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RESEARCH

Identification and evaluation of thermophilic bacteria isolated from Malaysian hot springs with potential for plastic polyvinyl chloride degradation

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Abstract

The biodegradation of polymeric plastics using biological agents has gained increasing interest. Thermophilic bacteria are particularly attractive as elevated temperatures weaken polymer structures, enhance substrate diffusion, and stimulate enzyme activity, thereby accelerating degradation. This study examined the thermophilic microbial degradation of polyvinyl chloride (PVC) by isolating, screening, and characterising effective PVC-degrading bacteria from three Malaysian hot springs. Sediment (50 g) and surface water (250 mL, 0–10 cm depth) were collected aseptically from three sites per hot spring. Physicochemical parameters (pH, temperature, salinity) were measured in triplicate. Samples were enriched in CFSM medium containing 0.1% (w/v) PVC powder and incubated at 55 °C for one week. Cultures were diluted and spread on carbon-free synthetic medium (CFSM) agar with PVC for viable counts. Isolates were screened using clear zone and bacterial adhesion to hydrocarbon (BATH) assays. Selected strains underwent 16 S rRNA sequencing, phylogenetic analysis, and RNA secondary structure prediction. Paku hot spring exhibited the highest bacterial density (5.49 ± 0.01 $\log_{10}$ CFU/mL), highest pH (6.40 ± 0.22), and lowest temperature (37.87 ± 1.21 °C), classifying it as a thermal neutral spring. Panchor was a hyperthermal weak acid spring (pH 5.36 ± 0.07; 42.33 ± 1.01 °C), while Ayer Hangat was hyperthermal neutral with the highest salinity (28.20–28.37 ppt). From 288 isolates, 264 produced clearance zones (10–25 mm). Hydrophobicity analysis revealed 167 hydrophilic, 85 moderately hydrophobic, and 10 strongly hydrophobic isolates. Molecular identification confirmed *Brevibacillus thermoruber* ($n=1$) and *Anoxybacillus rupiensis* ($n=9$). In conclusion, the thermophilic bacterial isolates from three Malaysian hot springs demonstrated preliminary indications of PVC-interacting capability via clear zone formation and hydrophobicity. These isolates require further validation through quantitative degradation assays to reveal the PVC-degrading potential, underscoring their promise for plastic biodegradation and environmental remediation.

Keywords Biodegradation · Hot springs · Microbial · Polyvinyl chloride · Thermophiles

Introduction

In recent years, the study of biodegradation and valorisation of plastic polymer wastes using organisms such as bacteria, fungi, thermophiles, and vermicompost has gained considerable attention in the research community. Biodegradation is recognised as a promising, cost-effective and efficient strategy for addressing the environmental challenges posed by persistent polymer wastes (Havaei et al. 2025). This process involves the decomposition of recalcitrant,

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hazardous, and large polymer molecules by living organisms under favourable conditions through physical, chemical, or enzymatic mechanisms to convert them into non-toxic, lower molecular weight oligomers and monomers that can be returned into the biogeochemical cycle (Atanasova et al. 2021). Despite its potential, previous studies have shown that the microbial degradation of synthetic polymers is often slow and inconsistent, making it less viable for immediate industrial applications compared to other waste management strategies. Hence, there is ongoing research to identify more efficient microbes and methods to tackle the bioaccumulation of polymer waste.

Thermophilic bacteria, capable of thriving at 45–122 °C, are promising candidates for plastic biodegradation (Toh et al. 2023). They occur in diverse high-temperature environments, including geothermal systems, hydrothermal vents, terrestrial hot springs, volcanoes, and even engineered sites like composting facilities and steam plants (Orellana et al. 2018). Elevated temperatures weaken polymer structures, increase organic compound diffusion, and enhance enzyme turnover, while also lowering culture viscosity and limiting contamination by mesophiles (Ahmed et al. 2018). Biodegradation typically initiates in the amorphous polymer regions, where chain mobility is restricted at lower temperatures (James-Pearson et al. 2023). The chain flexibility hypothesis suggests that higher temperatures increase polymer mobility, improving enzyme–substrate interactions and accelerating degradation (James-Pearson et al. 2023). These combined factors underscore the advantages of thermophiles in valorising recalcitrant polymers such as PVC waste.

Thermophilic bacteria have shown considerable promise in biodegradation research, offering valuable insights into their efficacy and underlying mechanisms. Thermophilic polymer bio-degraders have been isolated from various environment, including hot springs (Valdez-Nuñez and Rivera-Jacinto 2024), cow dung (Skariyachan et al. 2017), sewage treatment plants (Skariyachan et al. 2018), compost (Dang et al. 2018), soil (Mahdi et al. 2016), collection of microorganism (Jarerat and Tokiwa 2001), and plastic waste disposal site (Hadad et al. 2005). These thermophilic bacteria have demonstrated the ability to degrade hydrocarbons such as crude oils (Meintanis et al. 2006), low and high-density polyethylene (LDPE and HDPE) (Akarsu et al. 2023), and polyethylene terephthalate (PET) (Valdez-Nuñez and Rivera-Jacinto 2024). PVC is particularly difficult to degrade compared with other plastic such as PET or polyethylene (PE) due to its chlorinated polymer structure (Huang et al. 2025), which resists enzymatic attack and can generate inhibitory chlorinated by-products during breakdown. The resistance of PVC arises from the incorporation of chlorine within the polymer chain, which strengthens carbon–chlorine bonds, limits polymer flexibility, and can result in the release of degradation products that suppress microbial and enzymatic activity.

Despite the potential for biodegradation of PVC polymer wastes due to its high amorphous content, which enhances its susceptibility to enzymatic degradation by making it more loosely packed and water-accessible, research on the thermophilic biodegradation of PVC wastes remains scarce. A recent study on PVC biodegradation using vermibacteria from the guts of earthworms reported the highest chloride production at 50.0 °C in the presence of sucrose and yeast extract (Andleeb et al. 2025), suggesting the feasibility of PVC biodegradation at elevated temperature with thermophilic bacteria. Although earthworm-associated microbiota is typically mesophilic, the observed activity at elevated temperature indicates the presence of thermotolerant bacterial members or enzymes capable of functioning under near-thermophilic conditions, thereby supporting the possibility of extending PVC biodegradation strategies to thermophilic systems. Nonetheless, bacterial PVC biodegradation under mesophilic conditions remains limited, as PVC exhibits high resistance to microbial attack despite its amorphous fraction, owing to the absence of readily cleavable functional groups commonly found in other plastics. Consequently, thermophilic approaches employing elevated temperatures are explored to enhance polymer chain mobility and improve the accessibility of PVC to microbial and enzymatic action. Hence, this study aimed to address the knowledge gap in the thermophilic microbial biodegradation of PVC by isolating, screening, and characterisation of the effective PVC-degrading bacteria from hot springs in Malaysia.

Materials and methods/methodology

Materials and chemicals

Vinyl chloride (polymer) powder (average degree of polymerisation = 1000–1060) and solvents used for the enrichment and clear zone assays was acquired from Nacalai Tesque, Kyoto, Japan. The chemicals used in this study are ACS grade and were purchased from Sigma-Aldrich Merck (Darmstadt, Germany) and Thermo Fisher Scientific (United Stated).

Sampling sites

The hot springs selected were Panchor hot spring (1°15’14.8"N 110°27’04.4"E) and Paku hot spring (1°25’06.5"N 110°11’55.9"E) from Sarawak, Borneo and Ayer Hangat Village (6°25’20.4"N 99°48’45.9"E) from Kedah as shown in Fig. 1.

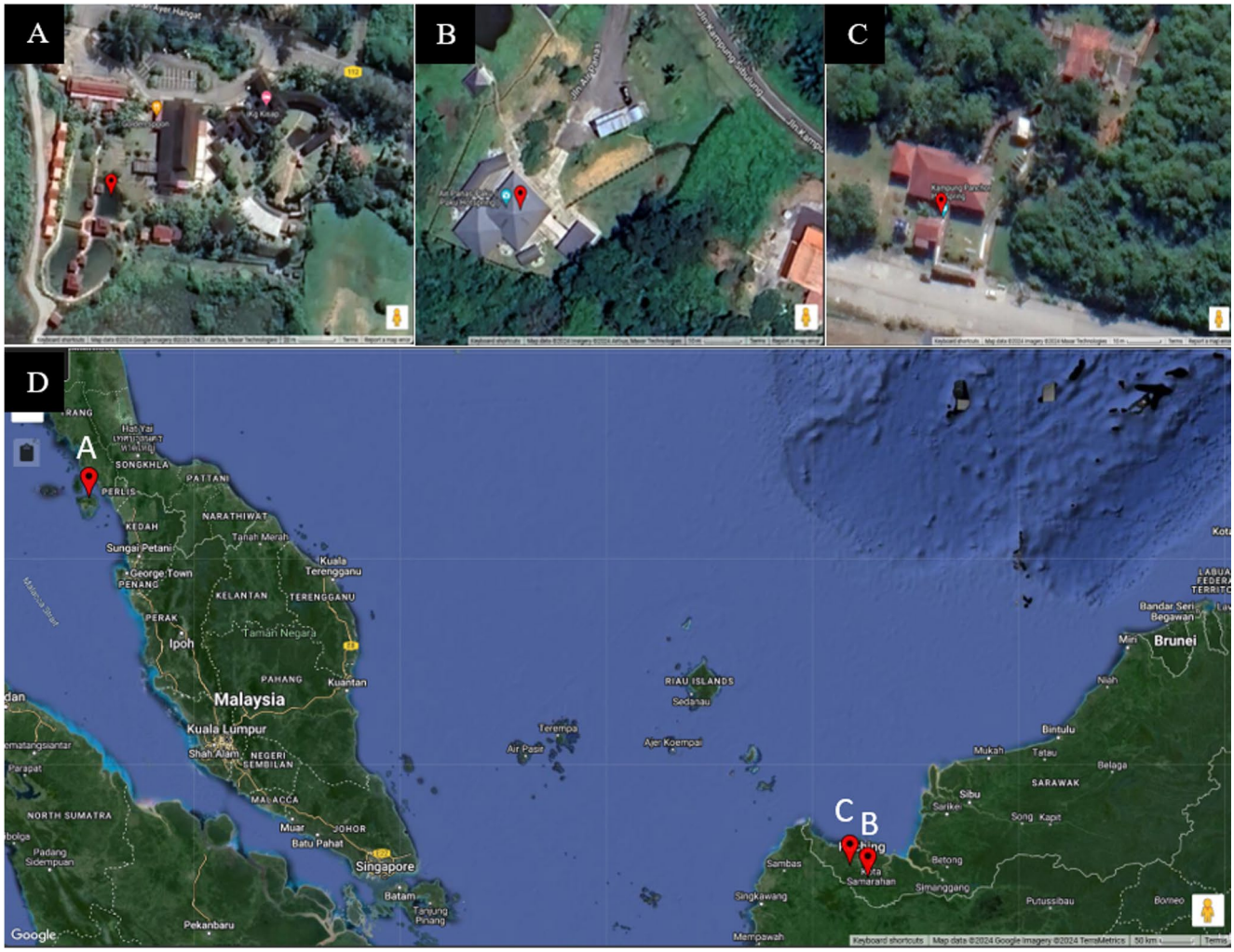

Fig. 1 Sampling sites for hot spring water and sediments. Red marks indicate the selected sampling sites in Langkawi, Kedah and Kuching, Sarawak. A, Ayer Hangat Village; B, Paku hot spring; C, Panchor hot spring; D, Satellite map of Malaysia.(Source: Map Maker)

Water and sediment soil sampling from hot springs

Approximately 50 g of sediment and 250 mL of surface water samples (0–10 cm depth) were aseptically collected at three random locations from both the heat source and discharge area of each hot spring (Leong et al. 2018). Physicochemical parameters of the hot springs, including pH, temperature, and salinity, were measured in triplicate on site to characterise the native habitat of the PVC-degrading thermophilic bacteria. Sediment samples were stored in sterile bags, while water samples were collected in insulated thermal to maintain their temperature during transport to the laboratory for further analysis.

Media preparation

The medium employed for the enrichment, isolation, and preservation of thermophilic bacterial strains was the customized liquid carbon-free synthetic medium (LCFSM), prepared in accordance with Maheswaran et al. (2024) as follow (g/L of distilled water): ammonium nitrate (NH_4NO_3), 1.0; magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), 0.2; potassium dihydrogen phosphate (K_2HPO_4), 1.0; calcium chloride, dihydrate ($CaCl_2.2H_2O$), 0.1; potassium chloride (KCl), 0.15; yeast extract, 0.1 and micronutrients for 1.0 mg L-1 of each of the following: iron(II) sulfate hexahydrate ($FeSO_4.6H_2O$), zinc sulfate heptahydrate ($ZnSO_4.7H_2O$) and manganese sulfate ($MnSO_4$).

Enrichment and isolation of PVC-degrading bacteria

About 1 g of sediment or 10 mL of water sample was inoculated into 90 mL of 0.1% (w/v) PVC powder-supplemented LCFSM in 250 mL Erlenmeyer flasks, prepared in triplicate (Maheswaran et al. 2024). Negative controls included uninoculated LCFSM or LCFSM inoculated with sterile distilled

water. Cultures were incubated at 55 °C, 100 rpm, for one week, then serially diluted (up to 10^{-2}) in PBS and spread on CFSM agar with 0.1% PVC powder. Plates were sealed with plastic wrap and incubated at 55 °C for five days with a beaker of distilled water to maintain humidity. Colony-forming units (CFU) were counted, and single colonies were purified on CFSM agar. Pure isolates were preserved on CFSM slants and in glycerol stocks for further analysis.

Screening of PVC-degrading bacteria

Clear zone assay

Thermophilic isolates were cultured in LCFSM with 0.1% PVC powder to early stationary phase, adjusted to 0.5 McFarland at OD600 (UV-1900i Plus, Shimadzu). CFSM agar plates (20 mL, 0.1% PVC) were sectioned and 8 mm wells prepared, each inoculated with 100 µL of culture; sterile LCFSM served as control. Isolates were also streaked on PVC-supplemented plates. Following incubation at 55 °C for 72 h, plates were stained with 0.1% Coomassie Brilliant Blue R-250 (40% methanol, 10% acetic acid) for 20 min and repeatedly de-stained. Clear zones were visualised against the blue background and measured with callipers (Gupta and Devi 2017).

Evaluation of cell hydrophobicity of the thermophilic bacterial isolates

Cell surface hydrophobicity of thermophilic isolates was assessed using a modified BATH assay (May et al. 2019). Strains grown to early stationary phase in nutrient broth were harvested (10,000 rpm, 15 min), washed thrice with PUM buffer (hydrogen phosphate (K_2HPO_4, 17.0 g/L), potassium dihydrogen phosphate (KH_2PO_4, 7.26 g/L), urea (1.8 g/L), and magnesium sulfate heptahydrate ($MgSO_4 \cdot 7\ H_2O$, 0.2 g/L); pH 7.1, 0.2 µm-filtered), and resuspended to OD_{400} = 1.0 (UV-1900i Plus, Shimadzu). Suspensions (1.5 mL) were mixed with 0.3 mL n-hexadecane, incubated at 55 °C for 10 min, homogenised 10 min, then incubated 1 h. After phase separation (15 min), OD_{400} of the aqueous phase was measured, with PUM as blank. Hydrophobicity (%) was calculated as [(ODinitial – ODaqueous)/ODinitial] × 100. Strains were classified as strongly hydrophobic (>50%), moderately hydrophobic (20–50%), or hydrophilic (<20%). All assays were triplicated, and results are mean values.

DNA extraction and full-length 16 S rRNA sequencing

Isolates meeting both selection criteria as clear zone formation and cell surface hydrophobicity exceeding 50% were proceed to DNA extraction and full-length 16S rRNA gene sequencing for taxonomic identification. About 0.25 mg of thermophilic biomass was suspended in DNA/RNA Shield and disrupted in 2.0 mL screw-cap tubes containing 0.1 mm glass beads by vortexing at maximum speed for 30 min, followed by centrifugation (10,000 × g, 5 min). DNA was extracted from the supernatant using the Zymobiomics DNA Extraction Kit (Zymo Research, USA). DNA integrity and quality were assessed prior to sequencing by agarose gel electrophoresis and spectrophotometric analysis (A260/A280 and A260/A230 ratios), and only samples showing intact high-molecular-weight DNA and acceptable purity were used for downstream sequencing. The 16S rRNA V1–V9 region was amplified with primers 27 F (5'-AGRGTTYGATYMTGGCTCAG-3') and 1492R (5'-GGYTACCTTGTTACGACTT-3'). PCR reactions (25 µL) contained 1 µL genomic DNA, 1× Rediant II PCR Master Mix (Apical Scientific, Malaysia), and 0.25 µM of each primer (Leong et al. 2020). Cycling was 95 °C (3 min), then 30 cycles of 95 °C (30 s), 50 °C (30 s), 72 °C (90 s), with final extension at 72 °C (1 min). Amplicons were checked on 1% agarose, purified with 0.6× SPRI beads, and prepared using the LSK114 kit (Oxford Nanopore Technologies, UK). Sequencing was performed on a Flongle R10.4.1 flow cell for 24 h. Base calling, adapter trimming, and demultiplexing were done with Dorado v0.8.3 (dna_r10.4.1_e8.2_400bps_sup@v5.0.0).

Consensus construction and phylogenetic analysis

Primer sequences were trimmed from the demultiplexed reads using Cutadapt v4.6, with a maximum permissible error rate of 0.15. After primer excision, the reads were processed using NGSpeciesID v3.0 to produce full-length 16 S rRNA consensus sequences for each sample. These consensus sequences were subsequently queried against the GreenGene2 database employing Nucleotide Basic Local Alignment Search Tool (BLASTn). The top BLASTn hits, along with the retrieved 16 S rRNA sequences, were aligned using MAFFT v7.0. The resulting alignment was utilised to construct a maximum-likelihood phylogenetic tree using FastTreeMP (-generalised time-reversible model -nucleotide).

RNA secondary structures prediction

The RNA secondary structure of the identified thermophilic bacteria was predicted (Skariyachan et al. 2017). The obtained 16 S rDNA sequences of the ten thermophilic bacterial isolates were folded using RNAfold web server version 2.6.3 (http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cgi) to examine the predicted secondary structure

and to analyse the structural stability in terms of Gibbs's free energy. The16S rDNA sequences were submitted to the RNAfold web server, and the parameters such as folding constraints, dangling end options, modified bases, fold algorithm and basic options were fixed and using the default settings. The folding process was carried out using RNA parameters (Andronescu Model) at 37 °C and under ionic conditions of 1 M NaCl deprived of divalent ions.

Results

Water physicochemical parameters of the hot springs

The Paku hot spring pool showed the highest pH (6.40 ± 0.22), whereas the Panchor source recorded the lowest (5.36 ± 0.07; $p < 0.05$) (Table 1). Temperature ranged from 37.87 ± 1.21 °C at Paku (the lowest) to 42.33 ± 1.01 °C at Panchor (the highest), with Paku differing significantly from both Panchor ($p = 0.043$) and Ayer Hangat Village ($p = 0.001$). Salinity was highest in Ayer Hangat Village (28.37 ± 0.06–28.20 ± 0.10 ppt; $p < 0.05$), contrasting with near-zero values in Paku and Panchor. According to the classification of Simon et al. (2019), Paku is a thermal neutral spring, Ayer Hangat Village a hyperthermal neutral spring, and Panchor a hyperthermal weak acid spring.

PVC-degrading bacteria isolation

The thermophilic bacterial counts, expressed in $\log_{10}$ colony-forming units per millilitre ($\log_{10}$ CFU/mL) for each sampling site, are presented in Table 2. The mean difference in $\log_{10}$ CFU/mL at Ayer Hangat Village hot spring was statistically significant at 0.05 level when compared to both Panchor ($p = 0.000$, $p < 0.05$) and Paku ($p = 0.005$, $p < 0.05$) hot spring. Among the sites, SPK(SD)M1 exhibited the highest $\log_{10}$ CFU/mL, with a count of 5.49 ± 0.01 $\log_{10}$ CFU/mL, followed by SPK(SD)M2 with 5.38 ± 0.01 $\log_{10}$ CFU/mL, and LAH(SD)B1 with 5.37 ± 0.01 $\log_{10}$ CFU/mL. Conversely, the site SPC(W)P3 exhibited the lowest bacterial counts, with 4.01 ± 0.06 $\log_{10}$ CFU/mL, followed by SPK(W)M1 and SPC(SD)M2, which recorded 4.26 ± 0.04 and 4.29 ± 0.06 $\log_{10}$ CFU/mL, respectively. The mean difference in $\log_{10}$ CFU/mL between water and sediment samples collected was statistically significant at 0.05

Table 1 Water physicochemical parameters of Panchor, Paku and Ayer Hangat Village hot spring, Malaysia

Water Physicochemical Parameters	Panchor hot spring		Paku hot spring		Ayer Hangat Village hot spring	
	Source	Pool	Source	Pool	Source	Pool
pH	5.36 ± 0.07 [a]	5.71 ± 0.19 [a]	6.15 ± 0.11 [b]	6.40 ± 0.22 [b]	6.26 ± 0.05 [b]	6.26 ± 0.06 [b]
Temperature (°C)	42.33 ± 1.01 [a]	39.20 ± 1.21 [a]	38.17 ± 0.06 [b]	37.87 ± 0.06 [b]	40.20 ± 0.85 [a]	40.78 ± 0.55 [a]
Salinity (ppt)	0.10 ± 0.00 [a]	0.13 ± 0.06 [a]	0.17 ± 0.06 [a]	0.20 ± 0.10 [a]	28.37 ± 0.06 [b]	28.20 ± 0.10 [b]

[ab] Different lowercase letters within a row indicate significant differences among the sampling sites for pH, temperature and salinity (Tukey HSD and Bonferroni post hoc tests: $P < 0.05$); Each value represents the average (± SE) of 3 replicates

Table 2 Bacterial counts expressed in $\log_{10}$ CFU/mL at 10^{-2} dilution factor after enrichment culture

Sample source	Panchor hot spring [a]	$\log_{10}$ CFU/mL	Paku hot spring [a]	$\log_{10}$ CFU/mL	Ayer Hangat Village hot spring [b]	$\log_{10}$ CFU/mL	Total
Water	SPC(W)M1	4.83 ± 0.01	SPK(W)M1	4.26 ± 0.04	LAH(W)J1	5.32 ± 0.01	4.84 ± 0.37[A]
	SPC(W)M2	4.88 ± 0.01	SPK(W)M2	4.64 ± 0.02	LAH(W)J2	5.18 ± 0.01	
	SPC(W)M3	5.01 ± 0.01	SPK(W)M3	4.40 ± 0.03	LAH(W)J3	5.34 ± 0.01	
	SPC(W)P1	4.47 ± 0.02	SPK(W)P1	4.68 ± 0.02	LAH(W)B1	5.19 ± 0.01	
	SPC(W)P2	4.72 ± 0.01	SPK(W)P2	5.01 ± 0.01	LAH(W)B2	5.11 ± 0.01	
	SPC(W)P3	4.01 ± 0.06	SPK(W)P3	5.05 ± 0.03	LAH(W)B3	5.06 ± 0.04	
Sediment	SPC(SD)M1	4.93 ± 0.01	SPK(SD)M1	5.49 ± 0.01	LAH(SD)J1	4.87 ± 0.02	5.08 ± 0.29[B]
	SPC(SD)M2	4.29 ± 0.06	SPK(SD)M2	5.38 ± 0.01	LAH(SD)J2	5.05 ± 0.02	
	SPC(SD)M3	4.75 ± 0.01	SPK(SD)M3	4.93 ± 0.01	LAH(SD)J3	5.16 ± 0.02	
	SPC(SD)P1	5.33 ± 0.01	SPK(SD)P1	5.26 ± 0.02	LAH(SD)B1	5.37 ± 0.01	
	SPC(SD)P2	4.77 ± 0.02	SPK(SD)P2	5.15 ± 0.01	LAH(SD)B2	5.20 ± 0.01	
	SPC(SD)P3	5.36 ± 0.01	SPK(SD)P3	5.03 ± 0.02	LAH(SD)B3	5.04 ± 0.01	

Note: SPC, Sarawak Panchor hot spring; SPK, Sarawak Paku hot spring; LAH, Langkawi Ayer Hangat Village hot spring; W, water; SD, sediment; M, main source; P, Pool; J, Jantan; B, Betina

[ab] Mean difference in $\log_{10}$ CFU/mL at Ayer Hangat Village hot spring is statistically significant at 0.05 level when compared to both Panchor and Paku hot spring using ANOVA Games-Howell post hoc test (equal variances not assumed from Levene test and further confirmed by equality of means using Welch test). [AB] Mean difference in $\log_{10}$ CFU/mL is statistically significant at 0.05 level when compared to both water and sediment samples. Each value represents the average (± SE) of 3 replicates

level. Subsequently, eight random bacterial colonies were isolated and purified from each sampling sites by streaking onto new CFSM agar plates supplemented with 0.1% (w/v) PVC powder. A total of 288 thermophilic bacterial colonies were isolated for further analysis.

Clear zone assay

In the present study, 264 out of 288 thermophilic bacterial isolates tested demonstrated clear zone formation around the bacterial colonies, confirming the PVC-decomposing capability of these bacterial strains. Figure 2A and C illustrated examples of clear zone formation observed around the thermophilic bacterial strains following the application of 0.1% (w/v) Coomassie blue R-250 solution to the agar plates, indicating the utilisation of PVC powder as the carbon source. Figures 2D and F and 3 illustrate the measurement of the clear zone size around the agar wells. As depicted in Fig. 3, most clear zone sizes were predominantly ranged from 10 to 25 mm range, with a median size of 14.67 mm. The largest clear zone was recorded by SPK(SD)P3(5), measuring 41.67 ± 0.90 mm in diameter, followed by SPC(SD)M2(4) at 28.00 ± 0.87 mm, SPK(W)M2(2) at 27.67 ± 0.42 mm, SPK(W)M2(3) at 27.33 ± 0.59 mm, and SPK(SD)P2(1) at 27.00 ± 1.91 mm.

Evaluation of cell hydrophobicity

Figure 4 illustrates that approximately 95 thermophilic bacterial isolates exhibited a cell hydrophobicity percentage exceeding 20%, as indicated in the labels. Among these labelled isolates, those with higher cell hydrophobicity are represented with thicker bar widths and higher radial label positions. The degree of cell hydrophobicity is categorised into three categories: strongly hydrophobic, moderately hydrophobic, and hydrophilic, corresponding to percentage adhesion values of > 50%, 20–50% and < 20%, respectively (De Souza et al. 2019). In this study, 167 thermophilic bacterial isolates with cell hydrophobicity below 20% were categorised as hydrophilic, 85 isolates as moderately hydrophobic, and 10 isolates with cell hydrophobicity percentages exceeding 50% as strongly hydrophobic. The highest cell hydrophobicity percentage was observed in SPK(SD) M3(6), which demonstrated a 79.2% reduction in culture turbidity, followed by SPK(W)M3(3) (77.15%), SPC(W) M3(3) (76.95%), LAH(W)J2(4) (76.40%), LAH(W)J2(6) (74.95%), and SPC(W)M2(3) (73.33%).

PVC-degrading bacteria identification

Total of 10 isolates with clear zone and cell hydrophobicity percentages exceeding 50% were undergone molecular identification. Full-length 16 S rRNA (V1–V9) sequencing of ten isolates (1476–1477 bp) identified one *Brevibacillus thermoruber* and nine *Anoxybacillus rupiensis* (Table 3). Phylogenetic analysis (MAFFT alignment, FastTreeMP) revealed polymorphic variations among the *A. rupiensis* isolates (Fig. 4).

16 S rDNA gene sequence folding prediction

The 16S rRNA folding was predicted to assess the thermodynamic stability of the gene sequence. The minimum free energy (MFE), ensemble energy, MFE frequency, ensemble

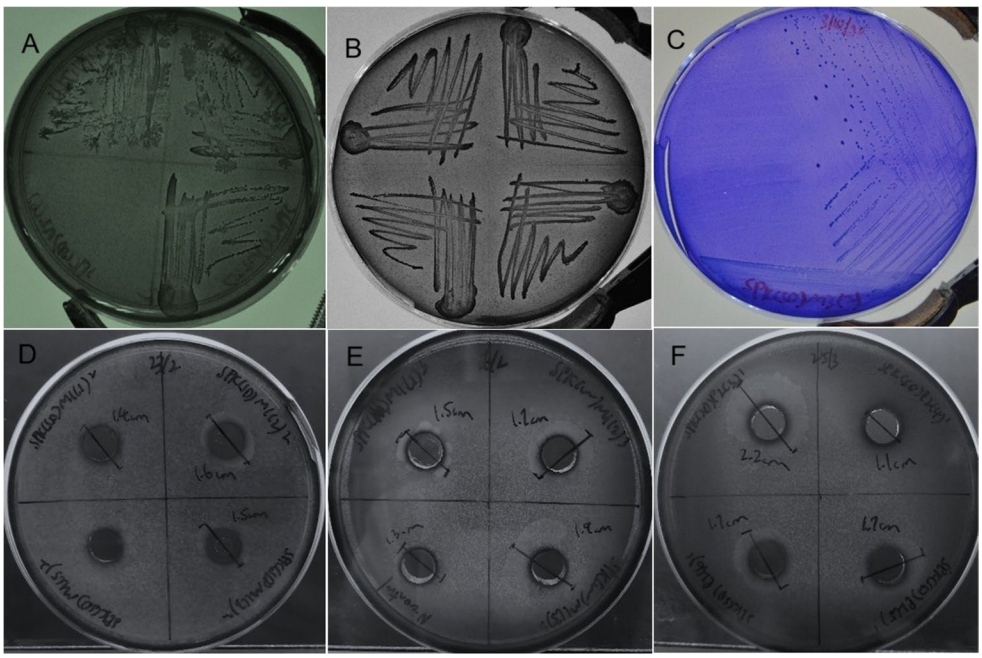

Fig. 2 Clear zone formation was observed around the thermophilic bacterial strains following the application of a 0.1% (w/v) CBB R-250 solution to the agar plates. A to C: clear zones of varying intensity were noted in contrast to the agar background surrounding the streaking lines and bacterial colonies; D to F illustrate examples of clear zone measurement around the agar well after inoculated with standardised thermophilic bacterial culture

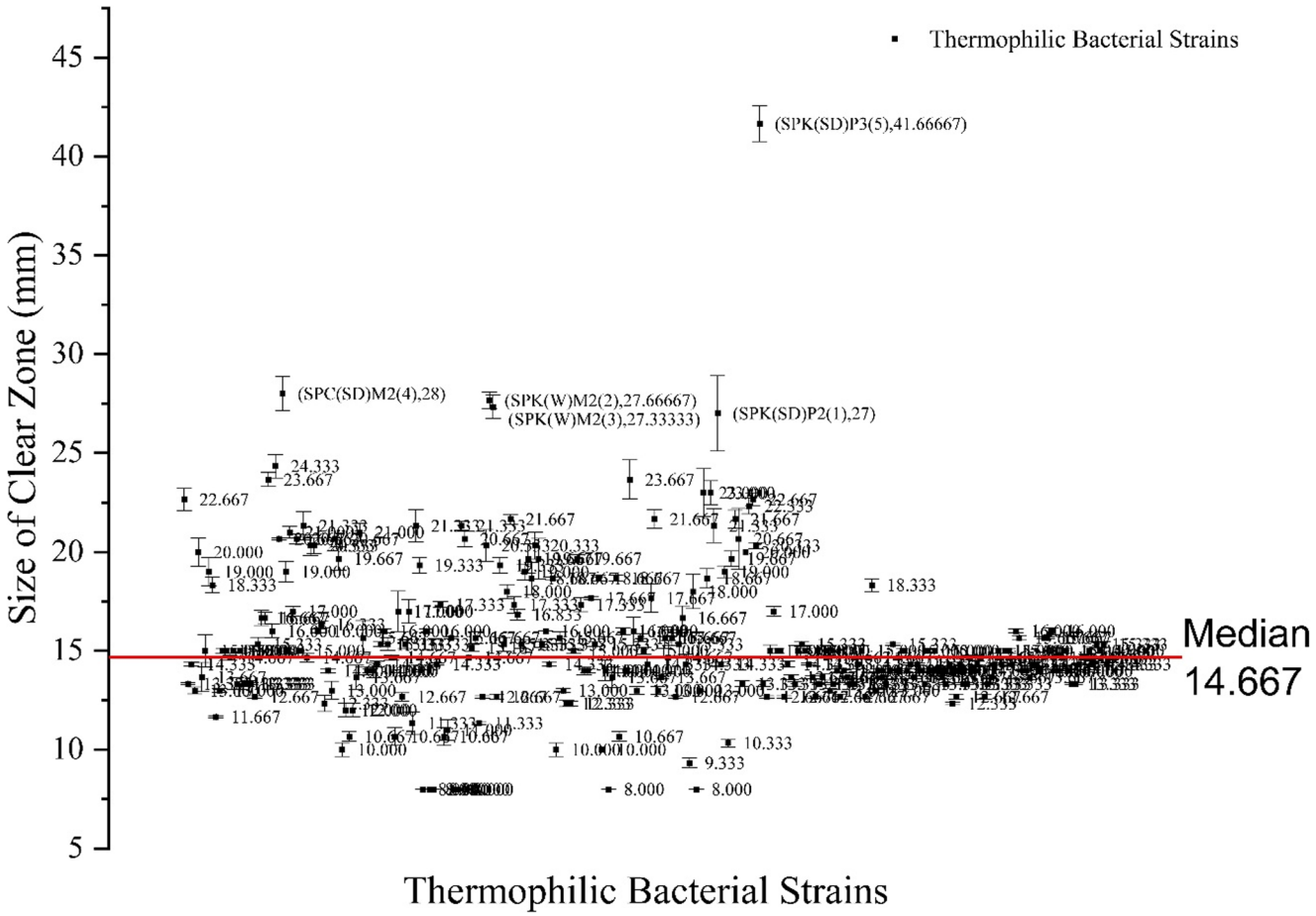

Fig. 3 Scatter plot for the size of clear zone formation around the agar well inoculated with the thermophilic bacterial isolates. The size of the clear zone formation recorded the median at 14.67 mm. The largest size of clear zone was observed in isolates SPK(SD)P3(5), followed by SPC(SD)M2(4), SPK(W)M2(2), SPK(W)M2(3), and SPK(SD)P2(1). Each value represents the average (±SD) of three replicates

diversity, and centroid energy of the predicted 16 S rRNA structure are detailed in Table 4. The prediction reveals that the Gibb's free energy for the 16 S rRNA folding of thermophilic bacterial isolates, despite the absence of a minimum free energy (MFE) representation due to a 0.000% MFE frequency. This 0.000% ensemble frequency of the MFE structure suggests that, although it is theoretically optimal, this structure cannot be sampled under thermodynamic equilibrium.

In this study, it was observed that the Gibb's free energy of 16 S rRNA folding for LAH(W)J2(4) was the lowest, predicted at −601.440 kcal/mol This was followed by SPC(W)M2(3) with a predicted value of −596.400 kcal/mol and LAH(W)J2(8) at −594.900 kcal/mol. Interestingly, both LAH(W)J2(6) and SPK(SD)M3(6) exhibited identical Gibb's free energy of −593.420 kcal/mol. Furthermore, the Gibb's free energy for LAH(WJ3(6), SPC(SD)M1(5), SPC(SD)P3(4), SPC(W)M3(3), and SPK(W)M3(3) was consistently predicted at −592.120 kcal/mol.

Discussions

Physicochemical parameters such as temperature, pH, and salinity are key determinants of bacterial community structure, as many taxa are restricted to specific ranges of these factors (Ortega-Villar et al. 2024). Understanding these natural conditions enables researchers to design culture media and incubation systems that mimic hot spring environments, which is essential for large-scale cultivation of thermophiles.

Unlike typical freshwater hot springs, the Ayer Hangat hot spring contains brackish to saline water due to its coastal proximity and low elevation (3 m above sea level) (Baioumy et al. 2015). Its salinity is considered rare among global hot springs and has been linked to seawater intrusion through the Kisap thrust fault, resulting in elevated sulfate, sodium, magnesium, potassium, chloride, and calcium compared with other Malaysian springs (Baioumy et al. 2015). The elevated salinity observed at Ayer Hangat Village (28.20–28.37 ppt) (Table 1) may act as an additional

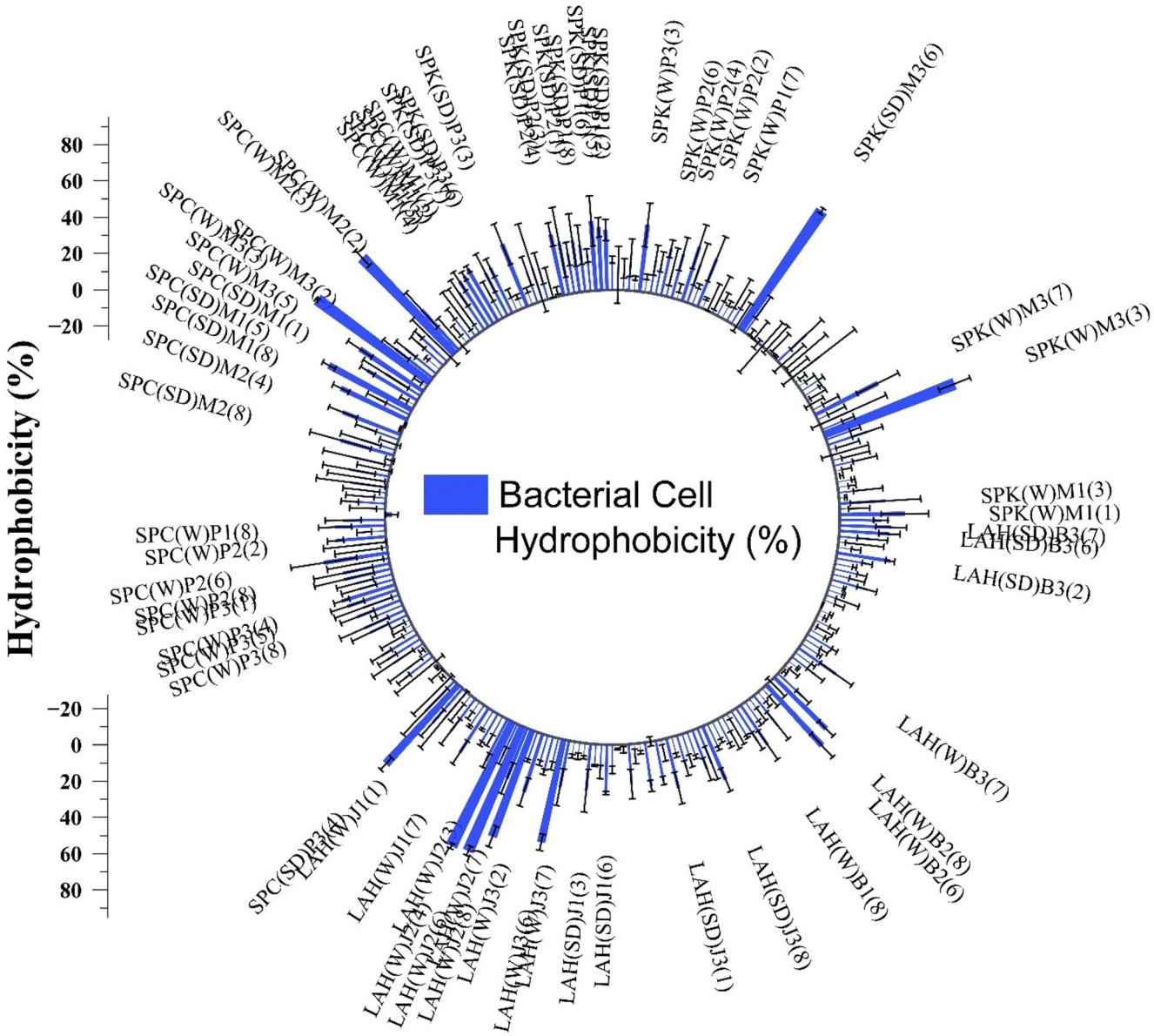

Fig. 4 Bacterial cell hydrophobicity percentage of thermophilic bacterial isolates. Thermophilic bacterial isolates with cell hydrophobicity below 20% are not included in the labelling. Conversely, those isolates exhibiting higher cell hydrophobicity are represented with a thicker bar width and a higher radial label position

selective pressure influencing the composition and diversity of thermophilic microbial communities by osmotic stress and altering microbial interactions (Gao et al. 2024). Halotolerant thermophiles are likely favoured under these conditions, which could affect both growth dynamics and metabolic capabilities, including polymer degradation. Consequently, isolates from high-salinity sites may possess adaptive traits, such as enhanced extracellular enzymes, that could influence PVC adhesion and biodegradation efficiency. These physicochemical insights are critical for both microbial ecology studies and the development of optimised laboratory protocols for thermophilic bacteria.

Spread plating of enriched cultures at a 10^{-2} dilution yielded relatively low bacterial counts on CFSM agar containing 0.1% (w/v) PVC, suggesting limited thermophile adaptation to PVC as a sole carbon source, consistent with reports on the rarity of PVC-degrading thermophiles (Patil and Bagde 2012). Sediment-enriched samples showed slightly higher counts than water-enriched ones, likely due to sediments providing organic matter, physical protection, and stable niches for bacterial growth. Elevated incubation also activated dormant thermophilic spores in sediments, enhancing bacterial proliferation and organic matter mineralisation (Hubert et al. 2010).

Table 3 Summary of top BLASTn hits against the GreenGenes2 database

Thermophilic Bacterial Strains	BLASTn Hit	% Identity	% query coverage	Accession Number
LAH(W)J2(4)	*Brevibacillus thermoruber*	96.307	97	VKJN01000044.1
LAH(W)J2(6)	*Anoxybacillus rupiensis*	100.000	100	CP047158.1
LAH(W)J2(8)	*Anoxybacillus rupiensis*	99.932	100	CP047158.1
LAH(WJ3(6)	*Anoxybacillus rupiensis*	100.000	100	CP047158.1
SPC(SD)M1(5)	*Anoxybacillus rupiensis*	100.000	100	CP047158.1
SPC(SD)P3(4)	*Anoxybacillus rupiensis*	100.000	100	CP047158.1
SPC(W)M2(3)	*Anoxybacillus rupiensis*	97.563	100	CP047158.1
SPC(W)M3(3)	*Anoxybacillus rupiensis*	100.000	100	CP047158.1
SPK(SD)M3(6)	*Anoxybacillus rupiensis*	100.000	100	CP047158.1
SPK(W)M3(3)	*Anoxybacillus rupiensis*	100.000	100	CP047158.1

The clear zone assay provided an initial indication of PVC-degrading potential among the thermophilic isolates, with zones reflecting extracellular enzymatic activity on polymer substrates. While this method is widely applied for polymer degradation screening (Gupta and Devi 2017), its interpretation is not straightforward. In this study, the smallest zone observed measured 8 mm (Fig. 3); however, clear zone formation can be influenced by technical artefacts. To minimize diffusion-related effects arising from inoculum variability, all isolates were standardised to the same cell density and inoculation volume prior to plating, and control plates without active cells were included (Lihan et al. 2021). For instance, the use of CBB R-250 dye requires methanol, acetic acid destaining, which may distort agar integrity or leave residual staining that conceals faint halos (McDonald et al. 2024). The CBB R-250 assay was used as a qualitative, preliminary screening tool rather than a definitive measure of PVC degradation. Furthermore, as the dye binds non-specifically to all proteins, and thus the formation of clear zones may reflect general extracellular protein secretion withmedium components rather than specific enzymatic activity associated with PVC degradation (Stochaj et al. 2006). The resulting zones size may therefore reflect protein composition rather than actual polymer utilisation. Given these limitations, the assay should be regarded as a preliminary tool, useful for excluding non-active strains, but insufficient for quantifying PVC degradation without complementary biochemical or molecular validation.

PVC is well recognised for its strong hydrophobicity, a property arising from its chemically inert C–C backbone and low surface wettability (Xu et al. 2023). This characteristic poses a major challenge for microbial colonisation, as effective interaction with the polymer surface generally requires bacteria to exhibit comparable levels of cell surface hydrophobicity (Gupta and Devi 2019). Since microbial degradation of plastics typically begins with adhesion and subsequent biofilm formation (Amobonye et al. 2021), the hydrophobicity of the bacterial cell surface is a key determinant of degradation efficiency. Previous studies have shown that reduced hydrophobicity often correlates with weaker

Table 4 Minimum free energy (MFE), ensemble energy, MFE frequency, ensemble diversity, and centroid energy of the predicted 16 S rRNA secondary structure for ten thermophilic bacterial isolates

Thermophilic Strains	Molecular Identity	Metric				
		MFE Energy (kcal/mol)	Ensemble Energy (kcal/mol)	MFE Frequency (%)	Ensemble Diversity	Centroid Energy (kcal/mol)
LAH(W)J2(4)	*Brevibacillus thermoruber*	−571.190	−601.440	0.000%	292.770	−455.370
LAH(W)J2(6)	*Anoxybacillus rupiensis*	−560.180	−593.420	0.000%	311.440	−465.050
LAH(W)J2(8)	*Anoxybacillus rupiensis*	−561.680	−594.900	0.000%	312.480	−466.550
LAH(WJ3(6)	*Anoxybacillus rupiensis*	−558.860	−592.120	0.000%	312.610	−463.730
SPC(SD)M1(5)	*Anoxybacillus rupiensis*	−558.860	−592.120	0.000%	312.610	−463.760
SPC(SD)P3(4)	*Anoxybacillus rupiensis*	−558.860	−592.120	0.000%	312.610	−463.760
SPC(W)M2(3)	*Anoxybacillus rupiensis*	−564.650	−596.400	0.000%	255.400	−505.860
SPC(W)M3(3)	*Anoxybacillus rupiensis*	−558.860	−592.120	0.000%	312.610	−463.760
SPK(SD)M3(6)	*Anoxybacillus rupiensis*	−560.180	−593.420	0.000%	311.440	−465.050
SPK(W)M3(3)	*Anoxybacillus rupiensis*	−558.860	−592.120	0.000%	312.610	−463.760

biofilm development, thereby limiting biodegradative potential (De Souza et al. 2019; Maheswaran et al. 2024).

In this study, the BATH assay, using n-hexadecane as the hydrocarbon phase, was applied to evaluate bacterial cell surface hydrophobicity. A higher affinity for the hydrocarbon phase reflects stronger hydrophobic interactions and a greater potential for stable attachment to hydrophobic polymers (Kavitha and Bhuvaneswari 2021). The results indicated that thermophilic isolates with hydrophobicity above 50% were most suitable for subsequent biodegradation studies. Interestingly, several isolates such as SPK(SD)P3(5), SPC(SD)M2(4), SPK(W)M2(2), SPK(W)M2(3), and SPK(SD)P2(1) produced large zones in the clear zone assay but displayed either hydrophilic or moderately hydrophobic characteristics in the BATH test (Fig. 4). Correlation analysis was conducted to examine the association between hydrophobicity values and clear zone diameters. The results revealed a statistically significant, weak positive correlation between the two variables ($r=0.120$, $P=0.001$; $n=792$). This discrepancy suggests that while extracellular enzyme activity can be detected in agar-based assays, effective colonisation and PVC surface degradation may be limited in isolates with insufficient hydrophobicity. Therefore, only strongly hydrophobic isolates were prioritised for further biodegradation experiments, as their enhanced ability to adhere and form biofilms on PVC surfaces is expected to support more consistent degradation.

The thermophilic isolate LAH(W)J2(4) displayed 96.3% identity with *Brevibacillus thermoruber* (Table 3), while other isolates aligned closely with *Anoxybacillus rupiensis*, albeit with varying identity percentages. Both species have been increasingly recognised for their biodegradation and bioremediation potential under elevated temperatures. *B. thermoruber* has been reported to degrade lignin through β-ketoadipate and benzoic acid pathways, with temperature being a key factor influencing efficiency (Niu et al. 2021). *B. thermoruber* demonstrated strong esterase activity, enabling the degradation of poly(ε-caprolactone) (PCL) by 63.6% within 28 days, supported by thermostable lipase activity optimised at 55 °C (Atanasova et al. 2021). In addition to polymer degradation, *B. thermoruber* is also known for producing thermostable enzymes such as keratinase and fibrinolytic enzymes, as well as functioning as an exopolysaccharide producer (Zilda 2021), suggesting wide applications in biotechnology and bioremediation. Similarly, *A. rupiensis* has demonstrated versatility in pollutant removal, particularly in the treatment of textile effluents and heavy metals. Strains have achieved up to 83% dye decolourisation at 60 °C and a 75% reduction of hexavalent chromium within 24 h (Gursahani and Gupta 2015). Genomic evidence further supports its role in environmental remediation, with genes encoding Azoreductases and metal resistance determinants identified in *(A) rupiensis* TPH1 (Mishra et al. 2023). Taken together, the identification of *(B) thermoruber* and *A. rupiensis* in this study underscores their potential contribution to thermophilic biodegradation processes and highlights their broader applicability in biotechnological and environmental management strategies.

Generally, bootstrap values above 90% are considered to indicate strong support, 70–90% moderate support, and below 70% weak support. In this study, the phylogenetic tree (Fig. 5) revealed consistently high bootstrap values for most thermophilic isolates. For example, strains LAH(W)J3(6), SPC(SD)M1(5), SPC(SD)P3(4), SPC(W)M3(3), and SPK(W)M3(3) clustered with *A. rupiensis* (Accession CP047158.1–2) with 100% support, while LAH(W)J2(8) showed 85% support to the same species. Similarly, SPK(SD)M3(6) and LAH(W)J2(6) demonstrated strong to moderate support (92%, 88%, and 77%) with different *A. rupiensis* clusters. Interestingly, SPC(W)M2(3) shared strong bootstrap values with both *(A) rupiensis* (76%) and *(B) thermoruber* (100%), indicating close relatedness. In line with this, LAH(W)J2(4) clustered with *B. thermoruber* at 100% support and 97% with a specific accession (VKJN01000044.1). The overall high bootstrap values confirmed the accuracy of molecular identification, while the short branch lengths (0.000–0.001 substitutions per site) indicated minimal genetic divergence among the isolates. Such patterns are typical of closely related strains or intraspecies variants, reflecting low evolutionary distance. These findings suggest that the thermophilic isolates identified in this study are genetically conserved and reliably clustered within *(A) rupiensis* and *(B) thermoruber* lineages, supporting their taxonomic placement and potential functional similarities.

The RNA secondary structure and Gibbs free energy analysis were not intended to directly predict PVC degradation capacity but were used to assess the structural stability and evolutionary adaptation of the identified thermophilic isolates. The prediction of RNA secondary structures provides valuable insights into bacterial adaptations to thermophilic environments, particularly for rRNA and tRNA, which must remain stable under high-temperature stress. Such structural adaptations ensure ribosome integrity, efficient translation, and overall cellular survival at elevated temperatures (Thomas et al. 2022). Thermophilic bacteria typically exhibit rRNA and tRNA sequences with enhanced thermal stability, reflected by increased melting temperatures, higher fractions of paired nucleotides, and G–C rich regions that resist denaturation (Jegousse et al. 2017). Lower Gibbs free energy values reflect more stable rRNA conformations, which are commonly associated with microbial survival and translational efficiency under elevated temperatures (Liao et al. 2021). Such thermal and structural stability is relevant

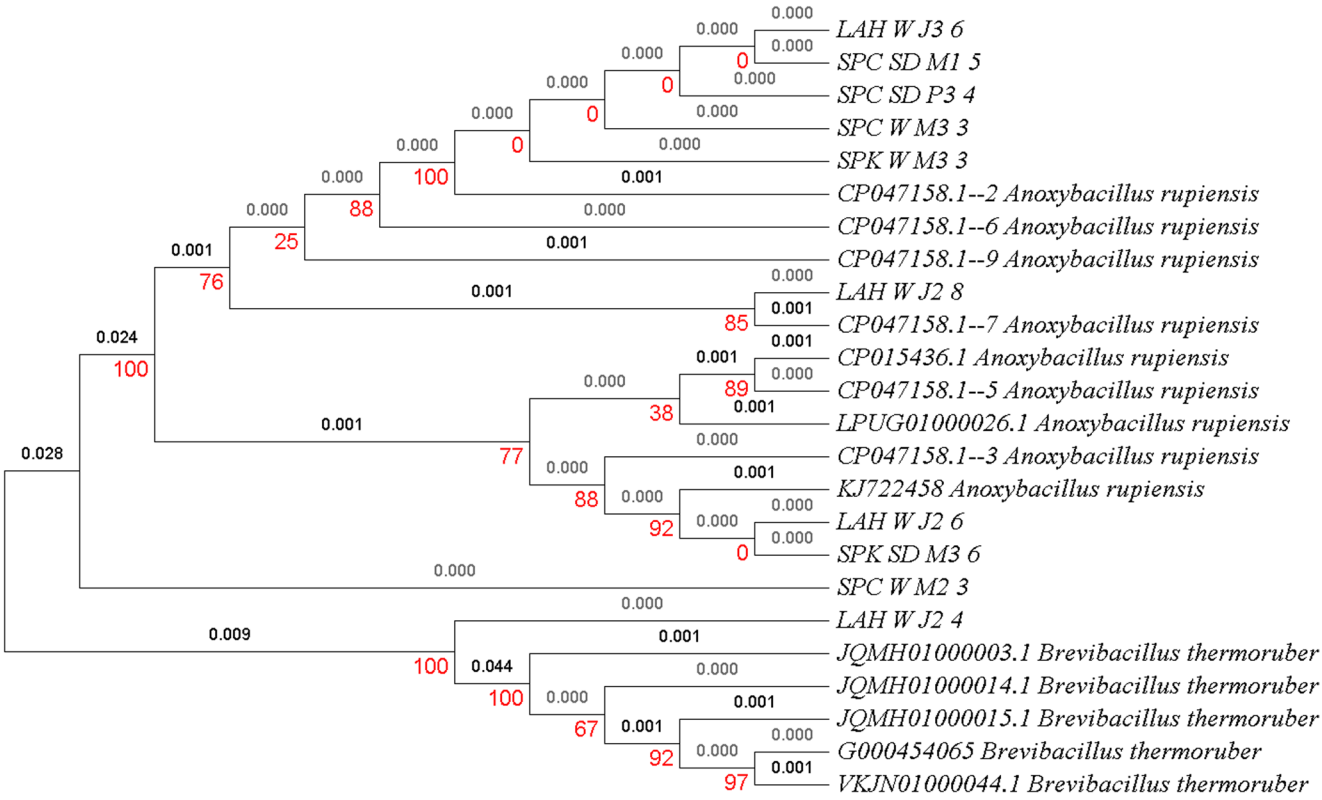

Fig. 5 Phylogenetic tree showing the maximum-likelihood phylogeny of sequenced strains with closely related species. Bootstrap value (red) evaluates the robustness or confidence of the branch supports in phylogenetic trees. Branch lengths (black) indicate number of substitutions per site

to sustaining metabolic activity in thermophilic conditions where PVC degradation is investigated.

Computational approaches predict the most stable RNA conformations by minimising thermodynamic free energy. Comparative analysis of 16 S rRNA secondary structures among isolates can highlight molecular signatures of thermophily, such as stable hairpins and conserved G–C stem regions (Jegousse et al. 2017; Yang et al. 2025). However, structural predictions are limited by algorithmic sampling, kinetic folding barriers, and the existence of multiple near-optimal conformations, often reflected in high ensemble diversity. Ensemble free energy provides a more accurate representation of RNA stability by considering all potential secondary structures, while centroid structures capture consensus folds, despite being less stable than minimum free energy (MFE) conformations (Ashok et al. 2025).

Our findings indicate that 16 S rRNA structures with lower Gibbs free energy exhibit stronger folding stability, which may also reflect nucleotide resilience in novel thermophilic organisms. Similar trends were reported in *Brevibacillus* and *Aneurinibacillus* isolates, where Gibbs free energy values correlated with thermotolerance (Skariyachan et al. 2018). These structural insights not only deepen the understanding of thermophilic adaptations but also hold promise for identifying strains with potential in polymer biodegradation. Given previous reports linking thermophiles to plastic degradation (Maheswaran et al. 2024; Skariyachan et al. 2018), genome-wide analysis of candidate strains will be essential to confirm the presence of genes associated with PVC biodegradation.

Although this study provides preliminary evidence of PVC-interacting potential among thermophilic bacterial isolates from three Malaysian hot springs, several limitations must be acknowledged. First, although clear zone and BATH assays are useful for high-throughput screening, they are indirect degradation proxies. The clear zones can arise from the release of non-specific protein or artefacts, and a weak positive correlation is found between hydrophobicity with zones ($r=0.120$, $P=0.001$; $n=792$), which limits predictive capability unless direct measurement such as gravimetric weight loss, FTIR, or SEM are employed. Second, PVC powder was used instead of commercial films or waste, which contain additives (plasticizers or stabilizers) that could inhibit or alter microbial interactions, reducing the material's applicability in the real world. Third, enrichment at 55 °C and short incubation periods may favour fast-growers over true degraders, whereas low CFU on PVC enriched media highlights adaptation challenges for recalcitrant PVC. In addition, the small number of sequenced isolates ($n=10$) and the lack of functional genomics might

overlook strain variability and PVC-specific enzymes. Variables such as salinity gradients specific to individual sites were treated as uncontrolled variables. These constraints position this study as foundational screening, necessitating quantitative degradation assays, enzyme characterisation, and real-waste testing in follow-up studies.

Conclusion

This study addressed the knowledge gap in thermophilic biodegradation of polyvinyl chloride (PVC) by isolating and characterising thermophilic bacteria from three Malaysian hot springs. Physicochemical analyses of the hot springs established baseline conditions for cultivating these thermophiles, while enrichment cultures with PVC as the sole carbon source enabled bacterial screening. Among the 288 isolates obtained, 264 demonstrated PVC-degrading potential through clear zone formation. Hydrophobicity assessment further narrowed the candidates to ten potential PVC-degrading isolates, primarily identified as *Anoxybacillus rupiensis* and *Brevibacillus thermoruber* via 16 S rRNA sequencing and phylogenetic analysis. The stable RNA secondary structures, reflected by low Gibbs free energy values, underscored their thermal resilience. Collectively, these findings highlight the potential of Malaysian hot spring thermophiles as candidates for PVC biodegradation. While this study focuses on preliminary screening, phylogenetic identification, and characterisation of thermophilic isolates, direct evidence of polymer degradation will be presented in subsequent work, which will include quantitative analyses of PVC breakdown and confirmation of enzymatic activity. This study provides a foundation for identifying thermophilic isolates with potential PVC-interacting capabilities. Future work will focus on genomic and proteomic characterisation of key isolates to identify the enzymes responsible for polymer interaction, followed by purification and functional testing of these enzymes. Additionally, the applicability of these thermophiles will be evaluated using real PVC waste materials, moving beyond powdered PVC, to assess their potential for practical biotechnological interventions in plastic waste management.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval Not applicable.

Declarations of Generative AI and AI-assisted technologies in the writing process The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript and no images were manipulated using AI.

Competing interests The authors declare no competing interests.

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