

Article

Production and Characterization of Poly(3-hydroxybutyrate) by Thermophilic *Geobacillus* sp. Strain SL28 Isolated from a Natural Hot Spring

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Abstract

Polyhydroxyalkanoates (PHAs) are biodegradable microbial polyesters that may reduce dependence on petroleum-derived plastics, but broader use remains constrained by production costs and tightly controlled cultivation. Thermophilic microorganisms have attracted increasing attention as promising PHA producers because they can be cultivated at elevated temperatures, reducing contamination risks and operational costs. In this study, a thermophilic *Geobacillus* sp. strain SL28 isolated from a Vietnamese hot spring was evaluated for its PHA-producing capability. Temperature, initial pH, carbon and nitrogen sources, C/N ratio, and cultivation time were examined. Intracellular inclusions were assessed by Sudan Black B and Nile Blue A staining and transmission electron microscopy, whereas the recovered polymer was characterized by FE-SEM, Fourier-transform infrared spectroscopy (FTIR), gas chromatography–mass spectrometry (GC–MS), ¹H- and ¹³C-nuclear magnetic resonance (NMR) spectroscopy, capillary viscometry, differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), and tensile testing. Under the optimized cultivation conditions identified by one-factor experimentation (50 °C, pH 7.0, sucrose, peptone, C/N 25:1, and 72 h), SL28 reached a dry cell weight of 2.215 ± 0.16 g/L and a PHA concentration of 1.383 ± 0.04 g/L, equivalent to $62.57 \pm 2.82\%$ of DCW. Structural characterization confirmed that the recovered polymer was poly(3-hydroxybutyrate) (PHB). The purified PHB also exhibited favorable thermal stability, a relatively high molecular weight, and satisfactory mechanical properties. These results demonstrate the potential of thermophilic *Geobacillus* sp. SL28 as a promising candidate for PHB production.

Keywords: *Geobacillus* sp.; polyhydroxyalkanoates; poly(3-hydroxybutyrate); hot spring; thermophilic bacterium; polymer characterization



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1. Introduction

The world produces over 400 million tons of synthetic plastic each year, yet recycling rates remain stubbornly below 10% [1,2]. The majority of these petroleum-based

plastics, such as polyethylene (PE), polypropylene (PP), polystyrene (PS), and polyethylene terephthalate (PET), are highly resistant to microbial degradation and can remain in the environment for centuries after disposal. Their breakdown into microplastics and nanoplastics has greatly contaminated our living environment, from deep-ocean sediments to agricultural topsoil [2,3], strengthening the search for materials that can enter biological degradation pathways.

Many bacteria synthesize PHAs as intracellular reserves of carbon and energy, particularly when an essential nutrient limits growth [3–5]. PHAs are highly diverse, with more than 150 hydroxyalkanoic acid monomers identified [6]. This compositional diversity enables the production of materials with a broad range of thermal, mechanical, and biodegradation properties, thereby expanding their industrial applications [7,8]. Poly(3-hydroxybutyrate) (PHB), a short-chain-length PHA, is relatively stiff and crystalline, whereas medium-chain-length PHAs are generally more flexible [5,6]. Microbial degradation of PHAs can yield carbon dioxide and water under aerobic conditions or methane under anaerobic conditions [2,5].

PHAs can be applied in a wide range of fields, including packaging, biomedical, and agriculture. Packaging remains one of the most important application areas for PHAs. Items such as food containers, carrier bags, and disposable cutlery made from PHAs can replace conventional plastics, especially in short-use products and in places where plastic collection and recycling systems are limited [2,5]. In biomedicine, the biocompatibility, non-toxicity, and controllable degradation properties make them suitable for tissue engineering scaffolds, drug delivery systems, wound dressings, cardiovascular patches, and absorbable surgical sutures [9,10]. PHB and PHBV blends can be used for both hard and soft tissue regeneration, and their mechanical properties can be adjusted by changing the monomer composition and processing conditions [9]. In addition, they can also be sterilized using common methods without causing major damage to the material [10]. PHAs are also useful in agriculture. PHA-based films can be used as biodegradable mulch films and coatings for fertilizers and pesticides, allowing the reduction in labor and investment costs [4]. Despite these advantages, feedstock costs, sterilization requirements, and downstream processing remain major economic barriers to large-scale commercialization of PHAs [11]. Currently, the manufacturing costs for PHAs are five to ten times more expensive than conventional plastics such as PE and PP [1–3]. In addition, many well-known PHA-producing bacteria, including *Cupriavidus necator*, *Alcaligenes latus*, and recombinant *Escherichia coli*, are mesophilic microorganisms that demand strict sterile cultivation conditions, which further increases production costs [3,5]. Several approaches have been proposed to reduce the production cost of PHAs, including the use of inexpensive feedstocks, metabolic engineering, and the exploitation of extremophilic microorganisms [12]. Among these strategies, thermophilic microorganisms have attracted increasing attention because of their potential to lower operating costs while maintaining high productivity [13,14].

Thermophilic cultivation is attractive for PHA production because temperatures of 45–70 °C can reduce contamination by mesophiles and may lessen sterilization demands [1,4,5]. In addition, thermophiles often exhibit rapid growth and efficient substrate utilization, which can improve productivity. Elevated temperature can also alter broth viscosity and oxygen transfer, although these benefits depend on the organism and process configuration [3–5]. Recent studies have demonstrated the potential of thermophilic bacteria for PHA production. For example, substantial PHA accumulation has been reported in *Cupriavidus cauae* PHS1 [1], while *Caldimonas thermodepolymerans* was shown to produce PHA directly from xylan-rich biomass [14]. Other thermophilic bacteria have also been found to utilize diverse monomer compositions for PHA production, thereby broadening the spectrum of polymer characteristics and applications [15]. These findings highlight thermophiles as promising candidates for sustainable and cost-effective PHA production [4].

Among thermophilic bacteria, the genus *Geobacillus* remains less explored for PHA production than several other bacterial groups. *Geobacillus* species are Gram-positive, spore-forming thermophilic bacteria commonly found in hot springs, geothermal soils, and other high-temperature environments, and are able to metabolize diverse carbon sources [16,17]. *Geobacillus stearothermophilus* K4E3_SPR_NPP reportedly accumulated PHA to 66.66% of dry cell weight (DCW) and utilized dairy wastewater as the carbon source [16]. These characteristics suggest that *Geobacillus* sp. may be a viable platform for cost-effective PHA synthesis using diverse and low-cost feedstocks [18].

In this study, we characterize a *Geobacillus* sp. strain isolated from a hot spring in Vietnam and systematically evaluate its PHA biosynthetic potential. Growth and polymer production were assessed under different cultivation conditions, including temperature, initial pH, carbon and nitrogen sources, C/N ratio, and cultivation time. Intracellular PHA accumulation was evaluated by Sudan Black B staining, Nile Blue A staining, and transmission electron microscopy (TEM), whereas the recovered polymer was characterized using FE-SEM, GC-MS/MS, FTIR, ¹H- and ¹³C-NMR spectroscopy, capillary viscometry, DSC, TGA, and tensile testing. The results provide further evidence that thermophilic *Geobacillus* strains are promising candidates for sustainable PHB production and offer valuable insights into the physicochemical properties of PHB produced by thermophilic bacteria.

2. Materials and Methods

2.1. Bacterial Strain

The bacterial strain *Geobacillus* sp. SL28 was isolated from water collected from a small natural thermal seepage spring in Phu Yen District, Son La Province, Vietnam (21°19′49.0″ N, 104°41′28.6″ E). The spring exhibited temperatures ranging from 45 to 55 °C and a pH of 6.5–7.2 and is associated with the Song Ma fault system. The water was reddish-brown, indicating iron-rich conditions. Such environments are known to harbor thermotolerant microorganisms with potential biotechnological applications.

The isolate was identified based on morphological characteristics and 16S rRNA gene sequence analysis. Genomic DNA was isolated with the Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA), and the nearly full-length 16S rRNA gene was amplified with primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) using procedures described previously [19]. Amplicons were purified and sequenced by 1st Base (Singapore). The SL28 sequence was compared by BLAST version 2.17.0 with GenBank accessions FN_428696.1, KC_252966.1, MW_413377.1, NR_116987.1, EU_477773.2, AF_326278.1, NR_113265.1, NR_043182.1, and NR_043424.1. A neighbor-joining tree was then generated in MEGA X version 10.0.

Prior to all fermentation experiments, the strain was purified by repeated streaking on LB agar plates until a single, morphologically uniform colony type was obtained. Culture purity was routinely confirmed by colony morphology and microscopic examination before inoculation into seed cultures and subsequent fermentation experiments.

The strain has been deposited in the VAST Culture Collection of Microorganisms (VCCM), Vietnam Academy of Science and Technology, under accession number VCCM 15717.

2.2. Detection of Intracellular PHA in *Geobacillus* sp. SL28

Detection of intracellular PHA accumulation in *Geobacillus* sp. SL28 was performed using Sudan Black B staining (Sigma-Aldrich), Nile Blue A fluorescence staining (Sigma-Aldrich, Sent Louis, MO, USA), and TEM. Colonies grown on Gelrite-solidified LB medium were exposed to the stain for 30 min and then washed with 70% (*v/v*) ethanol. Colonies retaining a dark blue–black color were recorded as positive.

For Nile Blue A screening, stained cells were incubated at 55 °C for 10 min and examined with a Nikon ECLIPSE Ti fluorescence microscope (Nikon, Tokyo, Japan) at an excitation wavelength of 460 nm. Orange fluorescence was taken as evidence of hydrophobic intracellular inclusion [20].

For ultrastructural observation, cells from liquid culture were centrifuged at 8000 rpm for 10 min, rinsed twice with sterile distilled water, and processed according to published TEM procedures [21–23]. The samples were examined using a JEOL JEM-1010 transmission electron microscope (JEOL Ltd., Tokyo, Japan). TEM revealed numerous electron-lucent intracellular inclusion bodies within the cytoplasm, supporting the staining-based evidence of PHA accumulation in *Geobacillus* sp. SL28.

2.3. Effects of Cultivation Conditions on Cell Growth and PHA Production

The influence of six cultivation variables, including temperature, initial pH, carbon source, nitrogen source, carbon: nitrogen (C/N) ratio, and fermentation time, on cell growth and PHA production by *Geobacillus* sp. SL28 was subsequently evaluated.

Seed cultures were generated from single colonies in 5 mL LB broth at 55 °C and 150 rpm for 12–16 h. A 5% (*v/v*) inoculum at OD₆₀₀ 0.8–1.0 was transferred to 100 mL mineral salt medium in 500 mL Erlenmeyer flasks. The basal medium initially contained glucose (30 g/L) as the carbon source and a mixed nitrogen source comprising (NH₄)₂SO₄, NH₄Cl, peptone, and yeast extract (1 g/L each), yielding a C/N ratio of 21.5:1 (mol/mol). The medium was supplemented with the following mineral components (g/L): Na₂MoO₄·2H₂O, 0.005; MnSO₄, 0.005; FeSO₄·7H₂O, 0.03; CaCl₂·2H₂O, 0.02; NaCl, 30; MgSO₄·7H₂O, 0.2; KH₂PO₄·2H₂O, 0.2; and K₂HPO₄, 1.6. The pH was set to 7.0 before sterilization.

For the temperature experiment, cultures were grown at 40, 45, 50, 55, or 60 °C, corresponding to the thermal conditions of the natural spring from which the strain was isolated, for 72 h with shaking at 150 rpm.

For the pH experiment, the initial medium pH was set between 5.0 and 8.5 before sterilization, and cultures were incubated for 72 h. The temperature and pH giving the strongest responses were used in the following experiments unless stated otherwise.

Carbon-source utilization was tested by replacing glucose with sucrose, maltose, arabinose, galactose, lactose, or glycerol at 30 g/L while retaining the original nitrogen formulation. A separate mixed-substrate treatment received 20 g/L glucose and 9 g/L 1,4-butanediol at inoculation, followed by 1 g/L valeric acid after 24 h.

To examine the effect of nitrogen sources on cell growth and PHA production, various inorganic and organic nitrogen sources were individually supplied to the basal medium, with glucose maintained at 30 g/L as the carbon source and other medium components kept constant. The inorganic nitrogen sources included (NH₄)₂SO₄, NH₄Cl, and NH₄NO₃, and the organic nitrogen sources consisted of peptone and yeast extract, each supplied at 4 g/L. The original four-component nitrogen mixture [(NH₄)₂SO₄, NH₄Cl, yeast extract, and peptone; 1:1:1:1, *w/w/w/w*; 4 g/L total] was included as a comparator.

To determine the effect of the C/N ratio on cell growth and PHA production, the most favorable carbon and nitrogen sources identified from the previous experiments were used. The C/N ratios of 15:1, 20:1, 25:1, and 30:1 (mol/mol) were generated by adjusting carbon and/or nitrogen concentrations without changing the mineral formulation. Cultures were incubated under the previously selected temperature and initial pH conditions for 72 h.

Cultivation time was evaluated by collecting cultures after 24, 48, 72, and 96 h under the selected conditions.

Cells were collected at 8000 rpm for 10 min after cultivation, and the biomass was held at 4 °C until analysis.

2.4. Dry Biomass Measurement

Biomass was rinsed twice with distilled water and dried at 100 °C to constant mass; the result was expressed as DCW (g/L).

2.5. PHA Extraction and Purification

PHA was recovered from dried cells by a modified hypochlorite–chloroform procedure [24]. Biomass was combined with solvent at a ratio of 1:35 (*w/v*) and held at 60 °C for 2 h with shaking at 100 rpm. After removal of the aqueous cell-debris layer, the chloroform phase was collected. Polymer was precipitated with methanol–water (7:3, *v/v*), pelleted at 12,000 rpm for 15 min, washed twice with 95% ethanol, and air-dried overnight.

2.6. Determination of PHA Concentration, PHA Content, and Semi-Quantitative Analysis of Monomer Composition

PHA concentration (g/L) was determined gravimetrically from the purified polymer obtained after solvent extraction. PHA content (%DCW) was subsequently calculated as the percentage of PHA concentration relative to DCW according to the following equation:

$$\text{PHA content (\%DCW)} = [\text{PHA concentration (g/L)} / \text{DCW (g/L)}] \times 100.$$

Monomer composition was examined by acidic methanolysis followed by GC–MS/MS. Dried biomass (0.100 ± 0.001 g) was placed in a 10 mL screw-cap tube with 0.2 mL toluene and 1.5 mL HCl/methanol (85:15, *v/v*), sealed, and heated at 100 °C for 2 h. After cooling, 1–2 mL hexane was added; the mixture was vortexed and centrifuged at 7000 rpm for 10 min. The organic phase was passed through a 0.45 µm solvent-resistant filter and transferred to a GC vial. Analysis used a TRACE 1300 gas chromatograph coupled to a TSQ 8000 Evo triple-quadrupole mass spectrometer (Thermo Fisher Scientific, USA) and an HP-5ms Ultra Inert column (30 m × 0.25 mm × 0.25 µm; Agilent, Santa Clara, CA, USA). Splitless injection was performed at 230 °C for 1 min, with helium at 1.5 mL/min. The oven was held at 40 °C for 2 min, ramped to 280 °C at 8 °C/min, and held for 5 min. Electron-ionization spectra were collected over *m/z* 33–500; the transfer line and ion source were set to 280 and 230 °C, respectively, with a 3.2 min solvent delay.

Compounds were identified against a PHB standard (Sigma-Aldrich) and the NIST 19 mass-spectral library. 3HB was quantified with an external calibration curve prepared from methanolized PHB standards. Because no dedicated 3HV calibration standard was used, the 3HV fraction was estimated semi-quantitatively from the relative peak areas of 3HB and 3HV.

2.7. Physicochemical Characterization of PHA

The purified polymer was comprehensively characterized to determine its surface morphology, chemical structure, molecular weight, thermal properties, and mechanical performance using FE-SEM, FTIR, ¹H- and ¹³C-NMR spectroscopy, DSC, TGA, capillary viscometry, and tensile testing.

Polymer surface morphology was observed with a Hitachi S-4800 field-emission SEM (Hitachi, Ibaraki, Japan). Dried material was mounted on gold-coated aluminum stubs and imaged at 5 kV, a 6.3 mm working distance, and 30,000× magnification.

Fourier-transform infrared (FTIR) spectra of the purified polymer were recorded using a Thermo Nicolet NEXUS 670 FTIR spectrometer (Thermo Scientific) over the wavenumber range of 4000–500 cm^{−1}. Prior to analysis, the purified polymer was dried to constant weight, finely ground, mixed with spectroscopic-grade KBr, and compressed into pellets for FTIR measurement. The FTIR spectra were used to identify the characteristic functional groups of PHA, particularly the ester carbonyl (C=O) stretching vibration and alkyl (C–H) stretching regions.

The purified polymer was dissolved in deuterated chloroform (CDCl_3), and ^1H - and ^{13}C -NMR spectra were recorded on a Bruker AVANCE NEO spectrometer (600 MHz, Bruker, Berlin, Germany). The ^1H - and ^{13}C -NMR spectra were acquired at 600.4 and 151.0 MHz, respectively. Broadband proton decoupling was applied during ^{13}C -NMR acquisition. The obtained spectra were used to confirm the chemical structure and monomer composition of the purified polymer.

Differential scanning calorimetry (DSC) analysis was performed using a NETZSCH DSC 204 F1 Phoenix instrument (NETZSCH, Selb, Bavaria, Germany). Approximately 3 mg of purified polymer was sealed in an aluminum DSC pan and heated from 30 to 200 °C at a heating rate of 10 °C min^{-1} under a nitrogen atmosphere (40 mL min^{-1}). The melting temperature (T_m) and melting enthalpy (ΔH_m) of the polymer were determined from the resulting thermograms.

Thermogravimetric analysis (TGA) was performed using a NETZSCH TG 209 F1 Libra thermogravimetric analyzer (NETZSCH, Germany). Approximately 3.8 mg of purified polymer was placed in an alumina (Al_2O_3) crucible and heated from 28 to 700 °C at a heating rate of 10 °C min^{-1} under a nitrogen atmosphere. The thermal degradation behavior of the polymer was evaluated from thermogravimetric (TG) and derivative thermogravimetric (DTG) curves, and the temperature of maximum degradation rate (T_{max}) and the residual mass were determined.

The viscosity-average molecular weight (M_v) of purified PHB was determined by capillary viscometry using an Ubbelohde viscometer (Capillary No. 1, $d = 0.8$ mm) at 30.0 ± 0.1 °C. PHB solutions in chloroform (0.10–0.30 g/dL) and pure solvent were measured in triplicate. Relative viscosity ($\eta_{\text{rel}} = t/t_0$) and specific viscosity ($\eta_{\text{sp}} = \eta_{\text{rel}} - 1$) were calculated from efflux times. Intrinsic viscosity ($[\eta]$) was obtained by extrapolating reduced viscosity (η_{sp}/c) to zero concentration ($c \rightarrow 0$) using a Huggins plot. M_v was derived via the Mark-Houwink-Sakurada equation $[\eta] = K \cdot M_v^a$ with $K = 7.7 \times 10^{-5}$ dL/g and $a = 0.82$ [25].

The tensile properties of the purified polymer film were evaluated in accordance with ASTM D882 method using a universal testing machine equipped with a 2 kN load cell. Rectangular film specimens were tested at a crosshead speed of 50 mm min^{-1} . The tensile strength (MPa) and elongation at break (%) were determined from the resulting stress–strain curves.

2.8. Statistical Analysis

One-way analysis of variance followed by Tukey's multiple-comparison test was conducted in OriginPro 2024 (OriginLab Corporation, Northampton, MA, USA). Results are reported as mean $\pm$ standard deviation (SD) from triplicate experiments, and $p < 0.05$ was used as the significance threshold.

3. Results and Discussion

Along with growing concern over environmental pollution from petroleum-based plastics, the exploration of microorganisms capable of synthesizing biodegradable biopolymers has attracted considerable attention [2,3]. Among these biopolymers, PHAs are considered promising alternatives to conventional plastics due to their biodegradability and biocompatibility. Thus, the discovery and characterization of innovative PHA-producing microorganisms, particularly thermophilic strains with prospective advantages for industrial fermentation processes, are vital for the evolution of sustainable bioplastic production techniques [1,4].

To date, studies on PHA biosynthesis by thermophilic bacteria remain relatively limited compared with those involving mesophilic microorganisms. Nevertheless, thermophilic bacteria have recently attracted increasing attention as promising candidates for

sustainable bioplastic production due to their ability to synthesize PHAs from diverse renewable substrates under elevated-temperature cultivation conditions [17,18]. Fermentation at high temperatures offers several advantages, including reduced contamination risk, lower sterilization requirements, improved substrate solubility, and facilitated downstream polymer recovery [5].

3.1. Characteristics of *Geobacillus* sp. SL28

The thermophilic bacterial strain SL28 formed creamy-white colonies on LB-Gelrite medium with a flat morphology, slightly rough surface, and undulate margins. Cells were Gram-positive, rod-shaped, and endospore-forming. The *Geobacillus* sp. SL28 strain was obtained as a natural isolate from water collected from a small natural thermal spring in Phu Yen District, Son La Province, Vietnam. The ability of the SL28 strain to grow over a temperature range of 40–60 °C, with optimal growth at 50 °C, confirms its thermophilic nature similar to other thermophilic bacteria [17,26,27], and suggests that the isolate is well adapted to the thermal conditions of its natural habitat. This finding indicates that local geothermal aquatic environments in Vietnam may represent an underexplored source of thermophilic bacteria with potential biotechnological value.

In the 16S rRNA gene tree, SL28 grouped within *Geobacillus* and was positioned closest to *G. thermoparaffinivorans* A1 and Aw3 (Figure 1). It formed a separate branch from *G. stearothermophilus*, *G. thermodenitrificans*, and *G. toebii*. However, species identification based solely on 16S rRNA gene sequences should be interpreted with caution because the related species in this genus shared high 16S rRNA sequence similarity; therefore, the isolate was conservatively designated as *Geobacillus* sp. SL28.

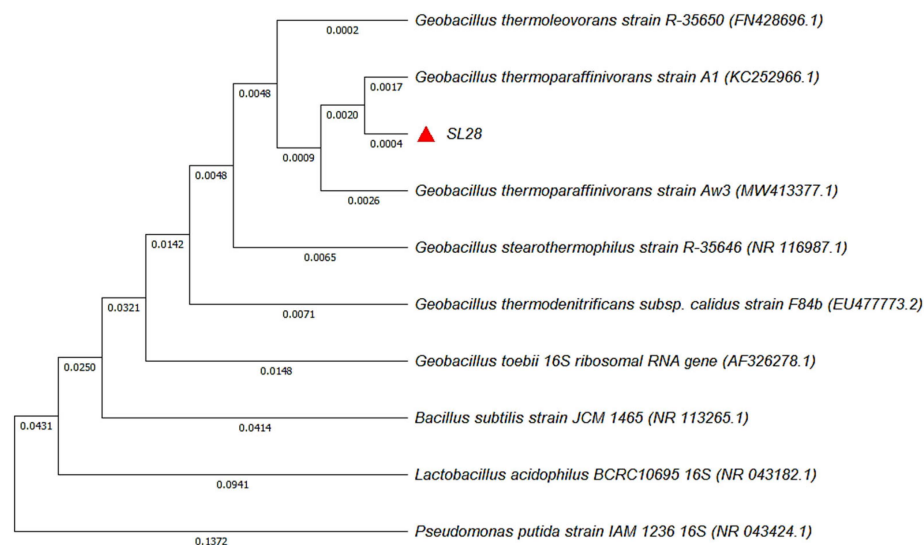


Figure 1. Neighbor-joining phylogenetic tree of strain SL28 among closely related *Geobacillus* species. GenBank accession numbers are given in parentheses. Numbers at branch nodes indicate bootstrap values based on 1000 replicates. Strain SL28 obtained in this study is marked with a red triangle.

The ability of *Geobacillus* sp. SL28 to produce PHA was initially screened using Sudan Black B staining, Nile Blue A fluorescence staining, and TEM observation. Sudan Black B and Nile Blue A staining are effective rapid-screening methods for identifying bacterial isolates capable of accumulating intracellular polyester granules prior to further quantitative and chemical characterization. Sudan Black B is a lipophilic dye commonly used for preliminary detection of hydrophobic intracellular inclusions such as PHAs, while Nile Blue A staining provides a more specific fluorescent indication of PHB/PHA granules [20,28]. In this study, *Geobacillus* sp. SL28 colonies stained with Sudan Black B

exhibited a dark blue–black coloration, while Nile Blue A staining revealed bright orange fluorescence under ultraviolet illumination, indicating the presence of intracellular polymer inclusions. The use of Sudan Black B and Nile Blue A staining in our study is consistent with previous reports on detecting PHA-producing bacteria. For example, Mohammed et al. (2019) applied these staining techniques to screen nearly 200 bacterial isolates obtained from a plastic waste landfill [29]. TEM observations further corroborated the staining results, revealing numerous electron-lucent inclusions distributed throughout the cytoplasm of SL28 cells (Figure 2) [30,31]. The combination of colony staining, fluorescence microscopy, and ultrastructural observation therefore strengthens the conclusion that SL28 is a potential PHA-producing thermophilic bacterium.

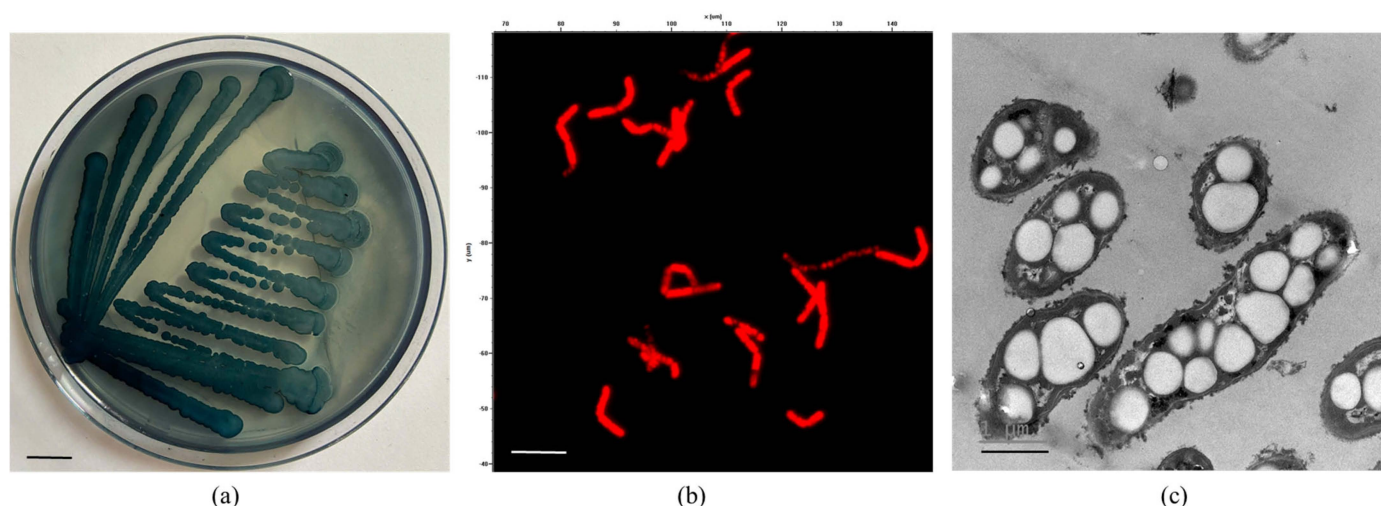


Figure 2. Screening of intracellular PHA accumulation in *Geobacillus* sp. SL28 by Sudan Black B staining, scale bar = 1 cm (a), Nile Blue A fluorescence microscopy, scale bar = 10 μ m (b), and transmission electron microscopy (TEM), scale bar = 1 μ m (c).

3.2. Influence of Cultivation Parameters on Growth and PHA Accumulation

Temperature, initial pH, carbon source, nitrogen source, C/N ratio, and cultivation time were assessed for their effects on DCW, PHA concentration, and PHA content during shake-flask cultivation. Variations in DCW, PHA content, and polymer composition were subsequently evaluated to identify favorable conditions for PHA biosynthesis by the thermophilic strain.

Since strain SL28 was isolated from a natural thermal spring, cultivation parameters such as temperature and initial pH were expected to strongly influence its growth and PHA biosynthesis. Nutritional factors, including carbon and nitrogen sources, the C/N ratio, and fermentation time, may also affect polymer accumulation. Each factor was varied sequentially, and the most favorable level was carried forward to the next experiment.

3.2.1. Temperature

Temperature significantly influenced both cell growth and PHA biosynthesis by *Geobacillus* sp. SL28 (Figure 3a). The strain grew over the temperature range of 40–60 °C, with DCW increasing from 1.28 ± 0.06 g/L at 40 °C to a maximum of 2.03 ± 0.16 g/L at 50 °C. PHA concentration increased significantly with increasing temperature, reaching a maximum value of 1.095 ± 0.06 g/L at 50 °C, which was significantly higher than those obtained at all other tested temperatures ($p < 0.05$). PHA content increased from $22.89 \pm 0.55\%$ at 40 °C to $54.07 \pm 3.10\%$ at 50 °C, although the value at 50 °C was statistically comparable to that at 45 °C ($46.27 \pm 4.46\%$). At temperatures above 50 °C, both biomass formation and polymer accumulation decreased markedly. At 55 °C, the DCW, PHA concentration,

and PHA content decreased to 1.19 ± 0.14 g/L, 0.428 ± 0.002 g/L, and $36.31 \pm 4.12\%$, respectively, while further reduction was observed at 60 °C. Under this condition, only 0.463 ± 0.02 g/L DCW and 0.080 ± 0.01 g/L PHA were obtained, corresponding to a PHA content of $17.58 \pm 2.83\%$. These results indicate that 50 °C was the most favorable temperature for growth and PHA accumulation by *Geobacillus* sp. SL28.

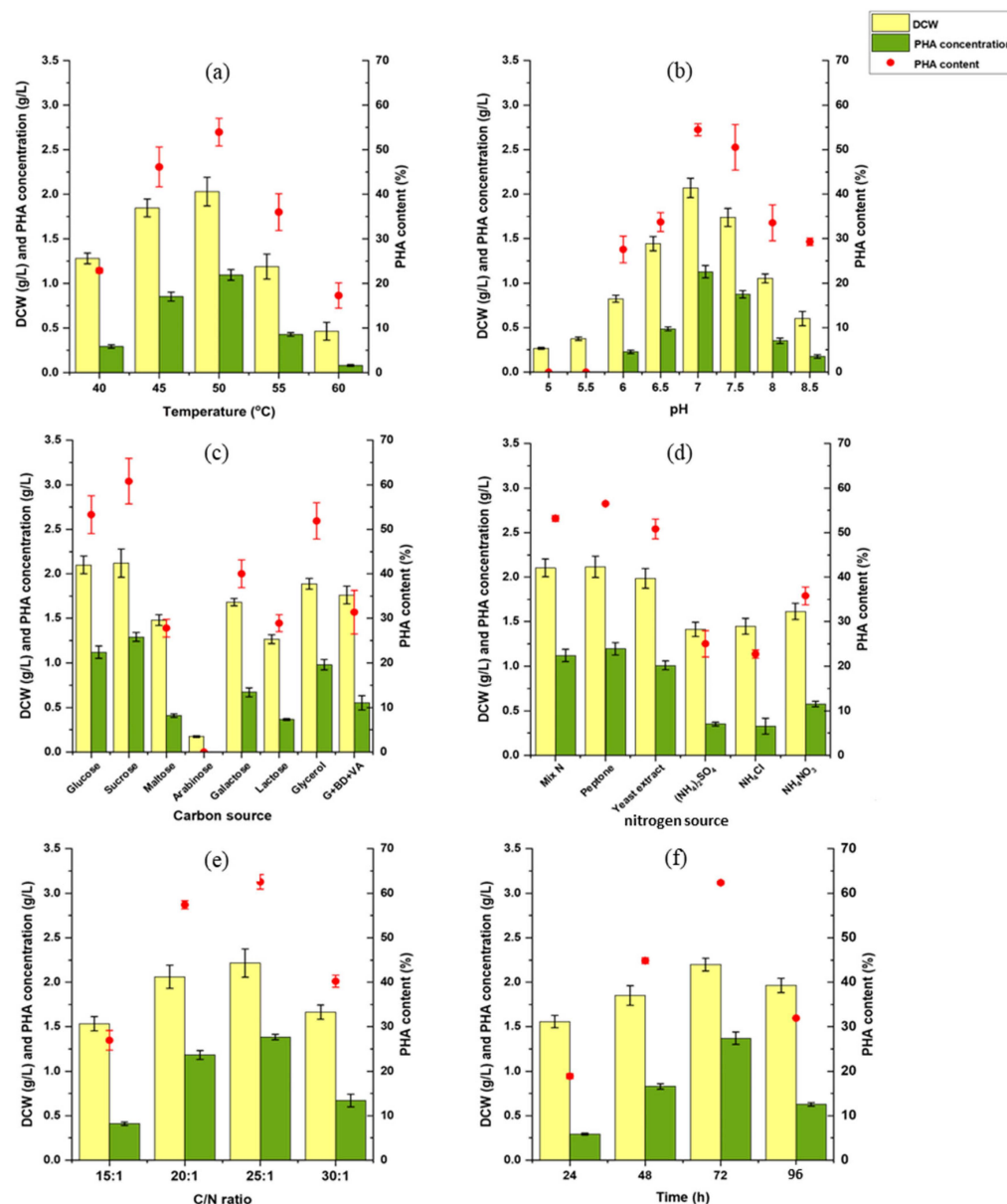


Figure 3. Effects of cultivation parameters on cell growth and PHA accumulation by *Geobacillus* sp. SL28 under different cultivation conditions, including temperature (a), initial pH (b), carbon sources, G: glucose, BD: 1,4-butanediol, and VA: valeric acid (c), nitrogen sources (d), C/N ratio (e), and fermentation time (f). Cell growth was expressed as dry cell weight (DCW, g/L), while PHA accumulation was evaluated based on PHA content (%DCW) and PHA concentration (g/L). Data represent the mean $\pm$ standard deviation (SD) of three independent experiments. Statistical analysis data are provided in Table S1.

The ability of strain SL28 to grow and accumulate PHA at elevated temperatures is consistent with its thermophilic nature and its isolation from a natural thermal spring. The optimal growth and polymer accumulation observed at 50 °C suggest that the metabolic activity of this strain is well adapted to moderately high-temperature cultivation. In contrast,

the reduction in both DCW and PHA accumulation at 55 and 60 °C may be associated with thermal stress, which can affect enzyme activity, membrane stability, nutrient assimilation, and overall cellular metabolism, thereby limiting biomass formation and polymer biosynthesis [32–34]. Comparable temperature optima have been reported for other thermophilic or thermotolerant PHA producers. For example, *Cupriavidus cauae* PHS1 was reported to efficiently synthesize PHB from acetate and phenol at 45 °C when gluconate was used as the carbon source [1]. Other thermophilic bacteria, such as *Aneurinibacillus* sp. H1 and *Bacillus thermoamylovorans* PHA005, have also been found to be able to produce PHA and related copolymers under optimal cultivation up to 55.3% of DCW at the temperature of 45 °C [35,36]. Compared with these previously reported thermophilic PHA producers, *Geobacillus* sp. SL28 showed favorable biopolymer accumulation at 50 °C, with a PHA content of $54.07 \pm 3.1\%$, indicating its potential as a promising thermophilic strain for PHA biosynthesis; therefore, this temperature was selected for subsequent experiments.

3.2.2. Initial pH

Initial pH strongly affected the growth and PHA accumulation of *Geobacillus* sp. SL28 (Figure 3b). The strain exhibited limited growth under acidic conditions, with DCW of only 0.267 ± 0.01 g/L and 0.376 ± 0.02 g/L at pH 5.0 and 5.5, respectively, and no detectable PHA accumulation. The poor growth and absence of detectable PHA accumulation under acidic conditions indicate that low pH may impose physiological stress on strain SL28, thereby limiting carbon assimilation and polymer formation. In particular, low pH values can disrupt cytoplasmic ionic homeostasis, membrane integrity, and metabolic regulation, thereby reducing enzyme activity and redirecting cellular energy toward stress adaptation rather than biosynthesis [37,38]. In contrast, as the initial pH increased from 6.0 to 7.0, both biomass formation and polymer biosynthesis increased markedly. The highest DCW, PHA concentration, and PHA content were observed at pH 7.0, reaching 2.27 ± 0.02 g/L, 1.128 ± 0.07 g/L, and $54.48 \pm 1.35\%$, respectively. However, this value was not significantly different from that observed at pH 7.5 ($50.53 \pm 5.13\%$) according to Tukey's multiple comparison test ($p > 0.05$). In contrast, PHA contents at pH 6.0, 6.5, 8.0, and 8.5 were significantly lower and fell within the same statistical group. The strong influence of initial pH on *Geobacillus* sp. SL28 suggests that maintenance of intracellular pH homeostasis is essential for both cellular growth and PHA biosynthesis. The highest PHA accumulation at pH 7.0 in strain SL28 indicates that a neutral initial pH provides the most favorable physiological condition for the activity of key metabolic enzymes and processes, as well as biopolymer accumulation [38]. For example, *Neobacillus niacini* and *Metabacillus niabensis* have been reported to show optimal growth and PHA production, with PHA production reaching 55.2% at a neutral pH [39]. In addition, PHA synthase, the primary enzyme responsible for catalyzing PHA biosynthesis, from the thermophilic bacterium *Thermus thermophilus* was reported to act optimally at approximately pH 7.3 [40]. This finding further supports the importance of near-neutral pH for PHA-related enzymatic activity. Based on these results, pH 7.0 was selected as the most favorable initial pH for subsequent cultivation experiments.

3.2.3. Carbon Source

Carbon source is among the most important nutritional factors affecting bacterial growth and PHA biosynthesis (Figure 3). In the present study, *Geobacillus* sp. SL28 was able to utilize several tested carbon sources, indicating a relatively broad substrate spectrum (Figure 3c). Sucrose and glucose supported the highest biomass production ($p < 0.05$), yielding DCW values of 2.12 ± 0.10 and 2.10 ± 0.13 g/L, respectively, with no significant difference between them ($p > 0.05$). However, sucrose gave the highest PHA concentra-

tion and PHA content ($p < 0.05$), reaching 1.289 ± 0.05 g/L and $60.93 \pm 4.41\%$ of DCW, respectively. Glucose also supported efficient PHA production, with 1.119 ± 0.08 g/L PHA and $53.58 \pm 7.17\%$ PHA content. These results suggest that readily metabolizable sugars were favorable for both cell growth and PHB biosynthesis in strain SL28. Similar observations have been reported in thermophilic PHA-producing bacteria, including *Geobacillus stearothermophilus* K4E3_SPR_NPP, in which sugar-based substrates supported high PHA accumulation after optimization [16]. Glycerol also supported substantial growth and PHA accumulation, with a DCW of 1.89 ± 0.06 g/L, PHA concentration of 0.979 ± 0.05 g/L, and PHA content of $51.95 \pm 3.85\%$. Although the results with glycerol were lower than those with sucrose, strain SL28 still accumulated more than 50% PHA, indicating that glycerol warrants further testing as an alternative carbon source [15,41]. In contrast, galactose, maltose, lactose, and the mixed substrate supported only moderate to low PHA accumulation, ranging from 27.83 to 40.00%, suggesting that strain SL28 utilized these sources less efficiently for polymer accumulation than sucrose. Despite the inclusion of valeric acid in the mixed substrate, only limited incorporation of 3HV units into the polymer was observed under the tested conditions. This finding suggests that *Geobacillus* sp. SL28 utilized valeric acid inefficiently as a precursor for 3HV biosynthesis under the cultivation conditions employed. However, the underlying metabolic mechanism remains unclear and may be associated with limited precursor uptake and/or inefficient metabolic conversion. Further genomic and enzymatic studies will be required to elucidate the metabolic pathways responsible for the limited incorporation of 3HV units into the polymer. Arabinose was the least favorable substrate, producing only 0.17 ± 0.01 g/L biomass and no detectable PHA accumulation. Based on these results, sucrose was chosen as the preferred carbon source.

3.2.4. Nitrogen Source

Nitrogen source strongly influenced biomass production and PHA biosynthesis (Figure 3d). Organic nitrogen sources were clearly more favorable than inorganic nitrogen sources. Peptone and the mixed nitrogen source supported the highest biomass formation, with DCW values of 2.12 ± 0.12 and 2.10 ± 0.10 g/L, respectively. Yeast extract also supported substantial growth at 1.98 ± 0.09 g/L. These results indicate that complex organic nitrogen sources provided a more suitable nutritional environment for strain SL28, probably because they supply not only nitrogen but also amino acids, peptides, vitamins, minerals, and other growth factors that enhance cellular metabolism and polymer biosynthesis [42,43].

In contrast, inorganic nitrogen sources resulted in lower PHA production, ranging from only 0.35 to 0.58 g/L. Among them, NH_4NO_3 gave the highest PHA content of $35.80 \pm 2.00\%$ ($p < 0.05$), whereas NH_4Cl and $(\text{NH}_4)_2\text{SO}_4$ resulted in lower PHA contents of $22.70 \pm 0.92\%$ and $25.02 \pm 2.96\%$, respectively. The poorer performance of inorganic nitrogen sources may be associated with their simpler nutritional composition, greater energetic assimilation cost, or changes in pH and ionic balance during cultivation [42,43]. Comparable benefits of complex nitrogen sources have been reported for other PHB-producing microorganisms, *Burkholderia cepacia*, *Priestia megaterium* GM-4 and *Pichia kudriavzevii* TSLS24, where complex organic nitrogen sources such as peptone and yeast extract often support higher biomass and PHB accumulation than some inorganic nitrogen sources [42–44]. However, Sriyapai et al. have found that, in *Paraburkholderia* sp. PFN29, inorganic nitrogen sources such as NH_4Cl and $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$, supported high PHB accumulation, reaching 3.90 ± 0.05 g/L and 3.50 ± 0.29 g/L, respectively [45]. Therefore, the lower performance of inorganic nitrogen sources in the present study should be interpreted as a strain-specific response of *Geobacillus* sp. SL28 rather than a general limitation of inorganic nitrogen sources for PHA production. Based on these results, peptone was carried forward as the nitrogen source.

3.2.5. C/N Ratio

The balance between intracellular PHA accumulation and cell proliferation is significantly influenced by the C/N ratio. In this study, increasing the C/N ratio from 15:1 to 25:1 enhanced both biomass formation and PHA production (Figure 3e). The highest DCW of 2.215 ± 0.16 g/L, PHA concentration of 1.383 ± 0.04 g/L, and PHA content of $62.57 \pm 2.82\%$ were obtained at a C/N ratio of 25:1 ($p < 0.05$). A relatively high performance was also observed at C/N 20:1, with 2.061 ± 0.13 g/L DCW, 1.182 ± 0.05 g/L PHA, and $57.40 \pm 1.64\%$ PHA content. These results indicate that moderate nitrogen limitation was favorable for PHB accumulation in strain SL28 while still allowing sufficient biomass formation. At the lower C/N ratio of 15:1, PHA production decreased markedly to 0.412 ± 0.02 g/L, with a PHA content of only $26.95 \pm 2.20\%$. Increasing the C/N ratio to 30:1 reduced DCW to 1.663 ± 0.08 g/L and PHA concentration to 0.670 ± 0.07 g/L, although the PHA content remained moderate at $40.23 \pm 2.34\%$. Therefore, a C/N ratio of 25:1 represented the most suitable balance between cell growth and carbon storage in *Geobacillus* sp. SL28. Under these conditions, the strain reached $62.57 \pm 2.82\%$ PHA content. This level of intracellular PHA accumulation is comparable to that reported for other thermophilic PHA-producing bacteria. For example, *Geobacillus stearothermophilus* K4E3_SPR_NPP achieved 66.66% PHA accumulation after optimization [16]. In addition, the thermophilic PHA producer *Schlegella thermodepolymerans* DSM 15344 has been reported to possess broad metabolic potential for using different substrates, including lignocellulose-derived sugars and glycerol, for PHA production [13,15]. These comparisons suggest that strain SL28 is a promising thermophilic PHB-producing bacterium.

3.2.6. Cultivation Time

Fermentation time strongly influenced cell growth and PHA biosynthesis by *Geobacillus* sp. SL28 (Figure 3f). Both biomass formation and polymer accumulation increased progressively from 24 to 72 h of cultivation. DCW increased from 1.557 ± 0.07 g/L at 24 h to a maximum of 2.198 ± 0.07 g/L at 72 h, while PHA concentration rose from 0.294 ± 0.01 to 1.371 ± 0.05 g/L over the same period. The highest PHA content was recorded at 72 h, at $62.37 \pm 0.36\%$, which was considerably higher than those observed at other cultivation periods ($p < 0.05$). Sustained cultivation over 96 h produced a substantial decrease in PHA accumulation, with PHA concentration and content reduced to 0.627 ± 0.02 g/L and $31.93 \pm 0.26\%$, respectively. The decline after 72 h may reflect substrate depletion, inhibitory metabolites, or intracellular PHA mobilization for maintenance [46,47]. A similar optimal cultivation time of 72 h has been reported for *Pseudomonas aeruginosa* EO1 isolated from edible-oil-contaminated soil [48], *Stenotrophomonas maltophilia* HA-16 isolated from paint industry soil [49], and *Halomonas ventosae* cultivated on algal biodiesel waste residue [50]. These results indicate that 72 h was the most favorable fermentation time for PHA accumulation by *Geobacillus* sp. SL28 under the tested conditions.

3.3. Characteristics of PHA Produced by *Geobacillus* sp. SL28

To verify the chemical structure and evaluate the physicochemical properties of the polymer produced by *Geobacillus* sp. SL28 under the optimized cultivation conditions, a series of characterization techniques, including Fourier-transform infrared (FTIR) spectroscopy, nuclear magnetic resonance (^1H - and ^{13}C -NMR) spectroscopy, differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), dilute-solution viscometry, tensile testing, and scanning electron microscopy (SEM), were employed. These complementary analyses provided comprehensive information on the chemical structure, thermal stability, molecular weight, mechanical properties, and surface morphology of the purified polymer.

3.3.1. Surface Morphology and Fourier-Transform Infrared (FTIR) Analysis

The purified polymer produced by *Geobacillus* sp. SL28 was obtained as a white powdered material after extraction and purification (Figure 4a). FE-SEM analysis revealed that the extracted polymer consisted of irregularly shaped particles with a rough and porous surface morphology (Figure 4b). Such surface features have previously been reported for PHB and have been associated with its relatively high crystallinity, as well as with rapid solvent evaporation and polymer solidification during sample preparation [51,52]. The porous and fragmented appearance may also have resulted from solvent evaporation and particle fracture during extraction, purification, and drying. Similar effects of preparation conditions on PHB morphology have been reported for PHB microparticles, in which solvent type and evaporation conditions markedly influenced particle shape, surface roughness, and porosity [53,54]. Overall, the absence of obvious cellular debris or other visible impurities suggests that the purification procedure effectively removed most non-polymeric components, producing a polymer with relatively high morphological purity prior to subsequent structural, thermal, and mechanical characterization.

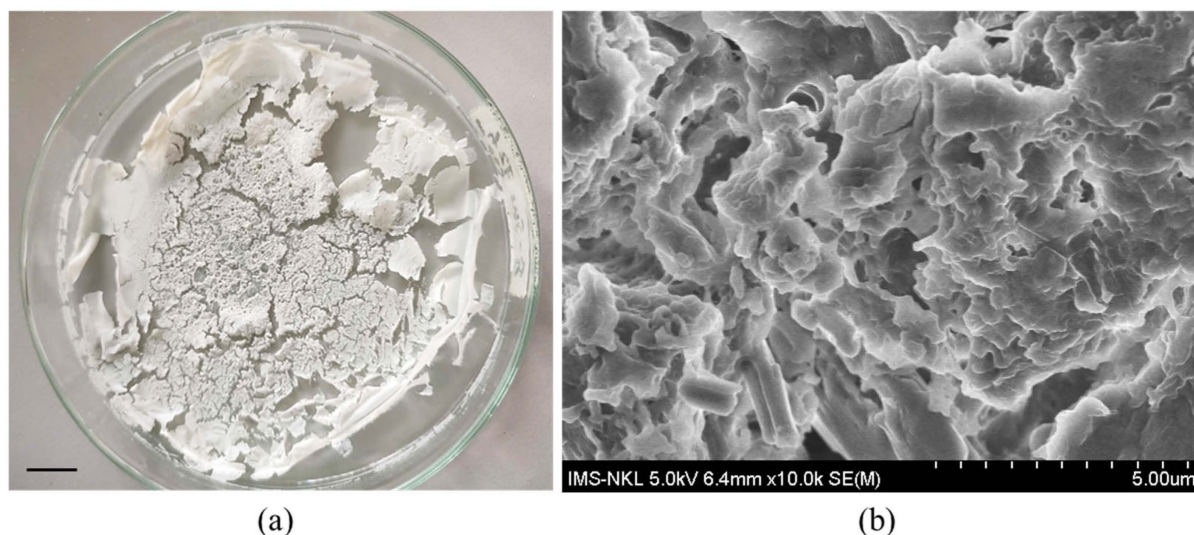


Figure 4. Purified PHB obtained from *Geobacillus* sp. SL28 after extraction and purification, scale bar = 1 cm (a) and SEM micrograph showing the surface morphology of the extracted polymer, scale bar = 5 μm (b).

The FTIR spectrum of the purified polymer exhibited the characteristic absorption bands of PHB and closely matched that of the commercial PHB standard (Figure 5). A strong absorption band at approximately 1720 cm^{-1} was assigned to the stretching vibration of the ester carbonyl ($\text{C}=\text{O}$) group, while the bands at 2973 and 2923 cm^{-1} corresponded to the asymmetric and symmetric stretching vibrations of methyl and methylene groups, respectively [51,55]. In addition, the absorption band at approximately 1270 cm^{-1} was attributed to the $\text{C}-\text{O}$ stretching vibration of the ester linkage. Similar absorption bands have been reported for PHB produced by thermophilic *Geobacillus* sp. AY 946034, with characteristic peaks at 1722 , 2983 , 2935 , and 1278 cm^{-1} , demonstrating excellent agreement with the spectrum obtained for strain SL28 [51,55]. The nearly identical spectral profiles of the purified polymer and the commercial PHB standard further indicate that the downstream purification process preserved the chemical structure of the biosynthesized polymer.

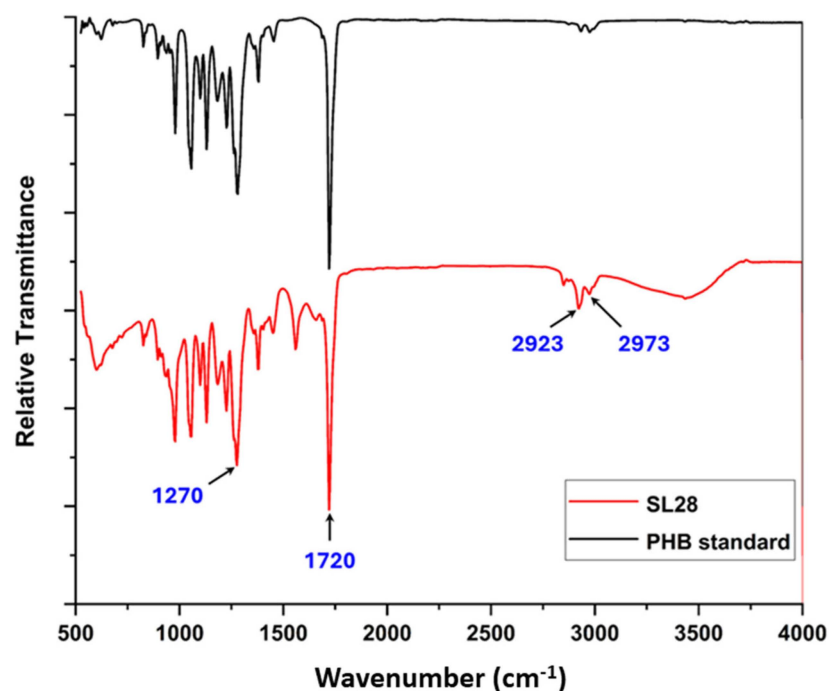


Figure 5. FTIR spectra of the polymer produced by *Geobacillus* sp. SL28 and the commercial PHB standard.

A weak broad absorption band was also observed in the region of approximately 3200–3600 cm^{-1} . This band is attributed to O–H stretching vibrations and is most likely associated with adsorbed moisture introduced during sample preparation or KBr pellet preparation rather than the PHB molecular structure, since PHB itself does not contain hydroxyl functional groups. The presence of adsorbed water in KBr pellets is well known to contribute to weak O–H stretching absorption in FTIR spectra and should therefore be considered when interpreting absorption bands in this spectral region [56]. Despite the presence of this weak absorption band, the overall FTIR spectrum remained highly consistent with that of the commercial PHB standard, confirming that the purified polymer possessed the characteristic chemical structure of PHB. However, because FTIR alone cannot unequivocally distinguish structurally similar short-chain-length PHA homopolymers and copolymers, complementary ^1H - and ^{13}C -NMR analyses were performed to further verify the chemical structure of the purified polymer.

3.3.2. Nuclear Magnetic Resonance (NMR) Spectroscopy

The ^1H - and ^{13}C -NMR spectra of the purified polymer produced by *Geobacillus* sp. SL28 are presented in Figure 6. The ^1H -NMR spectrum exhibited the characteristic proton resonance signals of PHB, including a doublet at δ 1.28 ppm ($-\text{CH}_3$), a multiplet at δ 2.45–2.65 ppm ($-\text{CH}_2-$), and a multiplet at δ 5.25 ppm ($-\text{CH}-$). Several weak and broadened signals were also observed in the region of approximately δ 3–5 ppm. These minor signals are not characteristic resonances of the PHB backbone and are most likely associated with trace residual impurities or exchangeable protons remaining after polymer extraction and purification. Because the identities of these minor components were not independently verified, their exact origin could not be conclusively determined. Nevertheless, their relatively low intensity did not interfere with the assignment of the characteristic PHB resonances. The ^{13}C -NMR spectrum further confirmed the polymer structure by displaying four characteristic carbon resonance signals at δ 19.8 ppm ($-\text{CH}_3$), δ 40.8 ppm ($-\text{CH}_2-$), δ 67.6 ppm ($-\text{CH}-$), and δ 169.2 ppm ($\text{C}=\text{O}$). No additional ^{13}C resonance signals attributable

to other structural components were observed, indicating that the purified polymer was predominantly composed of PHB.

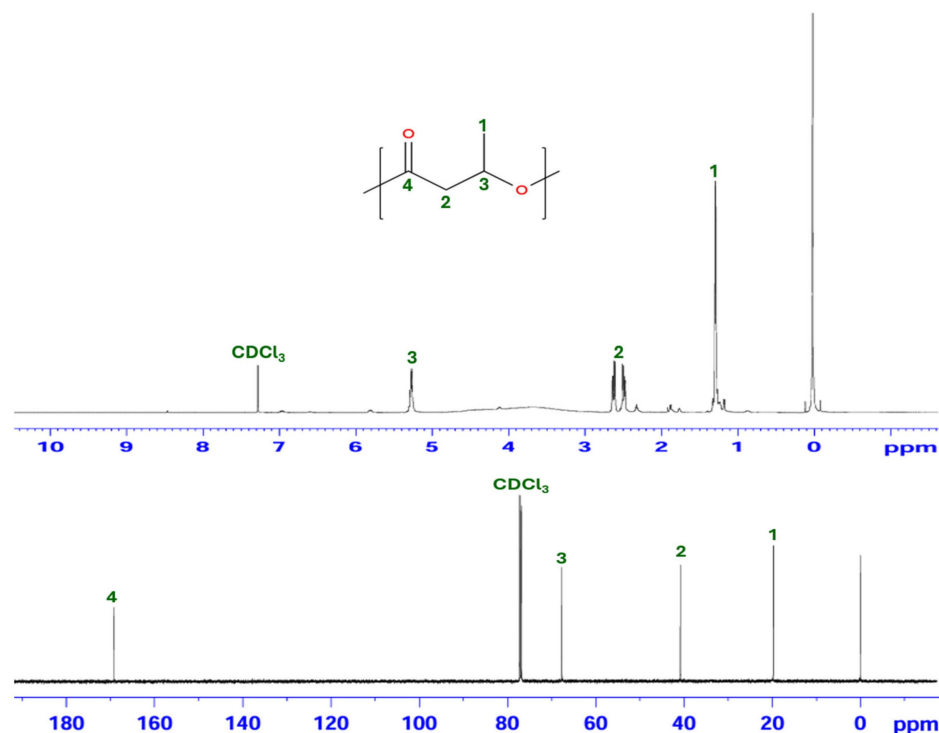


Figure 6. ^1H - and ^{13}C -NMR spectra of the purified polymer produced by *Geobacillus* sp. SL28, respectively. Spectra were recorded on a Bruker AVANCE NEO 600 MHz NMR spectrometer using CDCl_3 as the solvent.

These chemical shifts were in close agreement with those reported for PHB produced by *Pseudomonas plecoglossicida* [57], *Bacillus megaterium* [58], and other bacterial strains [59], supporting the assignment of the purified polymer as PHB. The close correspondence between the characteristic ^1H - and ^{13}C -NMRs obtained in the present study and previously published data further indicated that the polymer biosynthesized by *Geobacillus* sp. SL28 possessed the typical chemical structure of PHB. Overall, the FTIR and NMR analyses consistently supported the identification of the purified polymer as PHB. These findings provided a structural basis for subsequent evaluation of the polymer's thermal and physicochemical properties.

3.3.3. Thermal Properties of the Purified PHB

Differential scanning calorimetry demonstrated that the purified PHB possessed thermal characteristics typical of highly crystalline PHB, with a melting temperature (T_m) of 176.5 °C and a melting enthalpy (ΔH_m) of 93.29 J g $^{-1}$ (Figure 7). The observed melting temperature falls within the typical range (175–180 °C) reported for PHB, indicating that the purified polymer retained the characteristic thermal behavior of this biopolyester [60]. Furthermore, the relatively high ΔH_m indicates a relatively high degree of crystallinity, a characteristic feature of PHB that contributes to its thermal and mechanical performance. Hong et al. (2008) reported that residual cellular components and other impurities adversely affect the crystallization behavior and thermal properties of biosynthesized PHB, whereas purified PHB exhibits improved crystallization characteristics following the removal of non-polymeric constituents [61]. Accordingly, the thermal properties observed in the present study suggest that the extraction and purification procedures successfully preserved the crystalline organization of the polymer.

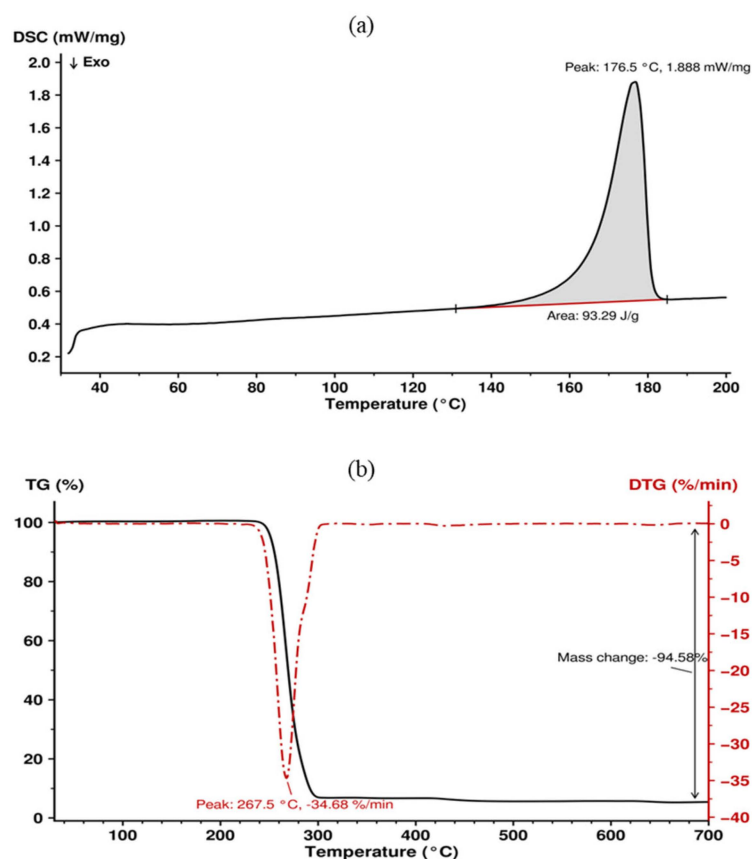


Figure 7. Thermal characterization of the purified PHB produced by *Geobacillus* sp. SL28. (a) The differential scanning calorimetry (DSC) thermogram. (b) The thermogravimetric (TG) and derivative thermogravimetric (DTG) curves.

Thermogravimetric analysis further revealed that the purified PHB exhibited good thermal stability, undergoing a characteristic single-step thermal degradation with the maximum degradation rate occurring at 267.5 °C (Figure 7). This degradation behavior is comparable to that reported for pilot-scale PHB produced by *Bacillus megaterium* cultivated on desugared sugar beet molasses, indicating that the thermal stability of PHB produced by *Geobacillus* sp. SL28 is comparable to that of PHB obtained under pilot-scale production conditions [62]. The observed degradation profile is also consistent with the well-established stereoselective cis-elimination mechanism of PHB, in which polymer chains thermally depolymerize to form crotonic acid and its oligomers [63].

Only a small residual mass (5.4%) remained after heating to 700 °C. Because neat PHB has been reported to undergo nearly complete thermal decomposition under an inert atmosphere, the residual fraction observed in the present study is unlikely to represent undegraded polymer. Instead, it may be attributed to trace inorganic constituents originating from the culture medium or purification process and/or the formation of a small amount of carbonaceous char during pyrolysis. The relatively low residual mass is therefore consistent with the high purity of the purified polymer, although additional compositional analyses would be required to determine the exact origin of the residue.

3.3.4. Viscosity-Average Molecular Weight

The viscosity-average molecular weight (M_v) of the purified PHB was determined from its intrinsic viscosity ($[\eta]$) in chloroform at 30 °C. The intrinsic viscosity ($[\eta]$) of the purified PHB was determined to be 2.12 dL/g by extrapolation of the reduced viscosity (η_{sp}/c) to zero concentration ($c \rightarrow 0$). Based on the Mark–Houwink–Sakurada equation

($K = 7.7 \times 10^{-5}$ dL/g, $\alpha = 0.82$ [25]), the M_v of the purified polymer was calculated to be approximately 2.55×10^5 g/mol (255 kDa). This value falls within the typical molecular weight range (200–600 kDa) reported for PHBs synthesized by wild-type *Bacillus* species and thermophilic bacteria. Although the M_v obtained in this study is lower than that reported for the thermophilic strain *Geobacillus* sp. AY 946034 (~556 kDa) [51], it is comparable to values reported for conventional *Bacillus* isolates (200–800 kDa) [64,65]. Differences in molecular weight among bacterial strains are generally attributed to variations in PHA synthase activity, intracellular substrate flux, and cultivation conditions. The relatively high M_v obtained in this study suggests that the downstream extraction and purification procedures effectively preserved polymer chain integrity with limited chain scission, thereby minimizing thermal and chemical degradation during polymer recovery.

3.3.5. Mechanical Properties

The purified PHB exhibited a tensile strength of approximately 22.5 MPa and an elongation at break of approximately 6.1%. A representative stress–strain curve is provided in Figure S1 of the Supplementary Materials. The stress increased continuously with increasing strain and reached its maximum value immediately before fracture, without a clearly defined yield plateau, indicating the limited ductility that is characteristic of PHB. These values fall within the typical range reported for neat PHB, which generally exhibits a tensile strength of 15–40 MPa and an elongation at break of 4–10%, indicating that the purified polymer retained the characteristic mechanical performance of PHB [60].

The relatively low elongation at break is consistent with the inherently brittle nature of PHB, which is commonly associated with its relatively high crystallinity and restricted molecular chain mobility [53,66]. This interpretation is consistent with the DSC results obtained in the present study, which showed a melting temperature of 176.5 °C and a relatively high melting enthalpy, suggesting the formation of a well-developed crystalline structure. Furthermore, the viscosity-average molecular weight determined in this study indicates that the extraction and purification procedures effectively preserved the integrity of polymer chains, thereby contributing to the observed tensile strength.

Although this study demonstrates the potential of *Geobacillus* sp. SL28 as a thermophilic PHB-producing strain, it has several limitations that should be acknowledged. The strain is a newly isolated natural bacterium, and PHB production was evaluated only at the shake-flask scale using a one-factor-at-a-time optimization approach. Further studies employing controlled bioreactor cultivation, statistical optimization methods such as response surface methodology, and fed-batch strategies are therefore required to improve PHB productivity and assess the scalability of the production process. In addition, evaluating low-cost renewable substrates and investigating the genetic and metabolic mechanisms governing PHB biosynthesis would provide a stronger basis for improving the economic feasibility and production performance of strain SL28. Although the chemical structure, molecular weight, thermal behavior, mechanical properties, and surface morphology of the purified PHB were characterized in the present study, further investigations into its biodegradation, long-term stability, processability, and performance in specific product formulations are required to determine its suitability for practical applications.

4. Conclusions

In conclusion, this study demonstrated that the thermophilic bacterium *Geobacillus* sp. SL28, isolated from a Vietnamese hot spring, is a promising producer of poly(3-hydroxybutyrate) (PHB). PHB production by strain SL28 was strongly influenced by cultivation conditions, including temperature, initial pH, carbon source, nitrogen source, C/N ratio, and cultivation time. Under the optimized conditions of 50 °C, pH 7.0, sucrose as the carbon

source, peptone as the nitrogen source, a C/N ratio of 25:1, and 72 h of cultivation, the strain achieved a maximum dry cell weight of 2.215 ± 0.16 g/L and a PHB concentration of 1.383 ± 0.04 g/L, corresponding to $62.57 \pm 2.82\%$ of DCW. Comprehensive physicochemical characterization by FE-SEM, FTIR, ^1H - and ^{13}C -NMR spectroscopy, capillary viscometry, DSC, TGA, and tensile testing confirmed that the recovered polymer was high-purity PHB with physicochemical and mechanical properties consistent with those reported for PHB produced by other *Geobacillus* and *Bacillus* species. These findings highlight the potential of *Geobacillus* sp. SL28 as a thermophilic microbial platform for sustainable PHB production and provide a foundation for future studies on bioreactor cultivation, process optimization, and industrial application.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/pr14162539/s1>. Table S1: Statistical analysis of the effects of cultivation parameters on cell growth and PHA accumulation by *Geobacillus* sp. SL28 under different cultivation conditions. Figure S1: Representative stress–strain curve of the purified PHB produced by *Geobacillus* sp. SL28.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

PHA	PolyHydroxyAlkanoates
3HB	3-HydroxyButyrate
3HV	3-HydroxyValerate
PHB	Poly(3-HydroxyButyrate)
PHBV	Poly(3-HydroxyButyrate-co-3-hydroxyValerate)
TEM	Transmission Electron Microscopy
FE-SEM	Field Emission Scanning Electron Microscopy
GC–MS/MS	Gas Chromatography–Tandem Mass Spectrometry
FTIR	Fourier-Transform Infrared Spectroscopy

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