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BACILLUS SP. FROM *POUZOLZIA ZEYLANICA*: A PROMISING SOURCE OF ANTIOXIDANTS OPTIMIZED FOR PRACTICAL APPLICATIONS

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ABSTRACT

Endophytic bacteria are increasingly being considered as precious sources of bioactive metabolites with diverse antioxidant properties, offering significant potential for medical and food applications. This study focused on identifying and characterizing antioxidant-producing endophytes from *Pouzolzia zeylanica*, a traditional herbal medicine commonly used in the Mekong Delta. A total of 26 endophytic bacterial strains were screened for antioxidant activity, and *Bacillus* sp. PZ-R1 emerged as the most promising candidate. The secondary metabolites of this strain were extracted using ethyl acetate and optimized for antioxidant compound production using response surface methodology. The optimized fermentation conditions for antioxidant metabolite production were: 28.37 g/L glucose, an initial pH of 7.89, a temperature of 31.36°C, and an incubation period of 45.09 hours Under these conditions, the strain produced remarkable antioxidant metrics, including total phenol content (71.69 mg GAE/mL), total flavonoid content (93.61 mg QE/mL), total antioxidant capacity (184.44 mg AAE/mL), reducing power (266.59 mg AAE/mL), and ferric reducing antioxidant power activity (91.01 mg AAE/mL). These findings underscore the remarkable potential of endophytic bacteria from *P. zeylanica* as a source of bioactive metabolites, paving the way for further research into their practical applications in the health and food industries.

Keywords: Antioxidant, *Bacillus*, Bacteria, Central composite design, Endophyte, *Pouzolzia zeylanica*

INTRODUCTION

Lipid oxidation occurs during food processing and within organisms, primarily through the oxidation of polyunsaturated fatty acids in cell-membrane phospholipids **(Kodali *et al.*, 2020)**. Fat-rich foods increase health risks due to oxidative stress and its associated diseases **(Barden & Decker, 2016; Reddy, 2023).** Noncommunicable diseases (NCDs)—including diabetes, cardiovascular diseases, and cancer—account for most deaths among people aged 30–70 years (**Razzaghi *et al.*, 2019; WHO, 2022**). These conditions are closely linked to free radicals, reactive oxygen species (ROS), and reactive nitrogen species (RNS) generated in aerobic organisms **(Zhang *et al.*, 2021)**. Although the body's antioxidant defense system helps neutralize these reactive species, excessive ROS can damage DNA, RNA, and membrane lipids, contributing to various diseases and NCDs **(Ruiz *et al.*, 2021)**. Therefore, adequate dietary intake of antioxidants is essential for mitigating the harmful effects of ROS and free radicals.

Antioxidants can neutralize free radicals and transform ROS into benign compounds. Some antioxidants are produced endogenously in the human body, whereas others must be obtained exogenously through dietary intake or supplementation, particularly in cases of illness or deficiency. Recent studies have shown a growing preference for natural antioxidants over synthetic antioxidants. Antioxidants derived from natural sources such as plants, animals, and microorganisms, including polyphenols **(Yan *et al.*, 2020)**, polysaccharides **(Zhong *et al.*, 2019)**, and peptides **(Wen *et al.*, 2020)**, have emerged as promising candidates owing to their antioxidant properties.

Plants are constantly exposed to abiotic challenges such as temperature fluctuations, salt stress, drought, and heavy metals. To cope with these stresses, plants rely on both non-enzymatic and enzymatic antioxidant defense mechanisms**(Kaur *et al.*, 2019)**. They also interact with a diverse range of endophytic microbes that enhance plant resilience by producing secondary metabolites **(Turner *et al.*, 2013)**. Plants are populated with a diverse range of endophytic bacterial species, which increase their biological activity by producing secondary compounds.

Endophytic bacteria have garnered considerable interest for their ability to generate structurally diverse bioactive compounds with antibacterial, antifungal, anticancer, anti-inflammatory, and antioxidant activities **(Rahman *et al.*, 2017; Singh *et al.*, 2017; Photolo *et al.*, 2020; Sithara K. Urumbil & Anilkumar, 2021; Nisa *et al.*, 2022; Damavandi *et al.*, 2023)**. In particular, endophytic Bacillus spp. produce metabolites with cytotoxic **(Matloub *et al.*, 2020)**, anti-inflammatory **,** antifungal fungi **(Sithara K Urumbil & Kumar, 2020; Moura *et al.*, 2021; Nisa *et al.*, 2022)**, and antidiabetic effects **(Chigurupati *et al.*, 2021)**. Owing to their stability and spore-forming ability, various Bacillus species are widely used in food, pharmaceuticals, cosmetics, and probiotic formulations **(N. K. Lee *et al.*, 2019; Na-Kyoung Lee *et al.*, 2023)(Sorokulova, 2013.** Thus, the identification of plant-associated Bacillus strains represents an important avenue for discovering new natural antioxidants.

Pouzolzia zeylanica (L.) Benn *(P. zeylanica* L.) Benn. is a medicinal herb commonly found in the Mekong Delta region of Vietnam and is traditionally used to treat diarrhea, indigestion, malnutrition, urinary disorders, and inflammatory injuries **(Hossain *et al.*, 2017)**. The plant is rich in phenolic and flavonoid compounds that exhibit strong antioxidant properties; however, extracting these compounds from plant biomass can negatively impact natural populations. Previous research has identified endophytic bacteria in P. zeylanica with antibacterial activity **(Huan *et al.*, 2022)**, but no study has examined their antioxidant potential.

P. zeylanica L. has been extensively studied for its antioxidant components and traditional medicinal use. Nevertheless, there is a paucity of data concerning its acute or subchronic toxicity as determined by standardized evaluations, such as those outlined in the OECD Guidelines for the Testing of Chemicals **(OECD, 2018, 2022)**, raising concerns regarding its safe oral consumption. Additionally, the composition and efficacy of plant-derived antioxidants are significantly influenced by environmental conditions, and the overharvesting of *P. zeylanica* threatens the ecological balance. To mitigate these challenges, controlled fermentation using endophytic *Bacillus* spp. offers a promising alternative.

Despite extensive research on antioxidant-producing *Bacillus* spp., the endophytic microbiome of *P. zeylanica*—a phenolic- and flavonoid-rich medicinal herb—remains completely uncharacterized. Given that endophytes often reflect or

complement the metabolic functions of their host plants, it is plausible that *P. zeylanica* harbors bacterial strains capable of synthesizing antioxidant metabolites similar to those found in the plant. To date, however, no research has investigated this metabolic potential. The present study therefore provides the first isolation, screening, and characterization of antioxidant-producing endophytic Bacillus strains associated with *P. zeylanica*, offering a sustainable microbial alternative to harvesting plant biomass. Although medium-only extraction controls were not included in this initial work, this limitation is acknowledged and addressed in the Discussion.

This study was conducted to screen, identify, and characterize bacterial endophytes with antioxidative properties from *P. zeylanica* isolated in a previous study **(Huan *et al.*, 2022)**. Ethyl acetate extracts of the bacterial endophytes were examined. Endophytic bacteria with the highest antioxidant activity were optimized for antioxidant compound generation using response surface methodology (RSM).

MATERIALS AND METHODS

Plant samples and endophytic bacteria strains

The study process was shown in the Figure 1. The *Pouzolzia zeylanica* (PZ) was collected in January 2022 in Can Tho City, Vietnam. The botanical properties of the plants were identified (voucher specimen, CT_Pz2022010001). *P. zeylanica* specimens in the Laboratory of Plants and Animals, Department of Biology, College of Natural Sciences, Can Tho University. After collection, the fresh plant samples were placed in sterile polyethylene bags, transported to the laboratory under cooled conditions, and stored at 4°C in a refrigerated chamber. All samples were processed for endophytic bacterial isolation within 24 hours to ensure tissue integrity and prevent microbial contamination.

The bacteria used in this study were obtained and 26 endophytic bacterial strains were isolated from *P. zeylanica*, as described in a previous study **(Huan *et al.*, 2022)** and stored at the Cell and Molecule Laboratory, Department of Biology, College of Natural Sciences, Can Tho University.

Culturing endophytic bacteria

Endophytes were initially cultivated in potato dextrose broth (PDB) following the method outlined by **Dai *et al.* (2024)**. They were agitated on an orbital shaker (New Brunswick™ Innova® 44, Eppendorf, USA) at 200 rpm and maintained at 30°C for 24 h. Sterile PDB medium was added or removed as needed to reach the desired optical density. For seed culture preparation, 10 mL of the OD-adjusted inoculum was transferred into a 250 mL Erlenmeyer flask containing 200 mL of sterile PDB medium. The flask was incubated at 30°C for 48 hours in an orbital shaker incubator (New Brunswick™ Innova® 44, Eppendorf, USA) at 200 rpm. After incubation, the cultures were centrifuged at 3000 rpm for 10 minutes at 4°C using the Kubota 5500 centrifugal (Kubota, Osaka, Japan), and the resulting cell-free supernatant was collected for further experiments.

Metabolite extraction

To obtain secondary metabolites, 200 µL of the bacterial suspension (OD_{600} = 0.5) was inoculated into 9.8 mL of PDB and incubated at 30°C for 24 hours on the orbital shaker at 200 rpm. The culture was then centrifuged at 3000 rpm for 30 minutes using the Hermle Z326K refrigerated centrifuge (Hermle GmbH, Germany). The supernatant was collected, and the pellet was discarded. For metabolite extraction, endophytic bacteria were cultured in 1 L of PDB under the same optimized conditions. After incubation, approximately 500 mL of cell-free supernatant was obtained and mixed with an equal volume (500 mL) of ethyl acetate. The mixture was left overnight at 4°C to allow phase separation. The ethyl acetate–supernatant mixture was transferred into a 1 L glass separating funnel (Duran®, Germany). After standing at 4°C, two distinct layers formed: an upper ethyl acetate layer containing the extracted secondary metabolites and a lower aqueous phase. Complete phase separation was confirmed when both layers appeared clear with no emulsion. The ethyl acetate layer was collected and concentrated using a rotary evaporator (Rotavapor® R-300, Büchi, Switzerland) at 50°C and 90 rpm, yielding crude extracellular metabolites. The remaining cell pellet was resuspended in DMSO for intracellular metabolite analysis. All concentrated extracts were stored at 4°C for later use **(Beiranvand *et al.*, 2017)**.

In vitro Determining of antioxidant activity of endophytic bacterial supernatant and crude extract

All 26 endophytic bacterial strains isolated from *P. zeylanica* were initially screened for antioxidant activity using their cell-free supernatants. Antioxidant capacity was quantified using five standard assays—TPC, TFC, TAC, RP, and FRAP—and expressed as mg of standard equivalent per mL of supernatant. Based on comparative antioxidant profiles, strains exhibiting the highest overall assay values were selected for further optimization.

Total phenolic content (TPC) was determined using the Folin–Ciocalteu method **(Matić *et al.*, 2017; Dai *et al.*, 2024)** with slight modifications. Briefly, 100 µL of sample was mixed with 500 µL of 10% Folin–Ciocalteu reagent and incubated for 5 min. Then, 400 µL of 7.5% Na_2CO_3 was added, followed by a 30-min incubation at room temperature. Absorbance was read at 765 nm, and results were expressed as mg gallic acid equivalent.

Total flavonoid content (TFC) was determined using an aluminum chloride colorimetric assay **(Matić *et al.*, 2017)**. A 250 µL aliquot of sample was mixed with 75 µL of 5% $NaNO_2$. After 5 min, 150 µL of 10% $AlCl_3$ was added. After an additional 6 min, 500 µL of 1 M NaOH was added, and absorbance was measured at 510 nm. Results were expressed as mg quercetin equivalent (QE).

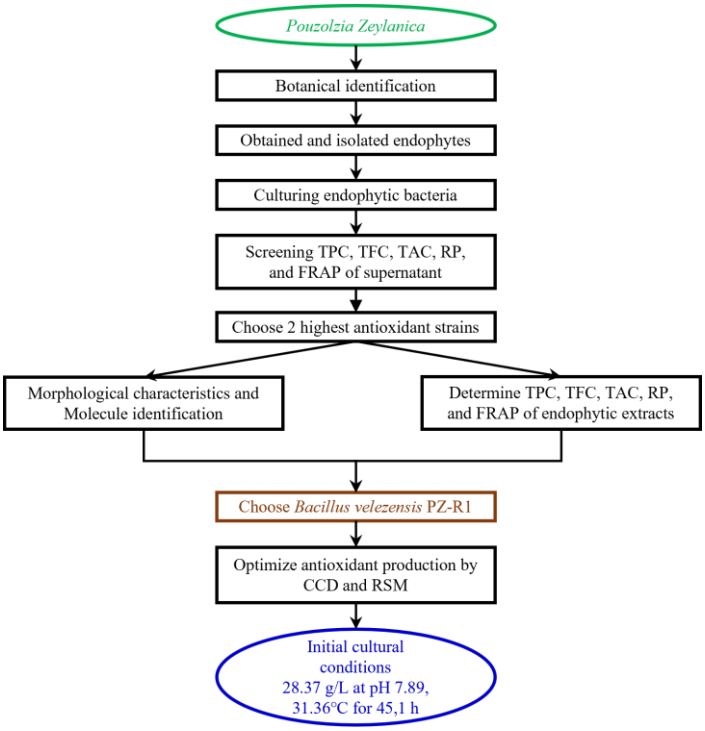


Figure 1 Flow chart of the experimental design.

Total antioxidant capacity (TAC) was evaluated using the phosphomolybdate method **(Matić *et al.*, 2017)**. A 100 µL sample was mixed with 1 mL of TAC reagent (0.6 M H_2SO_4, 28 mM Na_3PO_4, 4 mM ammonium molybdate), incubated at 95°C for 90 min, cooled to room temperature, and measured at 695 nm. Results were expressed as mg AAE equivalent (AAE).

Reducing power (RP) was assessed following **(Dai *et al.*, 2024)**. A 200 µL sample was mixed with 500 µL phosphate buffer (0.2 M, pH 6.6) and 500 µL of 1% potassium ferricyanide, then incubated at 50°C for 20 min. After adding 500 µL of 10% trichloroacetic acid, the mixture was centrifuged, and the supernatant was reacted with $FeCl_3$. Absorbance was read at 700 nm. Ferric reducing antioxidant power (FRAP) was determined by mixing 100 µL of sample with 900 µL of freshly prepared FRAP reagent (acetate buffer, TPTZ, $FeCl_3$) **(Benzie & Strain, 1996)**. The mixture was incubated for 30 min in the dark, and absorbance was measured at 593 nm. Results were expressed as mg Trolox equivalent (TE). All measurements were performed in triplicate to ensure reproducibility.

Identification of the endophytic bacteria

Identification of bacterial morphology and physiology

The morphology of bacterial cells was examined using an optical microscopy BX51TF (Olympus, Tokyo, Japan) at 1000× magnification were used to examine the bacterial cells. Gram staining followed the classical crystal violet–iodine–alcohol–safranin protocol. Crystal violet and iodine were applied sequentially, followed by ethanol decolorization and safranin counterstaining. Gram-positive cells appeared violet-blue, and Gram-negative cells appeared pink.

Scanning electron microscopy (SEM) was conducted using a Carl Zeiss EVO LS10 instrument (Carl Zeiss AG, Oberkochen, Germany). For SEM, cells were fixed in 2.5% glutaraldehyde, dehydrated through a graded ethanol series, gold-coated, and visualized at 7 – 10 kV electron beam and working distances between 6.0 and 7.5 mm, with the operating high vacuum pressure of the team was 1.97 – 3.36×10^{-4} Pa.

Molecule identification

Individual bacterial colonies were inoculated into PDB and incubated at 30°C in an orbital shaker incubator (New Brunswick™ Innova® 44, Eppendorf, Hamburg, Germany) at 200 rpm for 16–24 h. The incubation time reflects variation in bacterial growth rates; cultures were harvested once they reached mid-log phase (OD_{600} ≈ 0.8–1.0), ensuring sufficient biomass for DNA extraction. Cells were

collected by centrifugation at 10,000 rpm for 5 min using a Hermle Z326K refrigerated centrifuge (Hermle GmbH, Wehingen, Germany). Genomic DNA was extracted using an automated magnetic-bead extraction system, the Tanbead Maelstrom 4810 (Taiwan Advanced Nanotech Inc., Taoyuan City, Taiwan), following the manufacturer's protocol. Purified DNA was eluted in nuclease-free water and stored at –20°C until further analysis **(Anh *et al.*, 2021)**.
The 16s rRNA sequence of the bacterial strain was amplified by polymerase chain reaction (PCR) using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTTACGACTT-3'). The reaction was performed at 95°C (5 min), followed by 35 cycles at 94°C (1 min), 53°C (1 min), 72°C (1.5 min), and 72°C (5 min) **(Aravind *et al.*, 2009)**. PCR products were sequenced using a Macrogen sequencer (Macrogen Inc. Seoul, Korea). BLASTn searches were conducted using the National Center for Biotechnology Infor mation (NCBI) platform to determine sequence similarity with known bacterial taxa.
Phylogenetic trees were constructed using the neighbor-joining method in MEGA version 11 (Molecular Evolutionary Genetics Analysis, Temple University, Pennsylvania State University, USA). Multiple sequence alignments were generated using ClustalW, and evolutionary distances were computed using the Kimura 2-parameter model. Bootstrap analysis (1,000 replicates) was performed to assess the robustness of the phylogenetic relationships.

One-factor-at-a-time experimental design for optimization of culture condition

The primary factors influencing antioxidant generation are pH, culture temperature, time, and D-glucose concentration. Fermentation conditions were described in a previous study **(Dai *et al.*, 2024)**. The variable values of the factors in this study were initial pH (5, 6, 7, 8, and 9), D-glucose concentration (10, 20, 30, 40, and 50 g/L), culturing temperature (25, 30, 35, 40, and 45°C), and time (24, 36, 48, 60, and 72 h).
Design-Expert Software version 11 (Alfasoft AB, Göteborg, Sweden) was employed for the RSM experimental design and CCD. Optimal conditions were established using baseline parameters from one-factor experiments. Four variables influencing the production of antioxidant compounds were examined: pH ranging from 5 to 9 in increments of 1; glucose levels of 10, 20, 30, 40, and 50 g/L; growth temperatures of 25, 30, 35, 40, and 45°C; and incubation periods of 24, 36, 48, 60, and 72 h.
Optimization of the culture conditions for antioxidant production was achieved through a combination of one-factor-at-a-time experiments, central composite design (CCD), and response surface methodology (RSM). This process involves four key factors: pH (X1), glucose concentration (X2), cultivation temperature (X3), and cultivation time (X4). Each factor was evaluated on five coded levels (-a, -1, 0, +1, +a), as outlined in a previous study (Table 1) **(Dai *et al.*, 2024)**. The model was carried out with 30 experiments, including six center ones. Regression analysis was used to ascertain the coefficients of the RSM model, and mathematical analysis was applied to pinpoint the extreme points and their respective values of the independent variables. The equation below illustrates the connection between the coded and actual values, which is crucial for statistical computations.

$$x_i = (X_i - X_0)/\Delta X_i \quad (1)$$

where x_i represents the coded value of X_i. X_0 is the value of corresponding X_i and the central point and ΔX_i is the step change value.

Table 1 Factors affect the cultivation of the bacterium

Factors	Levels of experiment					
	-α	Lower level, (-1)	Medium, level (0)	Upper level, (+1)	+α	ΔX_i
pH (X_1)	5	6	7	8	9	1
Glucose concentration (X_2, g/L)	10	20	30	40	50	10
Culture temperature (X_3, °C)	25	30	35	40	45	5
Culture time (X_4, h)	24	36	48	60	72	12

The output data were analyzed using multiple regression analysis and a second-order polynomial model.

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i<j;i,j=1}^{4} \beta_{ij} X_i X_j + \sum_{i=1}^{4} \beta_{ii} X_{ii}^2$$

where Y is the predicted response of the antioxidant produced, β_0 is the intercept coefficient, β_i and β_{ij} are the linear coefficients.

The suitability and reliability of the fitted RSM models were evaluated through analysis of variance (ANOVA). Model significance was assessed using the model F-value and associated p-value, with $P < 0.05$ indicating a statistically significant model. The coefficient of determination (R^2) and adjusted R^2 were used to assess the proportion of variability explained by the model. A non-significant lack-of-fit ($P > 0.05$) indicated that the model adequately described the experimental data. Additionally, the agreement between predicted and experimental values, as well as diagnostic plots of residual distribution, were inspected to validate the assumptions of regression analysis. Where applicable, adequate precision values greater than 4 were considered to indicate an adequate signal-to-noise ratio for navigating the design space.

Validation of RSM-Optimized Conditions

To validate the predictive accuracy of the RSM model, *Bacillus* sp. PZ-R1 was cultured under the optimal conditions obtained from numerical optimization (pH 7.89, 28.37 g/L glucose, 31.36 °C, and 45.09 h). After incubation, the culture broth was centrifuged at 3,000 rpm for 10 min at 4 °C, and the resulting cell-free supernatant was collected. The antioxidant properties (TPC, TFC, TAC, RP, and FRAP) of the supernatant were quantified using the same analytical procedures described in Section "In vitro determination of antioxidant activity." These experimentally measured antioxidant concentrations were designated as "observed values" and compared with the "predicted values" generated by the quadratic regression model. This comparison was used to evaluate model accuracy.

Data analysis

GraphPad Prism software (GraphPad Software, LLC, US) were used for graphing and conducting ANOVA and Tukey's test for data of the corresponding figures. The tests were analyzed using Minitab 16 (Sydney, NSW, Australia) for date in tables. The data are expressed as the mean ±SD from three independent experiments, with differences considered statistically significant at $P^* < 0.05$.

Accession numbers of bacterial isolates

The nucleotide sequences of the endophytic bacteria identified in this study were submitted to the NCBI repository. These sequences will be available in the GenBank/DDBJ Nucleotide Sequence Database with accession numbers PQ328023 for PZ-R1 and PQ341171 for PZ-L9.

RESULTS AND DISCUSSION

Antioxidative activities of *P. zeylanica* endophytic bacteria

In this study, endophytic strains of *P. zeylanica* collected and preserved in a glycerol solution at –40°C were isolated from various parts of the plant, including the leaves (PZ-L, 9 strains), stems (PZ-S, 3 strains), and roots (PZ-R, 14 strains). Our investigation revealed that all strains exhibited the capacity to produce antioxidative compounds, albeit at varying levels of activity (Table 2). The antioxidant content varied depending on the microorganisms isolated from different parts of the PZ (Figure 2A and B). The correlation coefficients (Pearson's R) between the antioxidants and the variable were greater than 0.85 indicating a very strong linear correlation between the two corresponding variables and the antioxidant content in the same strain increase go together with the increase of the other antioxidants (Figure 2C).
Among the 26 strains of *P. zeylanica* endophytes screened, PZ-R1 and PZ-L9 exhibited the highest antioxidant activity. The TPC and TFC ranged from 11.38 to 35.23 mg GAE/mL and 9.36 to 25.31 mg QE/mL, respectively. Additionally, the TAC, RP, and FRAP activities ranged from 33.11 to 109.10, 72.82 to 227,28, and 19.40 to 62.20 mg AAE/mL, respectively. Notably, PZ-R1 displayed the highest levels of activity among the samples, which prompted further investigation into its potential applications.
Endophytes can synthesize antioxidants that are identical to those found in trees. To confirm the antioxidant activity of endophytic bacteria, ethyl acetate extracts from bacterial cell-free supernatants were evaluated for antioxidative activity. Moreover, **Hossain *et al.* (2017)** reported that *P. zeylanica* scavenges free radicals while producing antioxidants and phenolics. Thus, endophytic bacteria isolated from plants exhibit antioxidant activities. Our findings showed that the antioxidant activity of *P. zeylanica* endophytic bacteria was similar to that of their plant hosts. Although optimization of antioxidant production in Bacillus spp. has been widely reported, the endophytic microbiome of *Pouzolzia zeylanica* remains completely unexplored. *P. zeylanica* is a phenolic- and flavonoid-rich medicinal herb, suggesting that its endophytes may possess metabolic pathways analogous to the plant's antioxidant system. However, no previous study has characterized the plant–microbe relationship or assessed whether antioxidant-related metabolites could originate from its endophytic bacteria.
The present study provides the first isolation, antioxidant screening, and physiological characterization of *Bacillus* endophytes derived from *P. zeylanica.* The strong antioxidant activities observed in PZ-R1 and PZ-L9 support the hypothesis that the antioxidative potential traditionally attributed to the plant may, in part, involve contributions from its microbiota. This microbial source offers an

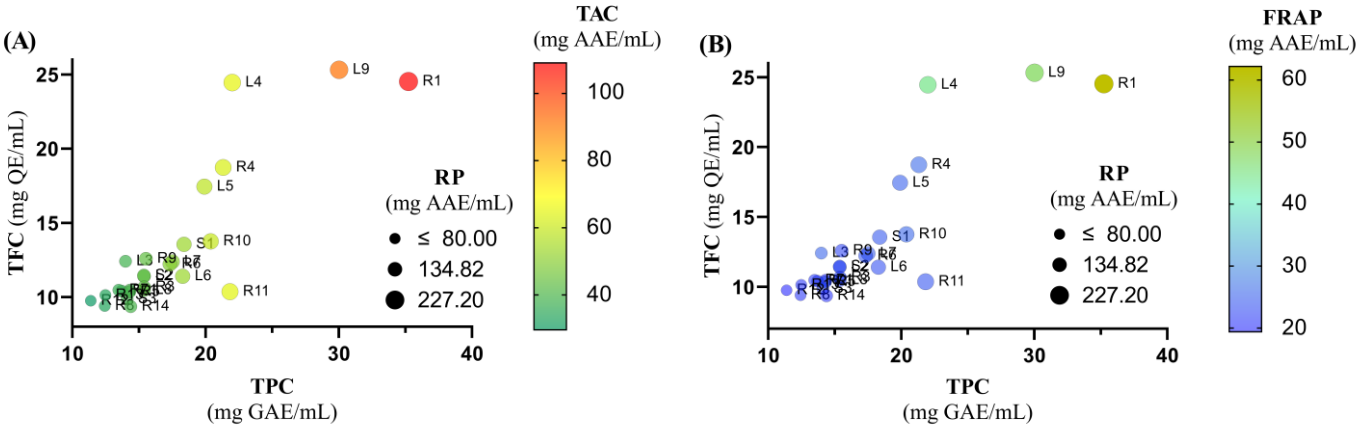
ecologically sustainable alternative to plant-based antioxidant harvesting, reducing pressure on *P. zeylanica* biomass.

A critical limitation of the study is the absence of cell-free uninoculated medium controls, which prevents definitive attribution of metabolites solely to bacterial biosynthesis. While subsequent experiments will incorporate these controls, this limitation is explicitly acknowledged here and addressed in the latter discussion.

Table 2 Antioxidant contents of cell-free supernatant endophytes

No.	Strain code	TPC (mg GAE/mL)	TFC (mg QE/mL)	TAC (mg AAE/mL)	RP (mg AAE/mL)	FRAP (mg AAE/mL)
1	PZ-L1	14.32±0.56^{hi}	10.46±0.37^{ef}	39.41±1.97^{hi}	102.24±5.11^{hi}	20.20±1.01^{fg}
2	PZ-L2	15.38±0.62^{gh}	11.40±0.40^{de}	42.94 ±2.14^{gh}	112.81±5.64^{gh}	23.40±1.17^{c-g}
3	PZ-L3	13.99±0.29^{hi}	12.41±0.47^{cd}	38.33±1.92^{hi}	98.98±4.95^{hi}	25.70±1.28^{cd}
4	PZ-L4	22.00±1.10^{c}	24.45±1.07^{a}	65.01±3.25^{c}	187.42±9.37^{b}	47.00±2.35^{b}
5	PZ-L5	19.91±0.84^{cde}	17.44±0.72^{b}	58.06±2.90^{cde}	158.17±7.91^{cd}	25.20±1.26^{cde}
6	PZ-L6	18.28±0.76^{def}	11.40±0.42^{de}	52.61±2.63^{def}	141.83±7.09^{de}	23.60±1.18^{c-g}
7	PZ-L7	17.51±0.72^{efg}	12.38±0.47^{cd}	50.06±2.50^{efg}	134.18±6.71^{ef}	24.70±1.23^{c-f}
8	PZ-L8	15.38±0.61^{gh}	10.47±0.37^{ef}	42.94±2.15^{gh}	112.82±5.64^{gh}	22.80±1.14^{c-g}
9	PZ-L9	30.02±1.50^{b}	25.31±1.12^{a}	91.76± 4.59^{b}	208.87±10.44^{a}	51.10±2.55^{b}
10	PZ-S1	18.38±0.76^{def}	13.55±0.52^{c}	52.94±2.65^{def}	142.81±7.14d^{e}	24.40±1.22^{c-f}
11	PZ-S2	15.37±0.61^{gh}	11.45±0.42^{de}	42.91±2.15^{gh}	112.72±5.64^{gh}	23.30±1.16^{c-g}
12	PZ-S3	14.11±0.55^{hi}	9.86±0.32^{f}	38.73±1.94^{hi}	100.18±5.01^{hi}	22.70±1.13^{c-g}
13	PZ-R1	35.23±1.76^{a}	24.52±1.22^{a}	109.10±5.46^{a}	227.28±11.36^{a}	62.20±3.11^{a}
14	PZ-R2	13.71±1.05^{hij}	10.40±0.37^{ef}	37.38±1.87^{hij}	96.13±4.81^{hi}	22.10±1.10^{c-g}
15	PZ-R3	15.39±0.62^{gh}	10.66±0.37^{ef}	42.97±2.15^{gh}	112.91±5.65^{fgh}	23.10±1.15^{c-g}
16	PZ-R4	21.32±0.91^{c}	18.73±0.77^{b}	62.74±3.14^{c}	172.21±8.61^{bc}	26.20±1.31^{c}
17	PZ-R5	14.30±0.56^{hi}	10.35±0.37^{ef}	39.34±1.97^{hi}	102.01±5.10^{hi}	23.80±1.19^{c-g}
18	PZ-R6	17.32±0.71^{fg}	12.22±0.47^{cd}	49.42±2.47^{fg}	132.25±6.61^{efg}	24.10±1.20^{c-f}
19	PZ-R7	13.48±0.52^{hij}	10.47±0.37^{ef}	36.61±1.83^{hij}	93.82±4.69^{hij}	21.80±1.09^{c-g}
20	PZ-R8	12.43±0.47^{ij}	9.40±0.32^{f}	33.11±1.66^{ij}	83.32±4.17^{ij}	21.28±1.06^{d-g}
21	PZ-R9	15.52±0.62^{gh}	12.58±0.47^{cd}	43.42±2.16^{gh}	114.27±5.71^{fgh}	22.78±1.14^{c-g}
22	PZ-R10	20.38±0.86^{cd}	13.75±0.52^{c}	59.61±2.98^{cd}	162.82±8.14^{cd}	25.90±1.29^{c}
23	PZ-R11	21.83±0.94^{c}	10.36±0.37^{ef}	64.44±3.22^{c}	177.32±8.87^{bc}	24.12±1.20^{c-f}
24	PZ-R12	11.38±0.40^{lj}	9.75±0.32^{f}	29.61±1.48^{j}	72.82±3.64^{j}	19.40±0.97^{g}
25	PZ-R13	12.48±0.47^{ij}	10.13±0.37^{ef}	33.27±1.66^{ij}	83.82±4.19^{ij}	21.30±1.06^{d-g}
26	PZ-R14	14.38±0.56^{hi}	9.36±0.32^{f}	39.61±1.98^{hi}	102.82±5.14^{hi}	20.90±1.04^{efg}

Legend: The values are expressed as mg/mL. The superscript different letters indicate the statistically significantly different (P* ≤ 0.05).



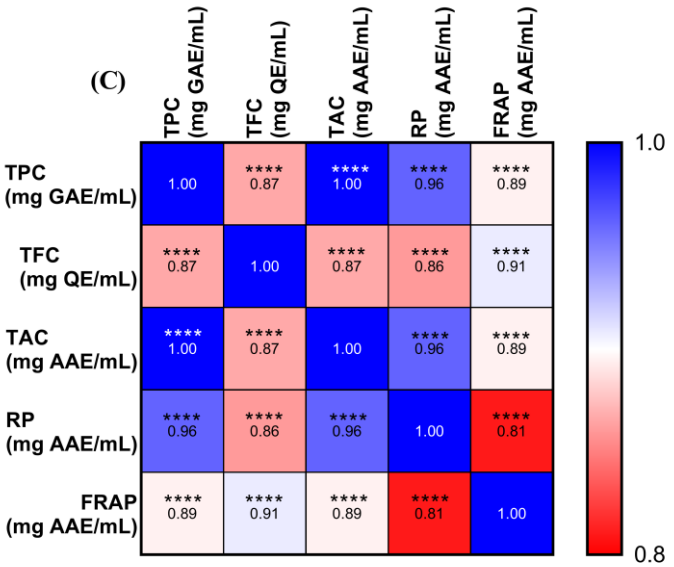


Figure 2 Relationship among antioxidants extracted from PZ supernatant by multi-variances analysis of TAC (A) and FRAP (B) with TFC, TPC, RP and (C) Person R of liner repression.

The values of RP are indicated by the numbers and the size of the circles. Person R was analyzed using correction matrix. The mean ± SD was calculated with triplicated experiments and the difference was expressed using the t–test and ANOVA (P**** ≤ 0.0001 < P*** ≤ 0.001 < P** ≤0.01 < P* ≤ 0.05, and ns: insignificant difference).

Morphological characteristics of the *P. zeylanica* endophytic bacteria

In this study, 26 endophytic bacteria from *P. zeylanica* were evaluated for antioxidant activity using TPC, TFC, TAC, RP, and FRAP. Among these bacterial strains, The PZ-R1 and PZ-L1 exhibited the highest activities. TPC, TFC, TAC, RP and FRAP of PZ-R1 were 35.23 GAE/mL 24.52 mg QE/mL, 24.52, 109.10, 227.28, 11.36 and 62.20 mg AAE/mL, respectively. Those of PZ-L1 were 30.02 GAE/mL, 25.31 mg QE/mL, 91.76, 208.87, and 51.10 mg AAE/mL, respectively. Therefore, the strains PZL9 and PZ-R1 were chosen for further experiments and identified the strains. The bacterial strains were cultivated on PDB medium for 48 h, and the morphological features of PZ-L9 and PZ-R1 strains were examined. Figure 3 and Table 3 show that the colonies of them were milky in color, round, glossy, and smooth. Both bacterial strains were gram-positive and rod-shaped.

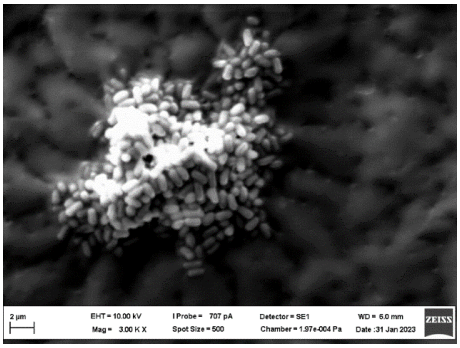
Molecule identification of the *P. zeylanica* endophytic bacteria

Molecular identification was performed using 16S rRNA sequencing (

Table **4**). The amplified 16S rRNA gene fragments were approximately 1400 bp in length. Phylogenetic reconstruction revealed that PZ-L9 (GenBank: PQ341171) and PZ-R1 (GenBank: PQ328023) clustered tightly within the *Bacillus safensis* lineage, supported by a high bootstrap value of 100 (Figure 3). Although phylogenetic analysis grouped PZ-L9 and PZ-R1 within the *Bacillus safensis* clade, this classification should be interpreted cautiously. Members of the *B. pumilus* group exhibit minimal divergence in 16S rRNA, and several species (including *B. safensis, B. altitudinis*, and *B. pumilus*) cannot be reliably distinguished using 16S alone. Therefore, the identification presented here is preliminary. Future studies incorporating gyrA/rpoB multilocus sequence analysis (MLSA) or whole-genome sequencing will be necessary to confirm the exact species status of these isolates.

The antioxidant properties of endophytic *Bacillus* species have been reported in other studies. **Akinsanya *et al.* (2015)** reported significant antioxidant activity of *Aloe vera* endophytic bacteria, including *Bacillus* such as *B. cereus, B. thuringiensis, B. aerophilus, B. anthracis,* and *B. stratophericus*. Similarly, endophytic *B. subtilis* isolated from the Chinese medicinal herb *Artemisia annua* produces exopolysaccharides that generate enzymatic and non-enzymatic antioxidants, thereby reducing oxidative DNA damage and cellular oxidation **(Zheng *et al.*, 2016)**. Additionally, *B. cereus* DCN1 isolated from *Syzygium aromaticum* leaves exhibited increased viability under severe oxidative stress, indicating that endophytes trigger and induce a significant oxidative stress response **(Larasati Arum Utami, 2023)**. Plants such as *Aloe vera*, *Artemisia annua,* and *Syzygium aromaticum* have been shown to contain natural antioxidants **(Bakour *et al.*, 2018; Hęś *et al.*, 2019; Amir Rawa *et al.*, 2022)**. Moreover, it has been reported that *P. zeylanica* may scavenge free radicals while producing antioxidants and phenolics **(Hossain *et al.*, 2017)**. Thus, the endophytic bacterium *Bacillus* sp. PZ-L9 and PZ-R1, isolated from plants, were chosen to evaluate and optimize antioxidant generation.

A)



(B)

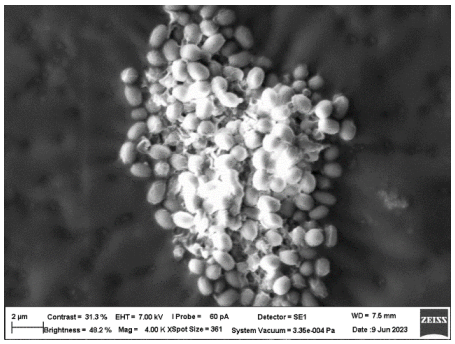


Figure 3 The shapes and sizes of the bacteria PZ-L9 (A) and PZ-R1 (B) captured by SEM.

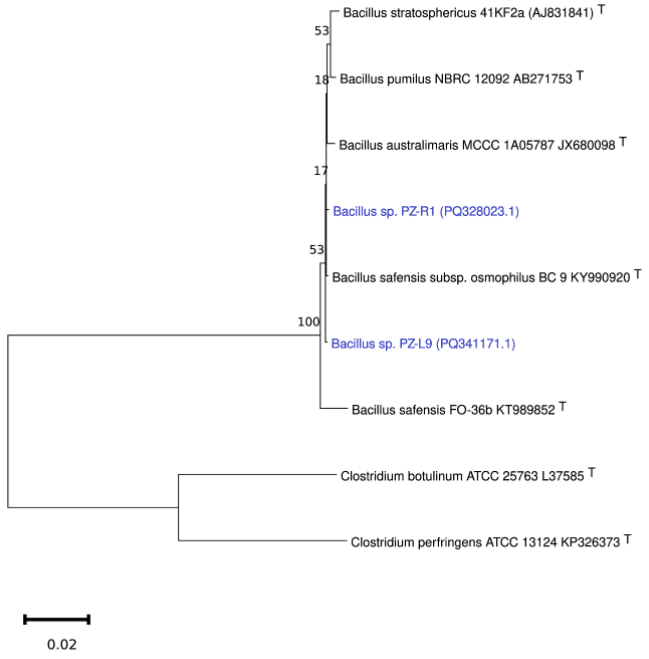


Figure 4 The phylogenetic tree of the PZ-L9, and PZ-R1strains based on 16S rRNA gene sequencing.

The rRNA was amplified by PCR single forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer 1492R (5'-TACGGTTACCTTGTTACGACTT-3'). Its products were sequenced by Macrogen and the BLAST comparisons were performed on NCBI.

Table 3 Phenotypic characteristics of strains with high antioxidants isolated from variant parts of *P. zeylanica*

No.	Strain code	Colony colors	Colony shapes	Colony surface	Colony size (mm)	Colony cover	Cell shapes	Gram
1	PZ-L9	Milky	Round	Glossy	2.0	Smooth	Rod	+
2	PZ-R1	Milky	Round	Glossy	2.0	Smooth	Rod	+

Table 4 NCBI BLAST *16s RNA* gene sequence of endophytic bacteria from *P. zeylanica*

Sample Name	Sample Accession Number	Sequence Length (bp)	Closest Related Type Strain	Type Strain Accession Number	Similarity to Type Strain (%)	E value
PZ-L9	PQ341171.1	1472	*Bacillus safensis* subsp. *osmophilus*	KY990920.1	100	0.0
PZ-R1	PQ328023.1	1472	*Bacillus safensis* subsp. *osmophilus*	KY990920.1	99.93	0.0

Antioxidative activities of the endophytic extracts

The cell-free supernatants of the PZ-R1 and PZ-L9 strains were extracted using ethyl acetate, and their TPC and TFC were determined. The endophytic bacteria PZ-R1 and PZ-L9 exhibited variations in TPC and TFC (Figure 5). The highest TFC was found in the PZ-R1 extract (39.74 mg QE/g extract), whereas the highest TPC was observed in the PZ-L9 extract (approximately 83.06 mg GAE/g extract). In the PZ-R1 extracts, the amount was less than 50% of that in PZ-L9 extract. Then, the antioxidant capacities of the extracts were evaluated using TAC, RP, FRAP, and DPPH assays. The antioxidant capacity of the extracts was positively correlated with bacterial concentration following first-order repression (Figure 6). The activities of the PZ-R1 extract were higher than those of the PZ-L9 extract, indicating that both extracts exhibited strong antioxidant activity.

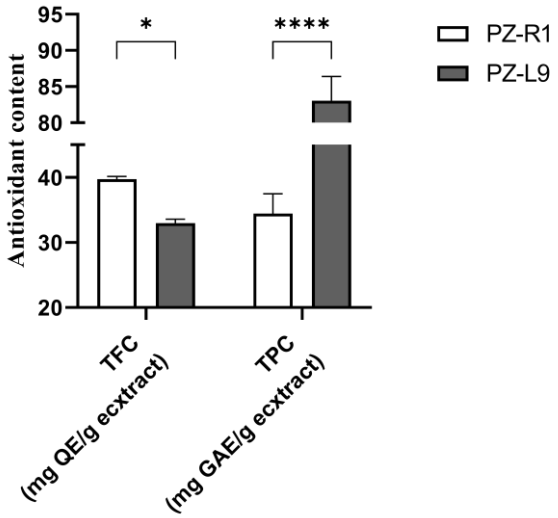


Figure 5 The total polyphenol and flavonoid contents of endophyte extracts from PZ-R1 and PZ-L9.

The TPC, TFC, and mean ± SD was calculated as described in the legends of Figure 2.

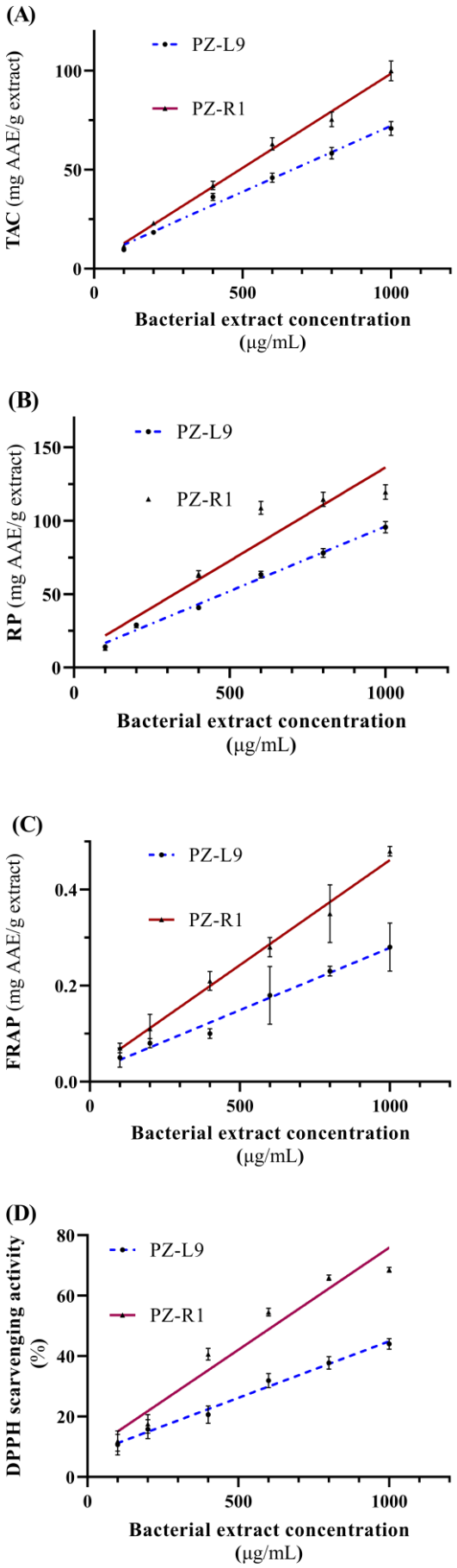


Figure 6 Effect of bacterial concentration to TAC (A), RP (B), FRAP (C), and DPPH (D) of PZ-R1 and PZ-L9.

The activities were examined with various bacterial extract concentration (100, 200, 400, 600, 800 and 1000 μg/mL). The antioxidant activities and mean ± SD were calculated as described in the legends of Figure 2.

The endophytic bacterium, *Bacillus* sp. PZ-L9 and PZ-R1 exhibited bioactivity. Research indicated that antioxidants from plant extracts, such as alkaloids, steroids, terpenoids, quinones, phenylpropanoids, phytohormones, isocoumarins, lignans, phenols, and lactones, can be derived from endophytic bacteria **(Singh *et al.*, 2017)**. These bacteria produce secondary metabolites with notable bioactivity **(Lotlikar & Naik-Samant, 2020; Makuwa & Serepa-Dlamini, 2021; Nisa *et al.*, 2022)**.

Ethyl acetate extracts of PZ-L9 and PZ-R1 displayed notable antioxidant capacities, with PZ-R1 consistently showing the highest values across all assays. This enhanced activity is likely due to strain-specific metabolic pathways and the ecological origin of PZ-R1 as a root-associated endophyte. Root endophytes are frequently exposed to oxidative and environmental stresses in the rhizosphere, which can stimulate higher production of antioxidant metabolites. The elevated TFC observed in PZ-R1 suggests that flavonoid-like compounds may contribute significantly to its reducing and radical-scavenging abilities. However, confirmation of the specific compounds responsible will require further chemical profiling using LC–MS or GC–MS.

Although our study focused on PZ-R1 bacteria, other studies have indicated that *Bacillus* may alleviate complications from free radical production or oxidative stress. Crude extracts of endophytic *B. safensis* from *Crinum macowanii* leaves exhibited anticancer activity against A549 lung carcinoma cells, containing compounds such as 3-O-methyl epimacowine, lycorine, and crinamidine **(Sebola *et al.*, 2020)**. Additionally, their anti-inflammatory properties were tested in mice with carrageenan- and formalin-induced inflammation **(Sithara K. Urumbil & Anilkumar, 2021)**. Endophytic bacteria isolated from ethnomedicinal plants possess significant antioxidant properties **(Photolo *et al.*, 2020; Nisa *et al.*, 2022; Damavandi *et al.*, 2023)**. Our findings suggest that the antioxidant properties of *Bacillus* extracts are linked to their bioactive metabolites, which are valuable in the pharmaceutical and food industries.

Conditions for antioxidants production of the *P. zeylanica* endophytic bacterium, *Bacillus* sp. PZ-R1

Independent effects of initial factors on fermentation to form antioxidants

The effects of pH, glucose concentration, cultivation temperature, and fermentation time on antioxidant production by *Bacillus* sp. PZ-R1 are shown in Figure 7A–D. pH strongly influenced metabolite synthesis, with antioxidant activity increasing from acidic to neutral conditions and reaching its maximum at pH 7.0. Activity declined under alkaline conditions. This trend is consistent with the fact that many *Bacillus* enzymes involved in secondary-metabolite biosynthesis exhibit optimal function near neutral pH, whereas alkaline environments can destabilize enzyme activity and reduce metabolite formation.

Temperature also had a pronounced effect, with peak antioxidant production observed at 35 °C. Higher temperatures caused a rapid decline, likely due to heat-induced stress that impairs bacterial growth and disrupts the stability of biosynthetic enzymes. Glucose concentration similarly modulated antioxidant output, with 30 g/L supporting the highest production. Excess glucose is known to shift bacterial metabolism toward biomass accumulation rather than secondary-metabolite synthesis, which explains the reduced activity at higher concentrations. Fermentation time showed that antioxidant production increased steadily and peaked at 48 h, after which activity declined, possibly due to nutrient depletion or feedback inhibition in the stationary phase.

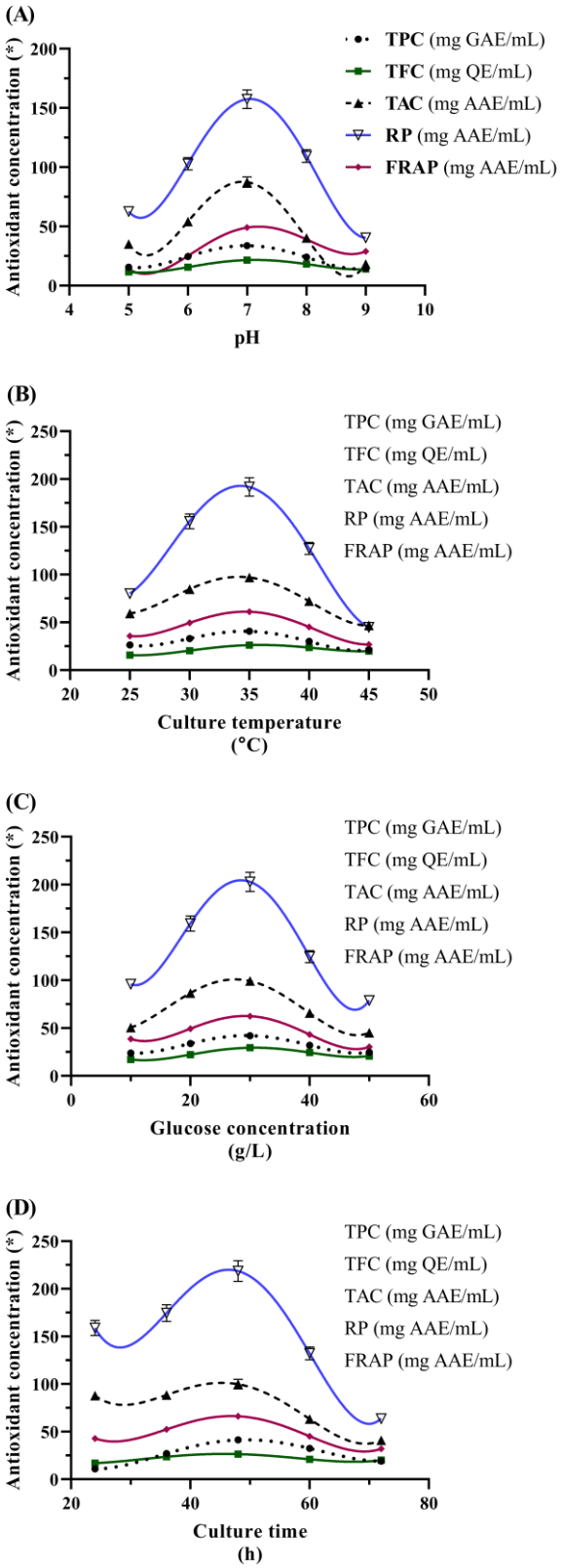


Figure 7 Effect of initial pH (A), glucose concentration (B), culture temperature (C), and culture time (D), on the formation of antioxidants during bacterial fermentation.
(*) the values were determined as mg of standards equivalent/mL supernatant. The antioxidant activities and mean ± SD were calculated as described in the legends of Figure 2.

Optimum culture conditions for antioxidants production

RSM design was based on the initial parameters of pH, glucose concentration, temperature, and time, which were derived from one-factor experiments. RSM was used to investigate the interaction between the variances that influence antioxidative capacity. This approach is consistent with previous optimization protocols **(Dai *et al.*, 2024; Dai *et al.*, 2025)**. The experimental matrix and corresponding antioxidant responses generated by the CCD are shown in Table 1. Regression coefficients obtained from the quadratic model, including linear (β_i), interaction (β_{ij}), and quadratic terms (β_{ii}), are presented in Tables 5–7. The optimum values of the antioxidants generated by *Bacillus* sp. PZ-R1 is shown in Figure 8.

A large F-value and extremely low p-value indicated that the model explained a very high portion of the variability in each response. The probability that these results occurred by random chance was <0.01%. Residual (unexplained variation) values (residual of TPC, TFC, TAC, RP, and FRAP were 9.90, 18.36, 57.85, 148.95, and 27.37, respectively) were compared to their model sum of squares (thousands to tens of thousands), meaning the model fits the data. The p-values of the lack-of-fit are larger than 0.05, meaning that the model adequately explains the data and there are no systematic errors remaining.

The linear coefficient β_1 (pH) is substantially larger than β_2–β_4, whereas β_2 (glucose) is the smallest among them. This indicates that pH exerts the strongest linear influence on the antioxidant responses, while glucose has the weakest effect. Similarly, for the quadratic coefficients, the magnitude of the pH-related term is the highest, suggesting that pH also contributes most strongly to the curvature of the model. Antagonistic effects were observed in the interaction terms β_{12}, β_{13}, and β_{14}, indicating that the interactions between pH and glucose, pH and temperature, and pH and time negatively affected the antioxidant activities. In contrast, the interaction terms β_{23}, β_{24}, and β_{34} exhibited positive coefficients, demonstrating synergistic effects between glucose and temperature, glucose and time, and temperature and time. The negative values of the quadratic terms β_{11}, β_{22}, β_{33}, and β_{44} confirm the presence of a maximum point within the experimental range, indicating that true optimal conditions can be identified by the model.

A negative coefficient β_{12} indicates that simultaneously increasing pH and glucose slightly reduces the antioxidant yield, compared with increasing each factor individually. Bacillus species often regulate carbohydrate uptake and secondary metabolism in a pH-dependent manner. Environmental pH can significantly alter glucose consumption rates and the balance between glycolysis and fermentation pathways in *Bacillus*, with optimal glucose utilization normally occurring near neutral pH and reduced efficiency under more acidic or alkaline conditions **(Singh *et al.*, 2017; Park *et al.*, 2022)**. Additionally, glucose is a preferred carbon source in Bacillus and exerts global metabolic control through catabolite repression, repressing secondary metabolic pathways when present. Higher pH combined with higher temperature exerts a negative effect on antioxidant production. Although temperature accelerates enzymatic reactions, the enzymes responsible for antioxidant synthesis only function optimally within a narrow pH–temperature window. Key enzymes (e.g., oxidoreductases, decarboxylases) involved in secondary metabolite synthesis become less stable when both pH and temperature deviate from their optimal range, leading to reduced antioxidant levels **(Vieille & Zeikus Gregory, 2001)**. Extending the cultivation time under high initial pH slightly decreases antioxidant production. As fermentation progresses, Bacillus may shift the environmental pH, creating conditions incompatible with sustained secondary metabolism. High glucose combined with elevated temperatures promotes rapid biomass formation rather than secondary metabolite production. Bacillus cells preferentially allocate carbon to growth under thermally favorable conditions. This suppresses the biosynthesis of phenolic, flavonoid, and other antioxidant-related compounds that typically accumulate during the stationary phase. Increasing glucose along with culture time slightly enhances antioxidant production. This means that the strain requires both adequate carbon and sufficient cultivation duration to effectively produce antioxidant metabolites. Many secondary metabolites are synthesized during the stationary phase. A sustained glucose supply ensures metabolic activity is maintained long enough for these pathways to operate efficiently. Maintaining the culture at a suitable temperature for an extended period enhances antioxidant production. This suggests that enzyme systems involved in antioxidant biosynthesis remain active when the strain is grown at a stable, optimal temperature for long enough. Bacillus sp. typically grows optimally at 30–37°C. Extended cultivation within this temperature range allows sustained enzyme activity and efficient conversion of substrates into antioxidant metabolites.

P. zeylanica is often used to treat ailments **(Hossain *et al.*, 2017)** , and the extraction of natural antioxidants from plants has been focused on. The results of acute or subchronic toxicity of the tree **(OECD, 2022))** indicated that the tree is safe for oral use. Furthermore, the composition, concentration, and activity of the antioxidants present *in P. zeylanica* can fluctuate depending on the living conditions of the plant, and overexploitation of *P. zeylanica* trees to extract their chemical components or for their medicinal properties can disrupt the ecological balance of the area. Thus, utilizing a controlled fermentation process with endophytic bacteria, specifically *Bacillus* spp., can help stabilize the concentration and activity of antioxidants and mitigate environmental imbalances.

Our primary objective was to identify bacteria in plant tissues capable of producing antioxidants outside the host plants. Several preliminary experiments were conducted to determine the initial factors affecting bacterial fermentation; however, the antioxidant activities were low. To utilize endophytic bacteria as natural antioxidants, it is essential to optimize their growth conditions to increase antioxidant levels. The CCD and RSM are the suitable methods to find the

optimum condition for bacterial fermentation which was reported previously **(Dai *et al.*, 2024; Dai *et al.*, 2025)**.

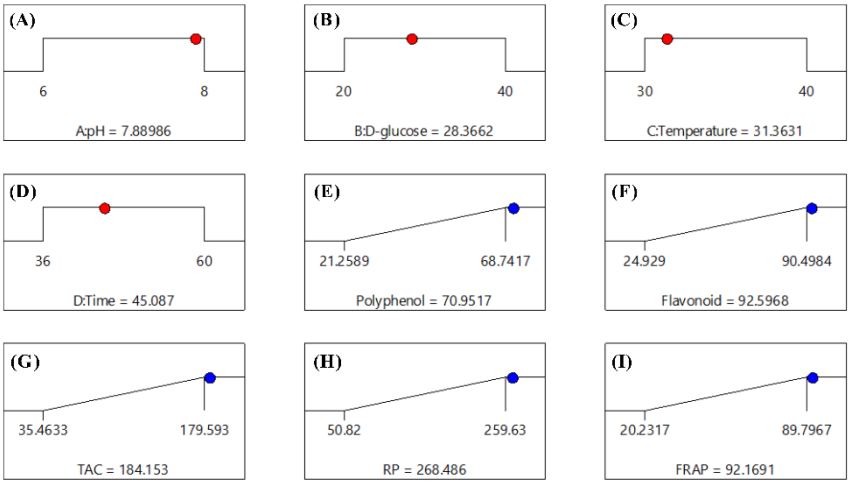


Figure 8 Optimal fermented variances (A) pH, glucose (B), temperature (C) and time (D) and objective functions, concentration of (E) TPC, (F) TFC, (G) TAC, (H) RP, and (I) FRAP, of the antioxidants produced by *Bacillus* sp. PZ-R1.

The antioxidant activities and mean ± SD were calculated as described in the legends of Figure 2.

Table 5 CCD for optimization of variables for antioxidant generation

Factors	X1	X2	X3	X4	TPC (mg GAE/mL)		TFC (mg QE/mL)		TAC (mg AAE/mL)		RP (mg AAE/mL)		FRAP (mg AAE/mL)	
					Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted
1	5	30	35	48	33.26±1.66	32.98	38.88±1.94	38.34	62.22±3.11	62.46	96.95±4.85	94.34	31.60±1.58	31.29
2	6	40	40	60	65.33±3.26	64.92	82.34±4.11	81.72	159.07±7.95	159.20	230.73±11.54	228.81	80.03±4.00	78.56
3	6	40	40	36	27.40±1.37	27.13	33.59±1.67	34.01	57.39±2.87	56.48	80.86±4.04	83.30	29.69±1.48	30.80
4	6	20	30	60	45.40±2.27	45.26	55.78±2.78	56.15	103.59±5.18	104.87	145.83±7.29	142.93	49.79±2.49	51.22
5	6	40	30	60	53.07±2.65	53.70	65.03±3.25	65.17	128.85±6.44	127.66	173.61±8.68	178.15	55.92±2.79	56.97
6	6	20	40	36	27.76±1.38	28.50	37.08±1.85	37.27	60.49±3.02	61.90	95.44±4.77	96.20	30.24±1.51	30.16
7	6	20	40	60	50.40±2.52	50.47	63.89±3.19	63.61	117.10±5.86	114.91	167.10±8.36	169.22	60.05±3.00	59.68
8	6	20	30	36	29.40±1.47	28.73	38.09±1.90	37.55	64.67±3.23	64.16	85.45±4.27	86.50	31.83±1.59	31.98
9	6	40	30	36	21.25±1.06	21.33	24.92±1.24	25.20	35.46±1.77	37.24	50.82±2.54	49.23	20.23±1.01	19.49
10	7	50	35	48	36.08±1.80	36.36	42.74±2.13	43.09	79.84±3.99	79.80	112.43±5.62	111.57	36.92±1.84	37.29
11	7	30	35	24	26.58±1.32	27.02	36.73±1.83	36.95	60.44±3.02	58.44	83.41±4.17	83.36	28.72±1.43	28.62
12	7	30	45	48	62.15±3.10	62.33	82.47±4.12	83.33	162.65±8.13	163.97	235.79±11.79	235.01	81.82±4.09	82.96
13	7	30	35	72	62.18±3.10	62.34	81.51±4.07	82.46	158.67±7.93	160.42	230.49±11.52	231.25	75.33±3.76	75.02
14	7	10	35	48	40.74±2.03	41.05	48.01±2.40	48.84	95.92±4.80	95.70	128.76±6.44	130.34	50.45±2.52	49.66
15	7	30	25	48	58.39±2.92	58.81	76.31±3.81	76.63	152.12±7.61	150.54	211.13±10.56	212.64	76.06±3.80	74.50
16	7	30	35	48	66.86±3.34	67.83	87.92±4.39	89.25	178.66±8.93	176.70	258.28±12.91	255.52	89.33±4.46	88.02
17	7	30	35	48	67.17±3.35	67.83	88.34±4.41	89.25	176.05±8.80	176.70	256.62±12.83	255.52	87.02±4.35	88.02
18	7	30	35	48	68.36±3.41	67.83	89.98±4.49	89.25	175.68±8.78	176.70	254.08±12.70	255.52	86.84±4.34	88.02
19	7	30	35	48	68.74±3.43	67.83	90.49±4.52	89.25	175.24±8.76	176.70	251.52±12.58	255.52	87.62±4.38	88.02
20	7	30	35	48	67.16±3.35	67.83	88.33±4.41	89.25	174.99±8.75	176.70	252.98±12.65	255.52	87.49±0.37	88.02
21	7	30	35	48	68.67±3.43	67.83	90.40±4.52	89.25	179.59±8.98	176.70	259.63±12.98	255.52	89.79±4.49	88.02
22	8	40	40	60	62.05±3.10	62.71	84.82±4.24	84.43	169.25±8.46	167.60	240.05±12.00	241.49	79.12±3.95	79.11
23	8	20	30	36	64.89±3.24	64.75	81.74±4.08	82.14	160.84±8.04	163.14	233.63±11.68	232.32	81.42±4.07	83.19
24	8	40	40	36	44.33±2.21	43.93	58.12±2.90	57.53	105.17±5.26	106.33	150.37±7.52	150.04	53.08±2.65	51.96
25	8	40	30	60	60.25±3.01	58.96	77.86±3.89	77.45	150.89±7.54	151.91	232.80±11.64	228.83	68.44±3.42	68.83
26	8	20	30	60	62.02±3.10	62.27	81.29±4.06	79.94	163.65±8.18	162.39	234.64±11.73	234.70	82.82±4.14	81.84
27	8	20	40	60	60.63±3.03	60.00	78.31±3.91	77.82	155.92±7.80	156.58	224.63±11.23	222.99	77.96±3.89	79.00
28	8	20	40	36	57.69±2.88	57.05	73.36±3.66	72.29	146.00±7.30	145.02	206.06±10.30	204.02	70.99±3.55	70.07
29	8	40	30	36	45.71±2.28	45.62	58.93±2.94	58.29	102.91±5.15	102.94	153.60±7.68	153.97	51.45±2.57	51.95
30	9	30	35	48	65.92±3.29	66.79	83.93±4.19	85.64	170.34±8.52	169.84	249.50±12.48	252.84	83.17±4.15	83.06

Table 6 Second-order polynomial model of coefficient values

Objective function	Coefficient values														
	β_0	β_1	β_2	β_3	β_4	β_{12}	β_{13}	β_{14}	β_{23}	β_{24}	β_{34}	β_{11}	β_{22}	β_{33}	β_{44}
Y_1	-665.55	112.17	3.672	5.89	5.59	-0.29	-0.37	-0.39	0.03	0.03	0.02	-4.48	-0.07	-0.07	-0.04
Y_2	-866.83	153.42	4.63	7.26	6.44	-0.29	-0.48	-0.43	0.05	0.04	0.03	-6.81	-0.11	-0.09	-0.05
Y_3	-1884.99	332.94	10.04	14.15	14.49	-0.83	-0.79	-0.86	0.11	0.10	0.05	-15.14	-0.22	-0.19	-0.17
Y_4	-2880.23	477.74	15.39	29.63	20.38	-1.03	-1.90	-1.13	0.12	0.15	0.07	-20.48	-0.34	-0.32	-0.17
Y_5	-976.72	175.34	5.54	6.86	7.37	-0.47	-0.57	-0.43	0.07	0.04	0.04	-7.71	-0.11	-0.09	-0.06

Legend: Y_1, Y_2, Y_3, Y_4, and Y_5 were TPC, TFC, TAC, RP, and FRAP, respectively.

Table 7 Analysis of variance of the experimental results for the quadratic model

Source	Dependent variables	df	Sum of squares	Mean square	*F*- value	*p*- value

Model	TPC	14	6826.75	487.63	738.7	<0.0001
	TFC		12594.95	899.64	735.16	< 0.0001
	TAC		61511.80	4393.70	1139.20	< 0.0001
	RP		13090.00	9349.13	941.51	<0.0001
	FRAP		14843.39	1060.24	581.01	<0.0001
Residual	TPC	15	9.90	0.66		
	TFC		18.36	1.22		
	TAC		57.85	3.86		
	RP		148.95	9.93		
	FRAP		27.37	1.82		
Lack of Fit	TPC	10	6.25	0.63	0.86	0.61
	TFC		11.51	1.15	0.84	0.62
	TAC		39.14	3.91	1.05	0.51
	RP		98.72	9.87	0.98	0.54
	FRAP		19.69	1.97	1.28	0.41
Pure Error	TPC	5	3.65	0.73		
	TFC		6.85	1.37		
	TAC		18.72	3.74		
	RP		50.23	10.05		
	FRAP		7.69	1.54		
Core Total	TPC	29	6836.65			
	TFC		12613.31			
	TAC		61569.65			
	RP		13100.00			
	FRAP		14870.77			

Legend: Coefficient of variations (CV%): TPC=1.55, TFC=1.64, TAC=1.52, RP=1.69, FRAP= 2.12. Coefficient of determination (R^2): R^2_{TPC}= 0.9986, R^2_{TFC} = 0.9985, R^2_{TAC} = 0.9991, R^2_{RP} = 0.9989, R^2_{FRAP} = 0.9982. Adjusted R^2: TPC=0.9972, TFC = 0.9972, TAC=0.9982, RP=0.9978, FRAP = 0.9964.

The optimal condition conformations for antioxidant generation

RSM optimization identified the optimal culture conditions for maximizing antioxidant production by *Bacillus* sp. PZ-R1, including pH 7.89, 28.37 g/L glucose, 31.36 °C, and 45.09 h fermentation (Figure 8). Under these conditions, PZ-R1 produced markedly higher antioxidant levels compared to non-optimized conditions, yielding 71.69 mg GAE/mL (TPC), 93.61 mg QE/mL (TFC), 184.44 µg AAE/mL (TAC), 266.59 µg AAE/mL (RP), and 91.01 µg AAE/mL (FRAP). The predicted values generated by the quadratic model closely matched the experimentally observed values (Figure 9), confirming the suitability and accuracy of the RSM. Furthermore, correlation analysis (Figure 10) showed that the antioxidant parameters increased concurrently under the optimized conditions, with Pearson R values exceeding 0.99, indicating strong positive associations among the antioxidant indicators.

The endophytes exhibited high antioxidant activity. The compounds demonstrated similar trends, positive correlation values, and high Pearson R-values. This indicates that *Bacillus* sp. is capable of producing antioxidants, and those within the same strain are correlated with each other and tend to covary with each other. Previous research has found that various genera with strong antioxidant and antibacterial activities, including *Bacillus, Enterobacter, Pantoe, Erwinia, Staphylococcus,* and *Stenotrophomonas,* have been isolated and identified from *Fagonia indica* **(Rahman *et al.*, 2017)** and *Dicoma anomala* **(Makuwa & Serepa-Dlamini, 2021)**. Numerous studies have demonstrated that *P. zeylanica* extracts possess antioxidant properties **(Hossain *et al.*, 2017; Wang *et al.*, 2018)**. Furthermore, bioactive compounds with cytotoxicity towards human cancer cell lines have been identified in one of the species of the *Pouzolzia* genus **(Chen *et al.*, 2015; Nhung *et al.*, 2022)**.

The optimal culture conditions for antioxidant production in *Bacillus* species vary considerably among strains, reflecting differences in their physiological traits and ecological origins. Previous studies have illustrated this variability in the literature. For example, *Bacillus subtilis* isolated from soil demonstrated maximal antioxidant exopolysaccharide production at pH 7 and 37 °C after 48 h **(Razack *et al.*, 2014)**, whereas *Bacillus aerophilus* achieved peak antioxidant activity at pH 7 and 30 °C after 72 h **(Gangalla *et al.*, 2021)**. These findings emphasize that even closely related species respond differently to environmental parameters, underscoring the need for strain-specific optimizations. In this study, the culture conditions were optimized for *Bacillus* sp. PZ-R1 fell within the commonly reported range for antioxidant-producing *Bacillus* strains, indicating that the physiological behavior of this endophytic isolate aligns with established microbial responses. Importantly, the use of moderate and easily maintainable conditions (near-neutral pH, mesophilic temperatures, and standard fermentation durations) confirms that the process is practical, reproducible, and readily scalable. Consequently, *Bacillus* sp. PZ-R1 represents a promising and feasible microbial platform for consistent antioxidant production, offering a sustainable alternative to plant-based extraction and reducing the environmental pressure associated with the overharvesting of *P. zeylanica*

Optimal conditions for antioxidant production by *Bacillus* sp. PZ-R1 were identified. High regression coefficients and adjusted R^2 in supplementary table (S. Tables) 1 to 5 (in the attached supplementary materials) indicated the accuracy of the prediction. The effects of additives and culture conditions emphasize the key variables in antioxidant production. Statistical optimization identified the optimal conditions, indicating the importance of the initial pH, glucose concentration, temperature, and culture time (Figure 8). Analysis of S. Tables 2 to 5 shows that the interactions of these factors influenced antioxidative compounds, resulting in variable optimal values in the CCD and one-factor time experiment. The optimal pH, glucose concentration, temperature, and culture time (Figure 8A–D) were notably different from those obtained in the preliminary experiments (Figure 7). This divergence in conditions (Figure 9) highlighted the effectiveness of our approach in identifying the most favorable culture conditions for bacterial fermentation.

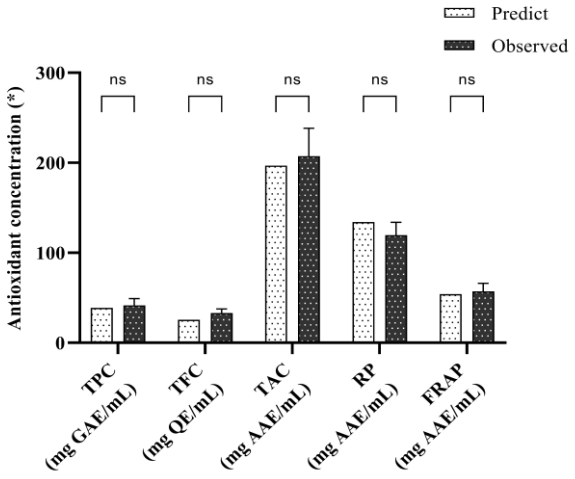


Figure 9 The antioxidant concentration of the *Bacillus* sp. PZ-R1 at optimum conditions.

The values were created by the quadratic regression equation (Predict) and measured by experiments (Observed) at the optimum conditions. (*) mg of standards equivalent/mL cell-free supernatant. The antioxidant activities and mean ± SD were calculated as described in the legends of Figure 2.

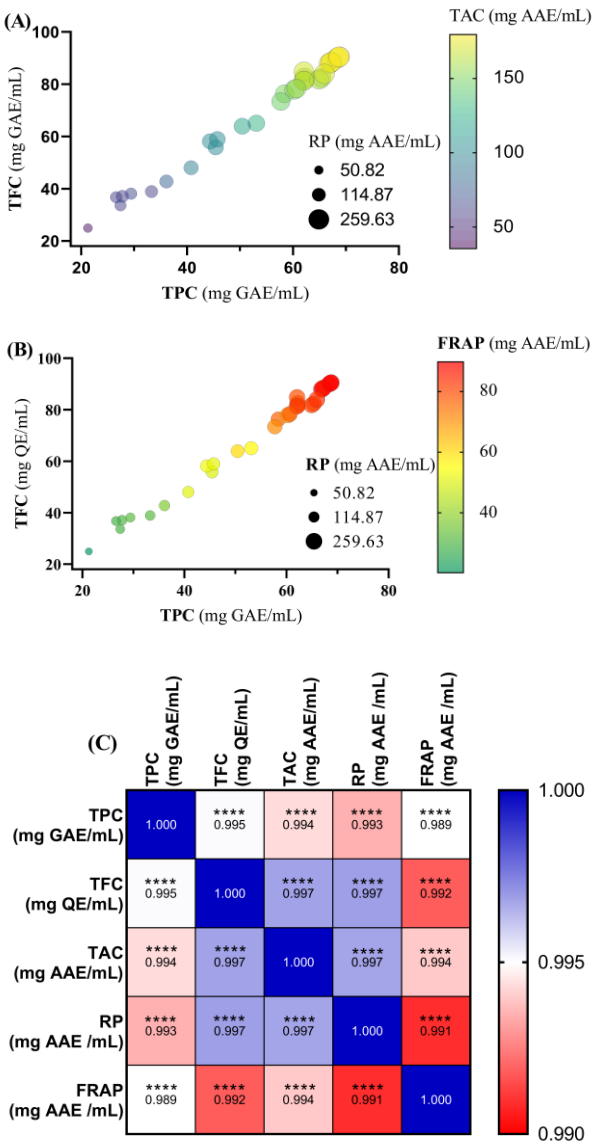


Figure 10 Multi-variances analysis of TAC (A), FRAP (B) with TFC, TPC, RP extracted from *Bacillus* sp. PZ-R1 and (C) Person R values of liner repression of antioxidants.

Size of circles indicate values of RP mg AAE/mL. The antioxidant activities and mean ± SD were calculated as described in the legends of Figure 2.

CONCLUSION

Bacillus sp. PZ-R1 emerged as a highly promising endophytic strain of *Pouzolzia zeylanica*, exhibiting robust antioxidant activity across multiple in vitro assays. Response Surface Methodology identified the optimized culture conditions—28.37 g/L glucose, pH 7.89, 31.36 °C, and 45.09 h—that markedly enhanced the production of phenolic, flavonoid, and reducing metabolites, with close agreement between predicted and experimental values validating the reliability of the quadratic model. The strain's ability to generate antioxidant compounds independent of its host highlights the potential of endophytic bacteria as sustainable and controllable microbial sources of natural antioxidants, offering an avenue for reducing harvesting pressure on *P. zeylanica*. However, these findings remain preliminary as they are derived solely from in vitro analyses. Comprehensive metabolite characterization, safety assessments, and *in-vivo* evaluations are essential to confirm the bioactivity and practical applicability of PZ-R1–derived metabolites. Overall, *Bacillus* sp. PZ-R1 represents a compelling candidate for biotechnological exploitation; however, further investigation is required before its use can be fully validated.

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