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Newly isolated halotolerant *Gordonia terrae* S-LD serves as a microbial cell factory for the bioconversion of used soybean oil into polyhydroxybutyrate

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Abstract

Polyhydroxybutyrate (PHB) is a class of biodegradable polymers generally used by prokaryotes as carbon sources and for energy storage. This study explored the feasibility of repurposing used soybean oil (USO) as a cost-effective carbon substrate for the production of PHB by the strain *Gordonia terrae* S-LD, marking the first report on PHB biosynthesis by this rare actinomycete species. This strain can grow under a broad range of temperatures (25–40 ℃), initial pH values (4–10), and salt concentrations (0–7%). The findings indicate that this strain can synthesize PHB at a level of 2.63 ± 0.6 g/L in a waste-containing medium containing 3% NaCl within a 3 L triangular flask, accounting for 66.97% of the cell dry weight. Furthermore, ^{1}H NMR, ^{13}C NMR, and GC–MS results confirmed that the polymer was PHB. The thermal properties of PHB, including its melting (T_m) and crystallization (T_c) temperatures of 176.34 °C and 56.12 °C respectively, were determined via differential scanning calorimetry analysis. The produced PHB was characterized by a weight-average molecular weight (M_w) of 5.43×10^5 g/mol, a number-average molecular weight (M_n) of 4.00×10^5 g/mol, and a polydispersity index (PDI) of 1.36. In addition, the whole genome was sequenced, and the PHB biosynthetic pathway and quantitative expression of key genes were delineated in the novel isolated strain. In conclusion, this research introduces the first instance of polyhydroxyalkanoate (PHA) production by *Gordonia terrae* using used soybean oil as the exclusive carbon source, which will enrich strain resources for future PHB biosynthesis.

Keywords Used soybean oil, Halotolerant, *Gordonia terrae*, Microbial cell factory, Polyhydroxyalkanoate (PHA)

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Introduction

Polyhydroxybutyrate (PHB) is a bioplastic synthesized by microorganisms, can be fully degraded in the natural environment, and possesses advantages comparable to those of traditional nondegradable plastics. These advantages make PHB a promising material for a variety of applications, such as medical devices, packaging materials, and disposable plastic products [1, 2]. Despite its many advantages, PHB faces significant challenges that hinder its widespread adoption. The primary issue is its high production cost, which stems from the complex fermentation processes required to produce PHB and the relatively low yields compared with those of traditional petroleum-based nondegradable plastics. The reasons for the high cost of PHB production are mainly due to the high cost of the carbon source required for production, and the limited number of strain species currently available for demonstration purposes. Most PHA producing bacteria usually take up simple sugars (e.g., glucose and fructose) as their main carbon source [3]. However, the cost of industrial grade glucose is approximately 35–50 USD/kg. Ongoing research aims to improve production efficiency and reduce the cost of the carbon source, with some studies focusing on the use of waste materials, such as oil-based substrates, agroindustrial residues, and domestic sludge, as feedstocks for PHB production [4].

Soybean oil consumption accounts for the highest consumption of edible vegetable oils in China, accounting for approximately 29.6%. Thus, waste soybean oil may be a cost-effective oil-based substrate for synthesizing PHB and has great potential for large scale applications. Studies have demonstrated that *Cupriavidus necator* can synthesize PHB using mixed waste cooking oil as the sole carbon source, yielding 7.6 g/L PHB [5]. This research not only offers a solution for mixed waste oil management but also contributes to sustainable plastic production. Researchers have successfully utilized active sludge from waste water treatment, which is rich in microorganisms capable of synthesizing PHB, to produce PHBV (a copolymer of PHB and hydroxyvalerate) [6, 7]. These studies highlight the advantages of using microbial processes to synthesize PHB using oil-based substrates as a carbon source, which can assist in solving environmental problems while reducing the cost of PHB production. However, many waste cooking oils or wastewaters have relatively high salinities or other extreme physicochemical properties, which strongly affect their bioavailability and bioconversion.

In recent years, *halophilic Halomonas* spp., which constitute the next generation of industrial biotechnology [8], have also been studied and reported to be useful for the large-scale production of PHA [9]. Extremely halophilic bacteria can grow under harsh conditions (such as extreme salinity or osmotic pressure), where most microorganisms are unable to proliferate, and consequently, they are more resistant to contaminants during fermentation. These methods allow for the open continuous production of PHA, reducing the sterilization process and energy consumption [10]. However, such extremely halophilic bacteria generally need to utilize sugars as a carbon source to synthesize PHB. In addition, the corrosion of fermentation equipment caused by high salt concentrations and the difficulty of treating large quantities of high-salt wastewater may also limit the development of PHB synthesis by extremophiles on a large scale [11–13].

In this study, *Gordonia terrae* is reported to synthesize PHB during cultivation on used soybean oil containing 3% NaCl. It can grow under a broad range of temperatures, initial pH values, and salt concentrations. The physio-chemical properties of PHB synthesized from used soybean oil were examined. Based on the whole genome, the PHB biosynthetic pathway and quantitative expression of key genes were delineated in the novel isolated strain. This provides strain resources and a process basis for future large-scale production applications of PHB synthesis using fried soybean oil as an economically feasible carbon substrate.

Materials and methods

Strain and cultivation

Soil samples (10 g) from the the temporary waste food storage area of out institute were collected in 250 ml triangular flasks, 90 ml of sterile water was added, and the flasks were placed on a shaker at 30 ℃ and 180 r/min for 30 min. Next, 1 ml of the upper layer of the suspension was added to 100 ml of LB medium, and incubated for 12 h at 30 ℃ and 180 r/min. Then, 1 ml of the upper suspension was diluted to 10^{-5} by gradient using sterile water, and 10^{-2}–10^{-5} dilutions were spread on the used soybean oil plates in triplicated and incubated at 30 ℃ for 72 h. The strains with larger transparent circles were selected from different plates for reisolation and purification, and then single colonies were selected and inoculated into used soybean oil medium. The culture was shaken at 30 °C and 180 r/min for 72 h, after which the OD_{600} was measured. The ability of the strains to synthesis PHA was preliminarily observed by transmission electron microscopy. The strain with the fastest growth and the largest white particles observed by transmission electron microscopy was selected as the target strain.

Luria–Bertani (LB) medium contains tryptone (10 g/L), yeast extract (5 g/L), and NaCl (10 g/L) with the pH adjusted to 7.0, and it was autoclaved at 121 ℃ for 20 min. Used soybean oil medium contains $(NH_4)_2SO_4$ (2.0 g/L), K_2HPO_4 (2.5 g/L), KH_2PO_4 (0.5 g/L), $MgSO_4$ (0.5 g/L), NaCl (30.0 g/L), and used soybean oil (20.0 g/L, pH 7.0),

and it was autoclaved at 121 ℃ for 20 min. The used soybean oil used in this study was obtained from the Tianjin Institute of Industrial Biotechnology canteen. Soybean oil (Arawana Brand) was used for frying for 30 min, after which the food materials were filtered out using filter mesh. For solid cultures, agar (15 g/L) was also added into the medium. The 16S rDNA gene sequence of the isolated strain was analyzed using the neighbor-joining method in MEGA (version 9.0) for phylogenetic analysis [14].

Determination of temperature, initial pH, and salt growth range

The strain was inoculated into used soybean oil medium at various temperatures (20 ℃, 25 ℃, 30 ℃, 35 ℃, 40 ℃, and 45 ℃) to determine the temperature growth range. The strain was inoculated into used soybean oil medium with various initial pH values of 3, 4, 5, 6, 7, 8, 9, 10, and 11 for determination of the pH growth range. The strain was inoculated into used soybean oil medium supplemented with varying concentrations of NaCl (0%, 1%, 3%, 5%, 7%, and 9%) for salt tolerance. The growth range of temperature, initial pH, and salt concentrations tolerated by the strain were determined on the basis of changes in the OD_{600} values. All experiments were performed in triplicated.

SEM and TEM imaging analysis

After 96 h of cultivation, the cell suspension was centrifuged and washed twice with 0.1 M PBS buffer solution (pH 7.0), and then added into a 2.5% glutaraldehyde solution overnight fixation at 4 °C before SEM and TEM imaging at the Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences.

PHB synthesis process analysis and extraction

The experiments were carried out at 30 ℃ and 180 rpm for 96 h with used soybean oil medium supplemented with 3% salt. Liquid samples were collected every 12 h for pH analysis and optical density (OD_{600}) measurement. The cell suspensions were collected for PHB extraction and structural analysis. All experiments were performed in triplicate. The cell suspension was centrifuged to collect the cells, which were then washed with 0.9% NaCl solution and acetone to remove any unused oil from the cell surface. The centrifuged cells were dried in an oven at −80 ℃ for 5 h to determine the cellular dry weight (CDW) and then extracted with chloroform to determine the PHB concentration and content [15]. PHB was isolated from the dried cell mass by chloroform extraction at 30 ℃ overnight at 200 rpm on a rotary shaker. Cellular debris was removed by rapid filtration using filter paper, after which the extract was concentrated by evaporating chloroform in a rotary evaporator and then precipitated via the dropwise addition of cold methanol to obtain purified PHB [16].

Medium-to-long-chain fatty acid detection

The supernatant of the fermentation broth of this strain utilizing used soybean oil medium to synthesize PHB was collected at different timepoints (0, 12, 24, 36, 48, 60, 72, 84, and 96 h) and then stored in a −20 °C freezer. All experiments were performed in triplicate. These samples were then sent to Shanghai Major Bio Company for fatty acid composition and content assessment under dry ice freezing conditions. Detecting the content of fatty acids in the fermentation broth provides more sufficient data to elucidate the PHB synthesis process.

PHB characterization analysis

NMR analysis: The chemical structure of purified PHB was detected by proton nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR) spectroscopy. Using tetramethylsilane as an internal chemical shift reference, approximately 5 mg of purified PHB was dissolved in 1 mL of deuterated chloroform ($CDCl_3$) at a concentration of 25 mg/mL [17].

GC–MS analysis: After 10 mL of fermentation broth was collected in a low-temperature centrifuge at 8000 rpm for 10 min and the supernatant was discatede, the organisms were resuspended in distilled water and centrifuged again under the same conditions, and the supernatant was discarded. The organisms were then stored at −80 ℃ overnight. The stored bacteria were placed in a vacuum freeze dryer for 24 h. A certain amount (≤50 mg) of dried bacteria was weighed into an esterification tube, to which 2 mL of esterification solution and 2 mL of chloroform were added. The mixture was subjected to esterification at 100 ℃ in a water bath for 4 h and then cooled, after which 1 mL of deionized water was added, and the mixture was mixed vigorously. After stratification, the lower layer of chloroform PHB was removed, filtered through a 0.22 μm filter membrane, and analyzed by gas chromatography. Mass spectrometry monomers were scanned and analyzed by mass spectrometry (MASPEC II system) and compared in a database to determine the individual components of the monomers [18].

Determination of PHB thermal properties: Differential scanning calorimetry (DSC) was used to analyze the thermal properties of the purified PHB samples and to measure their crystallization temperature (T_c) and melting temperature (T_m). The DSC curves were recorded on a TA instrument (Q2000 Differential Scanning Calorimeter) at a flow rate of 50 mL/min of nitrogen between −60 ℃ and 180 ℃.

Determination of PHB molecular weight: The molecular weight of PHB was determined using gel-permeation chromatography (GPC). The weight-average molecular weight (M_w), number average molecular weight (M_n), and polydispersity index ($PDI = M_w/M_n$) of the purified PHB samples were determined using gel permeation chromatography.

Genome sequencing and analysis

For the molecular characterization of the strain, whole genome sequencing was conducted. The whole genome sequencing and analysis in this study were performed on the platform at Major Bio. As a brief overview, the experimental procedure was performed according to the standard protocol provided by Oxford Nanopore Technologies (ONT), including the processes of sample quality testing, library construction, library quality testing, and library sequencing. The bioinformatics analysis included the following main steps: first, the raw data were quality controlled to filter low-quality and short reads using Canu v1.5 software. The genome was subsequently assembled, the filtered reads were assembled from scratch, and the assembled draft genome is corrected. The genome components were subsequently analyzed, including repetitive sequences, coding genes, noncoding RNA, prophages, gene islands, and CRISPR. Various databases were used to annotate functional genes, including general databases such as Nr (non-redundant protein database, nr-2019–03), KEGG (Kyoto Encyclopedia of Genes and Genomes, −201,703), eggNOG (evolutionary genealogy of genes: non-supervised orthologous groups, v4.0), as well as proprietary database annotations such as CAZyme (carbohydrate-active enzymes database, v6.0). The software Blast2GO v2.5 was used to perform functional annotation of the above database. Circos v0.66 software was used to draw and visualize the genomic maps.

Real-time PCR transcriptional profiling

The bacterial cells representing different growth conditions were collected at 24, 48, 72, and 96 h after the strain was inoculated on the used soybean oil culture medium. An RNAprep Pure Cell/Bacteria Kit (TIANGEN, DP430) was used for total RNA extraction, and the quality of the extracted RNA was determined by both gel electrophoresis and a Nanodrop 2000. TaKaRa's PrimeScript™ RT Master Mix (Perfect Real Time) was used as the reverse transcription reagent. The primer pairs used in this study are shown in Table 1. Three technical replicates were established for all treatment groups, the total reaction system was 10 µl, and the reaction procedure was the standard procedure for two-step PCR amplification: Stage 1: predenaturation repetitions: 1, 95 °C for 30 s; Stage 2: PCR repetitions: 40, 95 °C for 3 s, 60 °C for 30 s [19]. The 2 (-Delta Delta C(T)) method was used to determine the relative expression levels of key genes in the PHB biosynthesis pathway[20]. The 16S rRNA gene of the strains was used as the housekeeping gene for normalization via this method.

Statistical analysis

All data in this study were analyzed in triplicate using Excel 13.0 and are expressed as the mean ± standard deviation. Graphing in this study was performed using Graphpad Prism software (version 9.0) for analysis and plotting.

Results

Characterization of the strain

This strain was cultivated on used soybean oil agar medium at 30 ℃ for 3 days, resulting in the formation of round, regular-edged, orange–red colonies with a diameter of 0.5–1 mm (Fig. 1A). A phylogenetic analysis suggested that S-LD is a strain of *Gordonia terrae*, with 99% sequence similarity to *Gordonia terrae* ORE9 (Fig. 1B). Physiological tests indicated that S-LD thrived in the

Table 1 Primer sequences used in this study

Gene name	Sequence (5′– > 3′)
S-LD 16S	Forward primer GTGAGTGACGGTACCTGGAG Reverse primer ATTTCACAGACGACGCGACA
triacylglycerol lipase	Forward primer GCAGCTCTCAACACCCATGT Reverse primer CTGGTGTTCGATGCTCCACG
fadD	Forward primer CCTGCTCAAAGGACCGGTTG Reverse primer GGATGGTGTCCTCGAGTTGG
fadE	Forward primer CAGTTCCGACACCGCAGAA Reverse primer GGCCATCTCGATCAGTGTGT
fadJ	Forward primer TGACGCTCCAGCACTACAAG Reverse primer CAAGTCGGAACTCTCCCACG
fadB	Forward primer ACCCTGACCGATCTCAAGGA Reverse primer CACCTTCGTCCAGACACCTC
fadA	Forward primer GCGACCAAGAACGGACTCTT Reverse primer TCGTCTCGTCGAAATAGGCG
phaA	Forward primer GTTGAAGCCCGCCTATTTCG Reverse primer GTCCTCGATACGCAGTCCAC
phaC	Forward primer GTGAGTGGCACGAGACCC Reverse primer GGAACTGCACGACGGACA

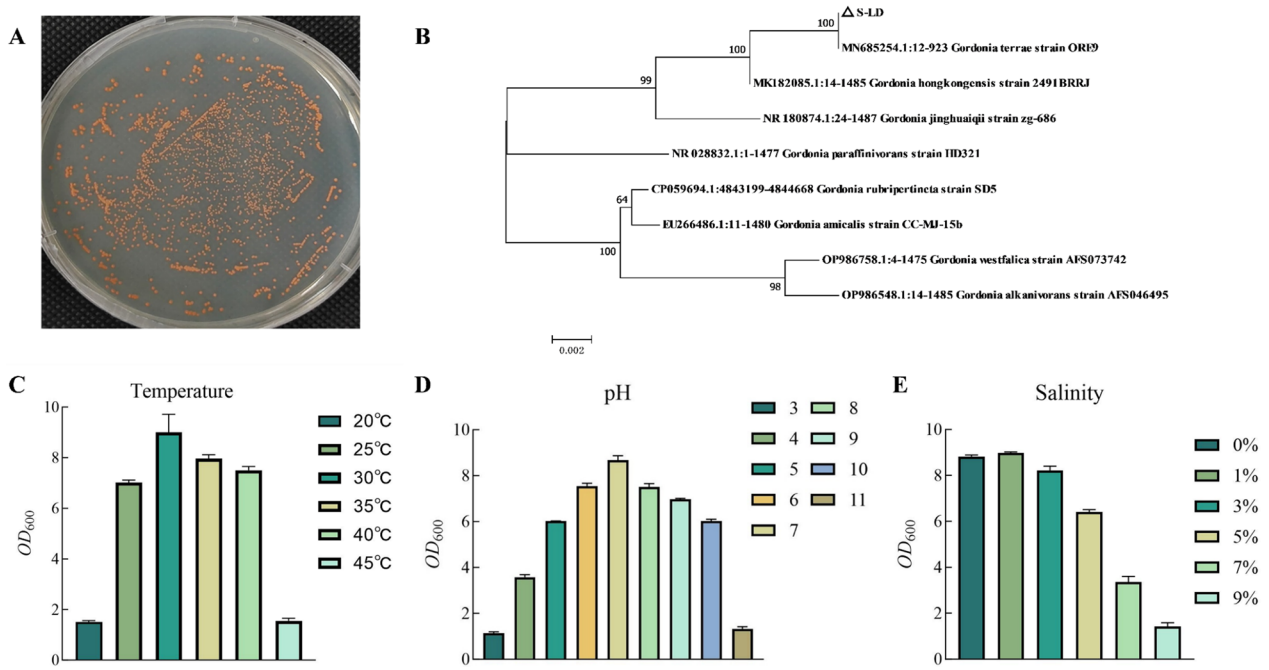

Fig. 1 Comprehensive characterization of *Gordonia terrae* S-LD. **A** Visual representation of the strain growth on used soybean oil medium plates. **B** Phylogenetic tree depicting the evolutionary placement of the strain, constructed via the neighbor-joining method. **C, D,** and **E** Variations in optical density (OD_{600}) measurements for the strain across a range of temperatures, initial pH levels, and salinity conditions

temperature range of 25℃ to 40 ℃, peaking at 30 ℃, and within a pH range of 4–10, with an optimum of 7 in used soybean oil medium (Fig. 1C, D). Given the high salt content in used cooking soybean oil, the salt tolerance of the strain was assessed, revealing that it could grow in the presence of up to 7% NaCl, with growth significantly inhibited at 9% NaCl (Fig. 1E). The optimal OD_{600} was observed at salt concentrations below 50 g/L (5%). This salt tolerance suggests that *Gordonia terrae* S-LD may be a suitable candidate for processes involving high salt concentrations from used cooking soybean oil.

As depicted in Fig. 2A, the medium was initially clear and became turbid post-fermentation, with the used soybean oil on the surface disappearing, indicating emulsification in addition to degradation. Scanning electron microscopy revealed the morphological traits of S-LD (Fig. 2B; scale bar, 1 μm). The strain identified is an obligate aerobe with a short rod-shaped morphology, measuring 0.4–0.6 μm, lacking a flagellum, non-motile. TEM examination of *Gordonia terrae* S-LD cells cultivated on used soybean oils post-fermentation revealed the presence of white particles, identified as PHB (Fig. 2C; scale bar, 500 nm). Moreover, the SEM and TEM results confirmed that during the bioconversion of used soybean oil into poly-β-hydroxybutyrate, the bacterial volume increased significantly to approximately three times greater than the initial bacterial volume. The strain was deposited at the China General Microbiological Culture Collection Center with the accession number CGMCC No. 27104 for patent preservation (202310832613.0).

pH, OD_{600}, CDW, PHB concentration and content variations during PHB synthesis

The cell density and pH variation during PHB synthesis are illustrated in Fig. 3A. Throughout PHB synthesis, the pH of the fermentation broth continuously decreased, reaching a minimum of approximately 3.0. This decrease may be associated with the production of fatty acids of various chain lengths from the degradation of used soybean oil. As microorganisms metabolize the triglycerides in used soybean oil, lipase breaks the ester bonds, yielding fatty acids and glycerol, increasing the acidity of the culture medium [21]. Over the 96-h fermentation period, the cell density at OD_{600} reached 9.8. The fermentation duration significantly influenced the accumulation of PHB synthesized by *Gordonia terrae* SL-D. The highest total dry weight, PHB concentration, and cellular content were observed after 96 h of fermentation culture, i.e., 3.92, 2.63 g/L, and 66.97%, respectively (Fig. 3B).

Trends of medium-to-long-chain fatty acid changes during PHB synthesis

Table S1 lists the medium-to-long-chain fatty acids identified during PHB synthesis of in *Gordonia terrae* S-LD.

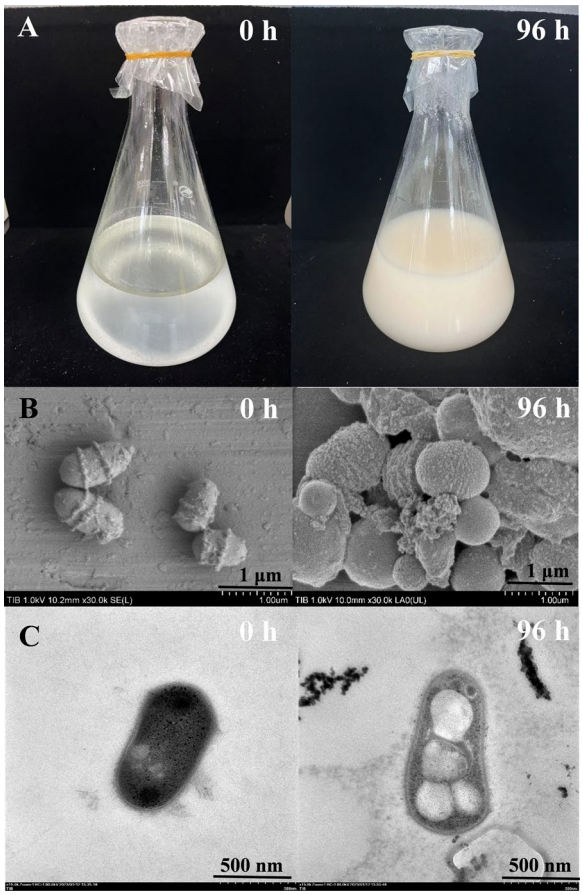

Fig. 2 PHB synthesis by *Gordonia terrae* S-LD utilizing used soybean oil. **A** Photographic documentation of fermentation broth evolution at 0 and 96 h, with used soybean oil as the sole carbon source. **B** SEM image highlighting the morphology of the strain at 0 and 96 h. **C** TEM analysis illustrating the accumulation of PHB at various incubation stages (0 and 96 h)

As shown in Fig. 3C, the main medium-to-long-chain fatty acids that exhibited changes during used soybean oil degradation were 18-carbon and 16-carbon fatty acids (Fig. 3C). The total content of medium-to-long-chain fatty acids peaked at 24 h under lipase degradation. This finding indicates that the hydrolysis rate surpasses the β-oxidation rate during fermentation before 24 h. From 24 to 96 h, the total fatty acid content decreased, suggesting that β-oxidation continued and that PHB synthesis was ongoing. In addition, the changes in the concentrations of fatty acid at different lengths over time indicate that PHB synthesis primarily occurred within the 24–96 h period. At 96 h, PHB production was maximized, and the concentration of medium-to-long-chain fatty acid was minimized.

Characterization of PHB synthesized by Gordonia *terrae* S-LD

The PHB extracted from *Gordonia terrae* S-LD during cultivation on used soybean oil was analyzed by ^{1}H NMR and ^{13}C NMR (Fig. 4A, B), which revealed the presence of methyl ($-CH_3$), methylene ($-CH_2$), and methane (–CH) groups, confirming its structure as PHB. Similarly, ^{13}C NMR revealed resonance between 22 and 34 ppm, indicating the presence of many carbon molecules in the methylene groups [22]. Spectroscopic characterization of the polymers obtained from *Gordonia terrae* S-LD during growth on used soybean oil revealed a strong presence of ester bonds and the presence of 3-hydroxybutyrate acids and confirmed it to be PHB. NMR characterization of the PHB produced by *Gordonia terrae* S-LD confirmed it to be a homopolymer of 3-hydroxybutyrate. Moreover, the GC–MS chromatogram confirmed the purified PHBs contained in this strain. The detection of hydroxybutyric acid is associated with the production of PHB (polyhydroxybutyric acid).

A DSC scan of the purified PHB revealed its molecular weight and thermal properties, with melting and crystallization temperatures of 176.34 °C and 56.12 °C, respectively (Fig. 4D, E). The melting temperature and crystallization temperature of PHB synthesized by this strain are consistent with those of PHB previously

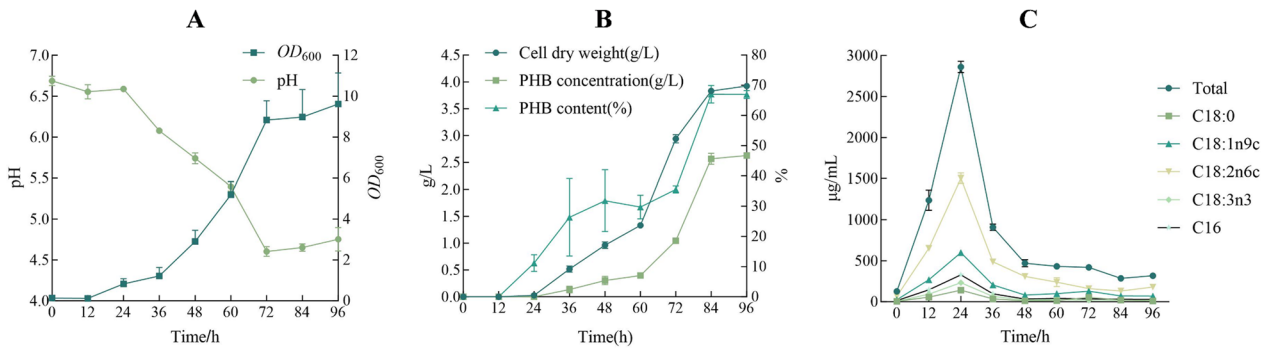

Fig. 3 Dynamics of PHB synthesis by the strain over 96 h. Trend plot depicting changes in pH, optical density (OD_{600}) (**A**), cell dry weight, PHB concentration and content (**B**), and medium-to-long-chain fatty acids (**C**) throughout the 96 h PHB synthesis process by the strain

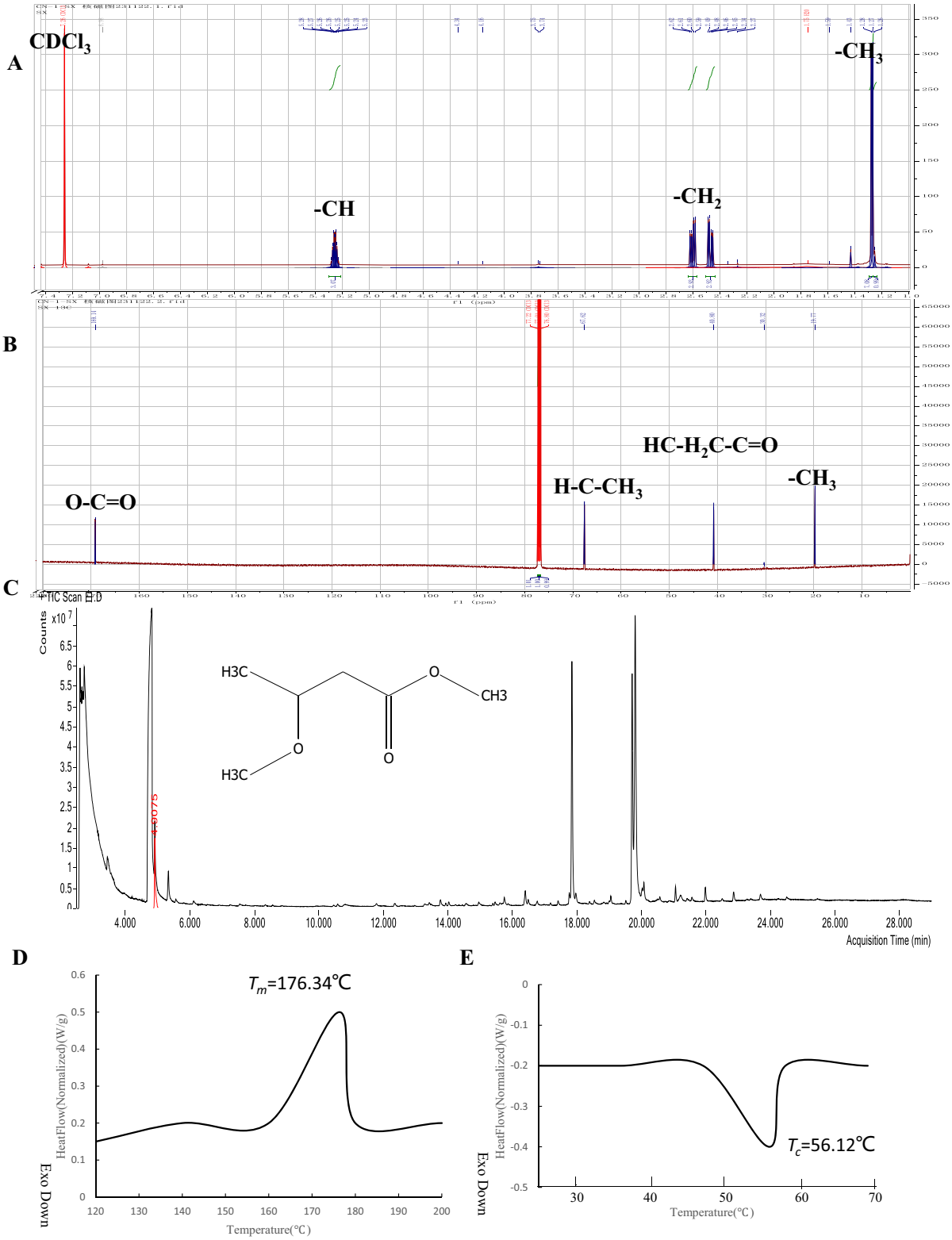

Fig. 4 Characterization of PHB accumulated from *Gordonia terrae* S-LD. **A**, **B** Proton nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) spectra confirming the structure of PHB synthesized by *Gordonia terrae* S-LD during cultivation on used soybean oil. **C** Gas chromatography–mass spectrometry (GC–MS) analysis characterizing the PHB produced by *Gordonia terrae* S-LD. **D**, **E**. Differential scanning calorimetry (DSC) analysis revealing the melting temperature (T_m) and crystallization temperature (T_c) of PHB synthesized by *Gordonia terrae* S-LD

reported in the literature [4]. The produced PHB was characterized by a weight-average molecular weight (M_w) of 5.43×10^5 g/mol, a number-average molecular weight (M_n) of 4.00×10^5 g/mol, and a polydispersity index (PDI) of 1.36.

Whole genome sequencing and analysis of the strain

Subsequently, the whole genome of the strain was sequenced with the goal of confirming the synthesis pathway of PHB by revealing all the key genes. Whole genome sequencing, assembly, and annotation were performed to gain a comprehensive understanding of the process of PHB synthesis by *Gordonia terrae* using used soybean oil and to expand the microbial strain resources for PHB production. Using the assembled and predicted genomic information, such as tRNA, rRNA, repetitive sequence, GC content, and gene function information, we were able to more clearly explore the positional relationships between genomic components in the genome (Fig. 5A). The genome size of *Gordonia terrae* S-LD was approximately 5.58 Mb, with a GC content of 67.96%. A total of 5034 genes were predicted, with an average gene length of 5, 113, and 191 bp. Among the 5034 predicted genes, 4992 genes (98.17%) were functionally annotated in the different databases (Fig. 5F), and enrichment analysis was performed using the Nr, KEGG, eggNOG, and CAZy databases, with a focus on fatty acid and butanoate metabolism. The genes are involved in the biodegradation of oils and the synthesis of PHB are involved mainly in fatty acid and butanoate metabolism. A comparison of the gene annotations with those in the Nr database (Fig. 5B), revealed that the highest percentage of genome annotations for the sequenced S-LD in this study was for *Gordonia terrae* (50.23%). After comparison with the KEGG database (Fig. 5C), we found that the genome of the S-LD strain has a complete pathway for lipid degradation and PHB synthesis. A comparison of the annotations with the eggNOG database (Fig. 5D), revealed that the genomic annotation information of this strain was related mainly to transcription (8.1%), energy production and conversion (7.19%), amino acid transport and metabolism (6.68%), and lipid transport and metabolism

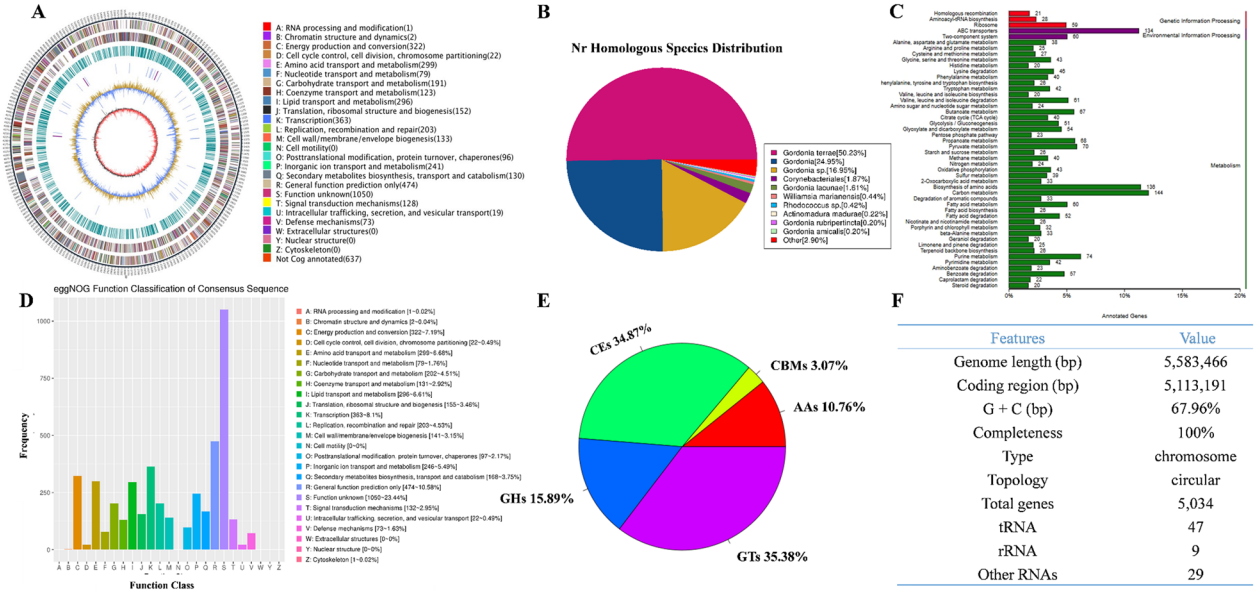

Fig. 5 Whole genomic insights into *Gordonia terrae* S-LD. **A** Circular genomic map of the strain, graphically detailing genomic features from the outermost to the innermost tracks: genomic size, coding DNA sequences, tRNAs, rRNAs, other RNAs, GC content, and GC skew values. Note: The outermost circle is the marker of genome size, with each scale mark representing 5 kb; the second and third circles are genes on the positive and negative strands of the genome, respectively, and different colors represent different COG functional classifications; the fourth circle is repetitive sequences; the fifth circle is tRNA (blue) and rRNA (purple); the sixth circle is the GC content, where light yellow indicates that the GC content of this region is higher than the average GC content of the genome, where the higher the peak value, the greater the difference from the average GC content, and blue indicates that the GC content of this region is lower than the average GC content of the genome; the innermost circle is GC-skew, where dark gray represents regions where the G content is greater than the C content, and red represents regions where the C content is greater than the G content. **B** Species distribution of sequences aligned to the Nr database. Note: This graph reflects the species distribution of matching sequences in the Nr database. Different colors represent different species. **C** Functional annotation and enrichment analysis of the strain genome using the KEGG database. Note: Vertical coordinates are KEGG secondary classifications and horizontal coordinates are percentages. **D** Statistical map of the functional classification of eggNOG functional genes. Note: The horizontal coordinate is the content of each eggNOG classification, and the vertical coordinate is the relative content of the number of corresponding functional genes. **E** Proportional distribution of carbohydrases. **F** Additional genomic features of the strain

(6.61%), excluding general function prediction and unknown functional genes. Through gene comparison and annotation using a proprietary database (CAZy database) (Fig. 5E), it was found that the gene information annotated in this strain genome consists of two main categories: glycosyltransferases (GTs, 35.38%) and carbohydrate esterases (CEs, 34.87%). In addition, three types of non-coding RNAs were identified in the *Gordonia terrae* S-LD genome, including 47 tRNA genes, 9 rRNA genes, and other RNA genes (Fig. 5F).

Elucidation of the PHB synthesis pathway and quantification of the expression of key genes

The biosynthesis of PHB in microorganisms is a complex metabolic process involving several key enzymes and pathways. As observed in *Gordonia terrae* S-LD, used soybean oil is utilized as the sole source of carbon/energy for PHB accumulation (Fig. 6). In the presence of lipase, used soybean oil is broken down into triglycerides and fatty acids of varying chain lengths. In this process, fatty acids of different chain lengths are metabolized through β-oxidation to produce acetyl-CoA. The enzyme acetyl-CoA C-acetyltransferase (phaA) catalyzes the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA, followed by conversion of 3-hydroxyacyl-CoA dehydrogenase (fadB) to (S)−3-hydroxybutyryl-CoA. (S)−3-hydroxybutyryl-CoA is converted into (R)−3-hydroxybutyryl-CoA under the action of 3-hydroxybutyryl-CoA epimerase (fadJ). Eventually, (R)−3-hydroxybutyryl-CoA is synthesized into PHB in the presence of PHB synthase (phbC). Based on the results of this study, we hypothesized that the pathway for synthesizing PHB in this strain can be roughly divided into three modules: the oil degradation module, the β-oxidation module, and the synthesis module (Fig. 6).

The PHB synthesis pathway elucidated in this study and the quantification of key genes via RT-qPCR indicated that PHB was not synthesized in large quantities during the initial 48 h. PHB biosynthesis primarily occurred from 48 to 96 h (Fig. 6). Since each round of β-oxidation releases a molecule of acetyl-CoA, the metabolism of the initially released acetyl-CoA through the tricarboxylic acid cycle may have provided the energy and metabolic intermediates required for the initial growth of the bacterium as well as for the accumulation of the precursors for PHB synthesis. It is speculated that *Gordonia terrae* S-LD degrades the medium-to-long-chain fatty acids (C18 and C16) in used soybean oil through β-oxidation and synthesizes (R)−3-hydroxybutyryl-CoA from the intermediates of this pathway, which can act as a substrate for PHB synthase.

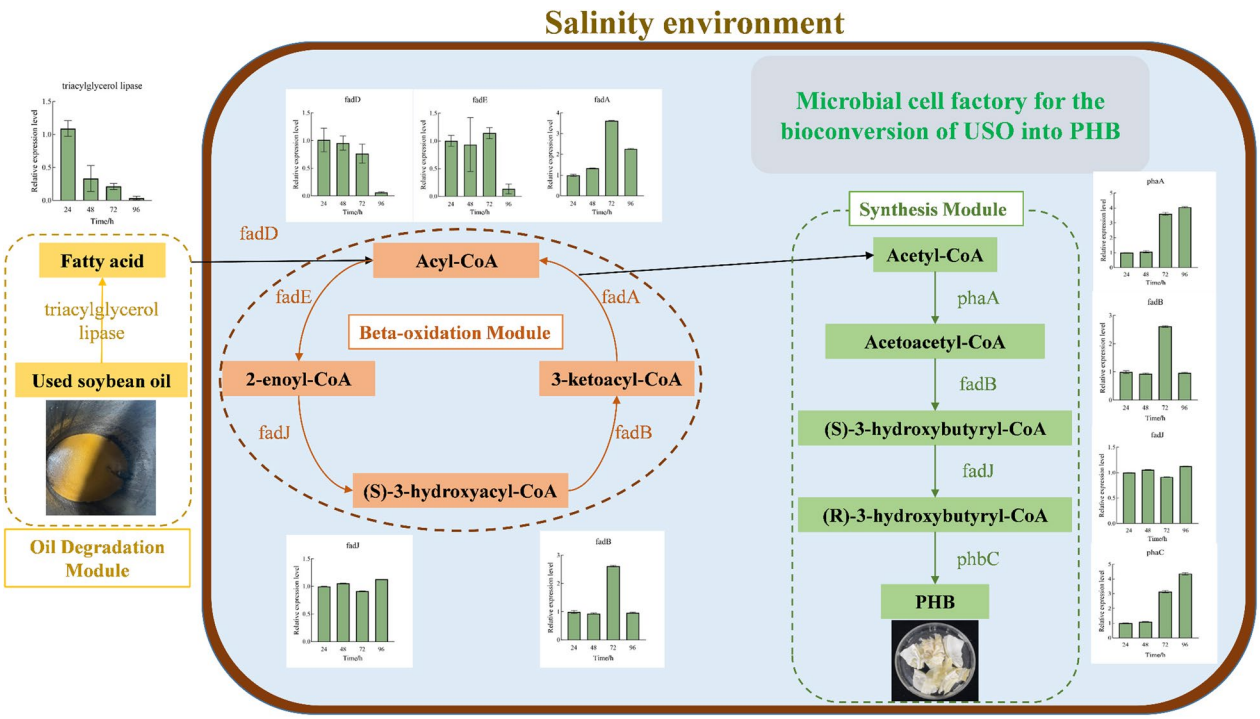

Fig. 6 Elucidation of the PHB synthesis pathway and the expression of key genes in *Gordonia terrae* S-LD. Schematic representation of the PHB synthesis pathway in *Gordonia terrae* S-LD, alongside a quantitative expression analysis of pivotal genes involved in this metabolic process. There are three main modules: the oil degradation module (yellow), the beta-oxidation module (orange), and the synthesis module (green)

Discussion

To the best of our knowledge, few studies on the production of PHB by the rare Actinomycete genus Gordonia have been conducted. Most studies on Gordonia sp. have focused on the degradation of alkanes, n-hexadecane, and benzenes, and the production of emulsifiers or carotenoids [23]. In this study, a salt-tolerant strain of *Gordonia terrae* that can utilize used soybean oil to synthesize PHB under 3% salt was successfully screened. Moreover, this strain has a wide range of growth conditions. This study represents the first report on PHB production by *Gordonia terrae*, particularly utilizing used cooking soybean oil as the sole carbon source. Numerous studies have confirmed that microorganisms can synthesize PHB using various carbon sources, such as sugars, corn, potatoes and more [4]. The use of inexpensive carbon and energy sources can significantly reduce the cost of synthesizing PHB while reducing environmental pollution problems [24], and used soybean oil is one of the best representatives of these inexpensive carbon and energy sources.

Cupriavidus necator H16, also known as *Ralstonia eutropha*, is currently the most extensively researched and typical example of a strain that can be used to synthesize PHA from waste oil [25]. The highest cell dry weight (CDW) of 9.44 g/L and the maximum PHA accumulation of 7.22 g/L were obtained with *C. necator* when low-cost vegetable oil was used [26]. Ruiz et al. used the *Pseudomonas chlororaphis* and used waste cooking oil (WCO) to synthesize PHA, reaching yields of 0.09–0.14 g PHA/g oil substrate [27]. Most of the conventional wild strains found thus far are essentially intolerant to salinity. However, most of the various waste oils or oily wastewaters produced in daily life have high salinities ($\geq$3%). Therefore, it is necessary to explore some salt-tolerant strains for potential application strains in the synthesis of PHA using waste oil-based substrates. The newly screened *Gordonia terrae* can not only synthesize PHB in the presence of 3% salt, but also tolerate a salt concentration gradient of up to 7%, which greatly broadens the scope of use of this strain in subsequent large-scale applications.

The molecular weight of PHB synthesized by *C. necator* using waste rapeseed oil as a carbon source was 5.77×10^5 g/mol and the PDI was 2.66 [28]. *C. necator* was grown on extracted spent coffee ground oil to synthesize PHB with a lower M_w of 4.27×10^5 g/mol and a PDI of 2.51 [29]. According to the literature, the molecular weight of PHB synthesized from different types of waste oil is generally between 0.4 and 3.0×10^5 g/mol, and the PDIs range from 1.2 to 3.0 [30]. The PHB produced by *Gordonia terrae* bioconverted from used soybean oil was characterized by a weight-average molecular weight (M_w) of 5.43×10^5 g/mol, a number-average molecular weight (M_n) of 4.00×10^5 g/mol, and a polydispersity index (PDI) of 1.36. The PDI of the PHB polymers produced in our study is within the range reported in the literature, whereas M_w is considered relatively high. A higher PHB molecular weight will increase the strength and hardness of the material and improve its physical and chemical stability, making it suitable for application scenarios that require high stability. Moreover, the PDI value of PHB synthesized by this strain is relatively smaller, indicating that the molecular weight distribution of the PHB synthesized by this strain is relatively uniform, which makes subsequent applications easier.

In addition, the microbial synthesis of PHB is a multifaceted process that can be influenced by various factors, including the type of carbon source and the metabolic pathways employed. Understanding and optimizing these pathways are crucial for improving the efficiency and cost-effectiveness of PHB production, especially for new strains and new synthetic pathways. Some bacteria, such as *Pseudomonas aeruginosa* and *Aeromonas caviae,* utilize the β-oxidation pathway to synthesize PHB. In this pathway, the intermediate enoyl-CoA is converted to (R)−3-hydroxyacyl-CoA by enoyl-CoA hydratase, which is then polymerized by PHA synthase (PhaC) into PHB. Bacteria such as *Pseudomonas putida* can synthesize PHB through the de novo fatty acid synthesis pathway. Acetyl-CoA enters the fatty acid synthesis pathway, and the intermediate (R)−3-hydroxyacyl-ACP is converted to (R)−3-hydroxyacyl-CoA by 3-hydroxyacyl-ACP: CoA transacylase, which is then polymerized into PHB by PHA synthase(PhaC) [31]. In this study, analysis of the changes in medium-to-long-chain fatty acids during PHB synthesis, revealed that soybean oil used for 24 h is decomposed into medium-to-long-chain fatty acids under the action of lipase. On the basis of our analysis of the genome of the strain and the elucidation of the synthesis pathway, it is speculated that these medium–long-chain fatty acids are decomposed into short-chain fatty acids through β-oxidation, and these short-chain fatty acids accumulate and subsequently synthesized into PHB. Notably, some strains, such as *Comamonas testosteroni* and *Pseudomonas putida*, can utilize other oils to synthesize medium chain length poly (3-hydroxyalkanoates) (mcl-PHAs). They can utilize the intermediate metabolites in the β-oxidation of fatty acids, such as 3-ketoacyl-COA and (R)-enoyl-CoA, to synthesize medium-chain PHAs. However, this strain was not able to directly convert medium-chain fatty acids into medium-chain PHAs. We speculate that this may be due to the substrate specificity of PHA synthase. However, this study provides a good

research point and possibility for our subsequent metabolic engineering efforts. In subsequent work, gene knockout and heterologous expression or modification of enzymes should be used to further verify the polymer synthesis pathway.

Advances in metabolic engineering have enabled increased PHB accumulation in microorganisms. The choice of carbon source significantly affects PHB production. Previous studies have compared the efficiency of glucose, acetic acid, and ethanol as carbon sources for PHB production, highlighting the importance of optimizing carbon source utilization in the production process. Moreover, recent studies have shown that recombinant *Escherichia coli* can use acetic acid and lactic acid as carbon sources for PHB production [32]. In this study, the used soybean oil employed as the substrate matrix, which is actually a relatively complex substance, mainly consisted of 18-carbon and 16-carbon fatty acids. This composition prompted us to pay attention to the issues of substrate selection and metabolic pathway diversification when we carry out future metabolic engineering of this strain. This study also demonstrates the flexibility of the metabolic pathways in the utilization of different carbon sources.

The transformation of waste into new raw materials is a fundamental aspect of the circular economy framework [33]. The higher cost of PHB synthesis impacts its market competitiveness, especially compared to less expensive, non-biodegradable alternatives. Addressing these challenges is essential for ensuring the commercial viability and environmental impact of PHB as a sustainable alternative to traditional plastics. Moreover, improper disposal of used soybean oil and other waste oils causes serious environmental pollution or endangers human health. For large-scale PHB production, the use of inexpensive carbon sources or energy is a critical prerequisite. This strain has good salt tolerance and can grow well over a wide temperature and pH range. Therefore, there is great potential for this strain to be scaled up for the bioconversion of waste oils. The ability of *Gordonia terrae* to degrade hydrocarbons and BTEX compounds has been reported [34]. Moreover, higher molecular weight PHB has better oil–water separation effect while improving its stability and pollution resistance. Since the molecular weight of PHB synthesized by this strain is high, it is easier to separate and purify it during subsequent large-scale application. These characteristics determine the great advantages of this strain in future large-scale applications. Of course, in subsequent research we must also consider the planned scale-up process and purification issues and even conduct a life-cycle assessment [35].

Conclusions

A newly isolated salt-tolerant *Gordonia terrae* S-LD can be utilized to biosynthesize PHB from used soybean oil with intriguing physicochemical properties. This finding is highly important, as it may favor process economics. Under waste-containing medium conditions, a maximum PHB concentration of 2.63 g/L was achieved after 96 h of cultivation at a 3% salt concentration. In addition, the whole genome and PHB synthesis pathway of this strain were sequenced and elucidated. These observations suggest that *Gordonia terrae* S-LD may be a promising candidate for PHB production using different oil-based substrates. Further investigation into *Gordonia terrae* is anticipated to determine whether PHB production can be more economically viable for subsequent large-scale industrial applications.

Abbreviations

DSC	Differential scanning calorimetry
M_w	Weight-average molecular weight
M_n	Number-average molecular weight
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PDI	Polydispersity index
T_c	Crystallization temperature
T_m	Melting temperature
USO	Used soybean oil
WCO	Waste cooking oil
Nr	Non-redundant protein database
KEGG	Kyoto encyclopedia of genes and genomes
eggNOG	Evolutionary genealogy of genes: Non-supervised orthologous groups
CAZyme	Carbohydrate-active enzymes database

Supplementary Information

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Supplementary Material 1.

Author contributions

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Data availability

Data will be made available on request.

Declarations

Ethics approval and consent to participate

Not Applicable.

Competing interests
The authors declare no competing interests.

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