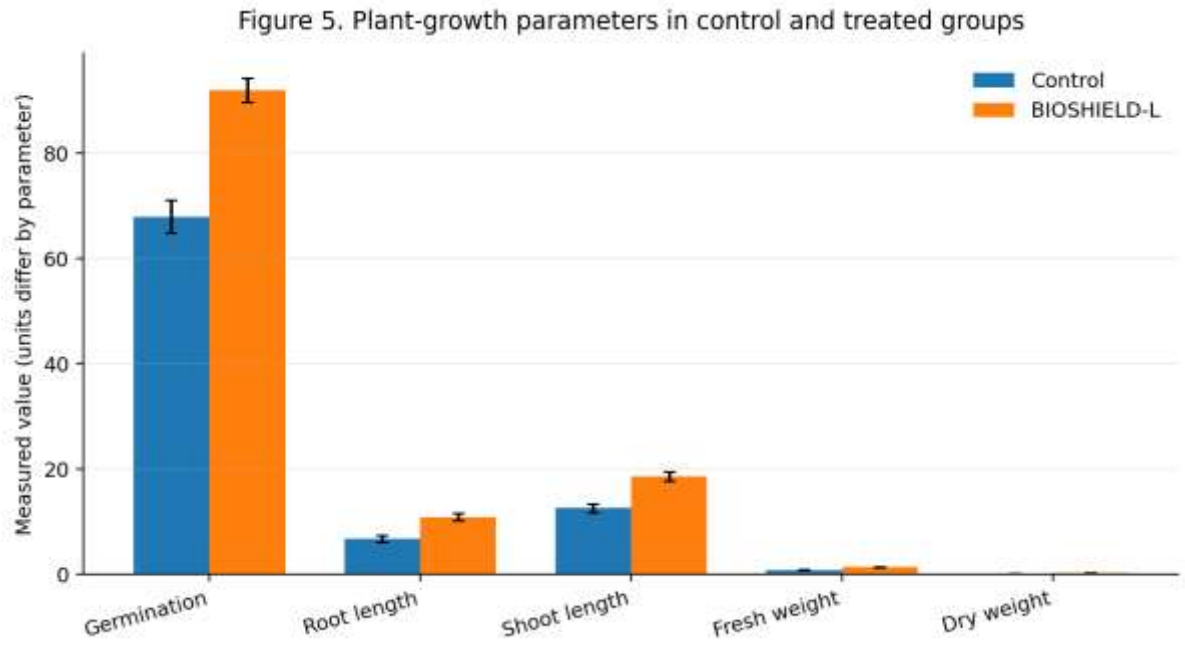


Figure 5. Control and BIOSHIELD-L treatment means for plant-growth parameters. Note that the parameters use different measurement units and should be interpreted individually.

4. Discussion

The recovery of B. pumilus BIOSHIELD-L from cow farmyard manure is consistent with the recognized role of manure as a reservoir of diverse bacteria, including spore-forming taxa with potential agricultural value [1–3]. The isolate's Gram-positive, endospore-forming morphology and biochemical profile supported its preliminary characterization. The 16S rRNA sequence similarity of 99.32% to B. pumilus provided molecular support for the species assignment. Because 16S rRNA similarity alone may not resolve all closely related Bacillus taxa, additional taxonomic confirmation, where required, could be obtained through genome-based analysis.

The cell-free supernatant and crude extract inhibited all six tested aquaculture pathogens under the assay conditions. The larger inhibition zones associated with the ethyl-acetate fraction suggest that inhibitory constituents were recoverable in the organic phase. Bacillus species are known to produce diverse antimicrobial metabolites, including lipopeptides and other secondary metabolites; however, the present GC-MS profile does not by itself establish which compounds were responsible for the observed inhibition. Further purification and compound-specific testing would be required to identify the active principles.

The crude extract also displayed measurable radical-scavenging and in-vitro anti-inflammatory activities. The DPPH and ABTS results indicate activity in two different radical-scavenging systems, while the protein-denaturation and membrane-stabilization assays provide additional preliminary evidence of in-vitro bioactivity. These assay outcomes should be interpreted as screening results and do not establish therapeutic efficacy in animals or humans.

HEK-293 viability remained at 86.7% at the highest tested concentration of 200 µg/mL. This finding indicates that the extract did not cause a marked reduction in cell viability under the reported in-vitro conditions. Nevertheless, evaluation in one cell line and over a limited concentration range is insufficient to establish overall safety. Additional cell models, dose ranges and exposure periods would be needed before considering applications involving animals, crops or environmental release.

The GC-MS profile contained fatty acid derivatives, a phenolic compound and phytol among the major reported peaks. Such chemical assignments provide a preliminary description of the extract; library-based identification should ideally be confirmed using authentic standards and complementary analytical methods. The relative peak areas describe chromatographic abundance and should not be interpreted as absolute concentrations.

In the pot experiment, the BIOSHIELD-L treatment group showed higher mean germination, root length, shoot length, fresh weight and dry weight than the untreated control. These observations are consistent with the potential of selected

Bacillus strains to support plant growth [13,14]. The present experiment demonstrates a growth-associated response but does not identify the mechanism. Future work could examine traits such as indole-3-acetic acid production, phosphate solubilization, siderophore production and root colonization, provided these are measured directly.

Overall, the results support further investigation of BIOSHIELD-L as a multifunctional bacterial isolate. Before product development, the isolate requires additional characterization, reproducibility testing, formulation assessment and controlled field validation. Its possible use in aquaculture should also be assessed through pathogen-specific efficacy, host safety and appropriate fish-based trials.

5. Conclusion

Bacillus pumilus BIOSHIELD-L, isolated from cow farmyard manure, exhibited antimicrobial activity against six selected aquaculture pathogens, antioxidant and in-vitro anti-inflammatory activity, comparatively high HEK-293 cell viability across the tested concentration range, and positive plant-growth responses in a pot experiment. GC-MS analysis provided a preliminary profile of the major constituents in the ethyl-acetate crude extract. These findings identify BIOSHIELD-L as a candidate for further research in plant-growth promotion and microbial bioactive-product development. Further taxonomic confirmation, metabolite characterization, safety evaluation, formulation studies and field-scale validation are required before practical application.

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