Psychrotrophic *Pseudomonas mandelii* CBS-1 produces high levels of poly-β-hydroxybutyrate

Rongpeng Li¹, Yuji Jiang², Xinfeng Wang³, Jingjing Yang¹, Yuan Gao¹, Xiaoli Zi¹, Xia Zhang¹, Haofeng Gao¹ and Nan Hu¹*

**Abstract**

A novel facultative psychrotroph (strain CBS-1), which accumulates poly-β-hydroxybutyrate (PHB), was isolated from soil samples taken from Changbai Mountain, China. Phylogenetic analysis based on 16S rRNA sequence data and Biolog analysis identified strain CBS-1 as *Pseudomonas mandelii*. Transmission electron micrographs revealed abundant electron-transparent intracellular granules. ¹H-nuclear magnetic resonance analysis revealed that the granules were composed of PHB. *P. mandelii* CBS-1 grew optimally at 20°C. When cultured aerobically for 48 h with sucrose as the sole carbon source, strain CBS-1 yielded a maximum cell density of 29.3 g/L cell dry weight and synthesized 22.3 g/L of PHB. The ability of strain CBS-1 to grow at a low temperature and rapidly synthesize high levels of PHB may reduce the costs of industrial PHB production.

**Keywords:** PHB; *Pseudomonas mandelii* CBS-1; Facultative psychrotrophs; ¹H- nuclear magnetic resonance; Fermentation

**Background**

Polyhydroxyalkanoates (PHAs) are biodegradable polyelecters composed of (R)-3HA monomers, which are ubiquitously synthesized by microorganisms. Many bacteria store carbon as PHAs to protect themselves from nutrient limitation, including lack of nitrogen, phosphorus, and magnesium (Dowes and Senior, 1973). PHAs are synthesized by most genera of Bacteria and (Archaea Steinbuchel and Byrom, 1991). The best-characterized microorganisms that produce PHAs are *Alcaligenes* sp. (Linko et al., 1993), *Azotobacter* sp. (Page, 1992), *Bacillus* sp. (Huang and Reusch, 1996), *Rhodopseudomonas* sp. (Yue et al., 2007), and *Pseudomonas* sp. (Kimura et al., 1992).

Poly-β-hydroxybutyrate (PHB), the least complex PHA biopolyester, offers distinct advantages for polyester production because of its biodegradability, lighter mass, thermoplasticity, and resistance to abrasion (Steinbuchel and Valenii, 1995). PHB shows great potential for manufacturing products such as degradable plastics, medical implant materials, and fine chemicals (Holemes, 1985). Unfortunately, available bacterial strains synthesize low levels of PHB even after prolonged fermentation. Therefore, PHB biosynthesis is more expensive than standard chemical synthesis and limits its use (Choi and Lee, 1999).

Therefore, the goal of our research was to isolate bacteria capable of producing high levels of PHB at low temperatures. We report here the isolation of a facultative psychrotrophic strain (CBS-1) from the soil of Changbai Mountain, China, and its identification as *Pseudomonas mandelii*. It is the first report of PHB accumulation in *P. mandelii*. Strain CBS-1 grew aerobically to a dry-cell weight of 29.3 g/L and synthesized 22.3 g/L of PHB in 48 h, which is higher compared with other reported strains (Kimura et al., 1992; Huang and Reusch, 1996; Yue et al., 2007).

**Material and methods**

**Strains, growth media, and culture condition**

A soil sample from Changbai Mountain (altitude 2183 m, N 42°08’19.7″, E 128°10’8.3″), Jilin Province, China, was suspended in sterile H₂O, vortexed thoroughly, and cultured in an enriched medium containing 5 g/L yeast extract, 10 g/L tryptone, and 2.5 g/L NaCl at 20°C for 2 days. A 50-μL aliquot was spread on PHB detection agar (20 g/L glucose, 2 g/L (NH₄)₂SO₄, 13.3 g/L KH₂PO₄, 1.2 g/L MgSO₄·7H₂O, 1.7 g/L citric acid, 1.7 g/L trace elements solution, 15 g/L agar, and 0.5 μg/mL Nile blue A

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* Correspondence: hunnanjut126.com
**College of Biotechnology and Pharmaceutical Engineering, Nanjing University of Technology, Nanjing 211800, People’s Republic of China
Full list of author information is available at the end of the article

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(Spiekermann et al., 1999). After overnight incubation at 20°C, plates were observed under ultraviolet (UV) light. Fluorescent colonies were purified by streaking on the same agar plates, and the isolates were further characterized by staining with Sudan Black B (Lee and Choi, 2004).

Morphological characteristics
Cell morphology was examined using transmission electron microscopy as follows: a 2-mL aliquot of cells in late exponential phase (optical density OD₆₀₀ 55–80) was fixed with 4% glutaraldehyde and 1% osmium tetroxide and dehydrated by sequential treatment with 30%, 50%, 70%, 80%, 90%, and 100% acetone. The dehydrated cells were immersed in epoxy resin and then transferred into sample boats. The resin was polymerized for 24 h at 30, 45, and 60°C. Ultra-thin sections were prepared using an ultramicrotome, stained sequentially with uranyl acetate and lead citrate for 22 min and 5 min, respectively, and observed using an H-7650 transmission electron microscope (Hitachi, Tokyo, Japan).

Biolog Gram-negative (GN) assay
Gram staining was performed as described by Magee et al. (1975). A pure culture was grown on a BÜG agar plate (Biolog Catalog #70101). Biolog™ GN 96-well microtiter plates contain 95 different carbon sources, a negative control, and a tetrazolium dye (Biolog Inc., Hayward, CA, USA). Cells were swabbed from the surface of the plate and suspended to a specific density in GN Inoculating Fluid (Biolog Catalog #72101). The well of the Biolog GN2 MicroPlate (Biolog Catalog #1011) was inoculated individually with 150 μL of diluted suspensions and then incubated at 30°C for 24 h (Garland and Mills, 1991). The MicroPlate was inspected either visually or analyzed using the Biolog MicroStation™ and the results compared with the GN III Database according to the manufacturer's instructions (Biolog Catalog #22730D).

Polymerase chain reaction (PCR) amplification of the 16S rRNA gene
The 16S rRNA gene was amplified using PCR with the universal bacterial primers 27F (5′-agacctgttggcatcag-3′) and 1492R (5′-ggttaccttacctgctcag-3′) (Lane, 1991), and the product was sequenced by Takara Corp. (Dalian, China). The sequence was compared with 16S rRNA gene sequences in the GenBank database (http://www.ncbi.nlm.nih.gov) using the BLAST algorithm (Altschul et al., 1990). A neighbor-joining tree was constructed using the MEGA 4.0 program (Kumar et al., 2004).

Poly-β-hydroxybutyrate extraction
Cells were harvested and treated with 10% sodium dodecyl sulfate at 100°C for 15 min. After centrifugation at 10,000 rpm for 20 min, the pellets were washed twice with H₂O, dried at 40°C, and extracted with chloroform for 1 h at 60°C. Insoluble material was removed by filtration, and the soluble PHB was separated from the chloroform phase by evaporation, washed twice with methanol, filtered, dried at 60–70°C, and weighed using an electronic balance (Sartorius, BSA224S).

Nuclear magnetic resonance (NMR) spectroscopy
The NMR spectra of 1-mL samples were recorded using a BRUKAV AV-400 spectrometer with a 5-mm ¹H-probe, and deuterated chloroform (CDCl₃) was used as a solvent at a final concentration of 10 g/L. The ¹H-NMR spectrum for PHB was recorded at 400 MHz. A PHB standard was purchased from Sigma-Aldrich (St. Louis, MO, USA, #MSDS 363502).

Effect of culture conditions on cell growth
The effects on cell growth of carbon and nitrogen sources, C/N ratio, temperature, initial pH, and NaCl concentration were investigated using single-factor tests (Tang and Luo, 2008). Specifically, yeast extract, glucose, sucrose, fructose, glycerol, and ethanol were used as carbon source. Nitrogen sources included tryptone, peptone, beef extract, monosodium glutamate, urea, and NH₄Cl. All media contained 1.0 g/L KH₂PO₄, 2.5 g/L Na₂HPO₄, pH 7.0, and cells were cultured at 20°C, 200 rpm. To test the effect of the C/N ratio, sucrose and monosodium glutamate served as the sole carbon and nitrogen sources and were added at the C/N ratios as follows: 1, 5, 10, 15, 20, 25, and 30. Other conditions were the same as those described above. The optimum C/N ratio was used to test the conditions described next. NaCl concentrations were varied from 0–50 g/L (5-g/L intervals), and at initial pH values from 5.0 to 9.0 (0.5 pH unit intervals). Cultures were incubated from 5°C to 40°C (5°C intervals). After 3 days, cells were collected by centrifugation, and the pellets were dried at 70°C to a constant weight.

PHB production by aerated cultures
Cells were cultured at 20°C in medium containing 1.0 g/L KH₂PO₄, 2.5 g/L Na₂HPO₄, with optimal 1% (w/v) sucrose as the carbon source and monosodium glutamate as the nitrogen source. The pH was adjusted to 7.0 with 0.5 M NaOH. A 10-mL inoculum from a CBS-1 starter culture (OD₆₀₀ 0.5) was added to a 150-mL minimal medium. The 250-mL flasks were incubated at 20°C for at least 3 days and shaken at 200 rpm. Samples were collected at 8-h intervals from 0–72 h, centrifuged, and dried at 70°C to a constant weight.

Statistical analysis
All results are shown as the average and standard deviation of at least three independent experiments. Student's t-test, analysis of variance (ANOVA), and Duncan's
multiple range test were used to determine the statistical significance of differences in the nitrite-to-nitrogen conversion rate. Statistical significance was defined as $P < 0.05$. All analyses were performed using SPSS for Windows version 11.0.

Results

Isolation and characterization of strain CBS-1

Oxazine dye Nile blue A was used to develop a simple and highly sensitive staining method to directly detect poly-3-hydroxybutyric acid and other PHAs in growing bacterial colonies (Spiekermann et al., 1999). A 50-μl soil sample from Changbai Mountain, Jilin Province, China, was spread on PHB-detection agar containing Nile blue A stain (Lee and Choi, 2004). After incubation and observation under UV light, 59 fluorescent colonies were purified, and 27 were stained by Sudan Black B. A clone isolated from the largest and most intensely stained colony was named strain CBS-1. After incubation for 2 days on LB plates, CBS-1 formed white, circular colonies of semitransparent slabs that were wet with smooth surfaces. The bacilliform cells were GN, 3–4 mm in diameter × 5–7 mm long, and did not form spores. The cells occurred singly or in clusters and contained large granules of PHB (Figure 1).

A stretch of 1335 nucleotides of the 16S rRNA gene was PCR amplified and sequenced. The sequence was submitted to GenBank under the accession number KC778401. BLAST analysis indicated that strain CBS-1 is most closely related (99% identity) to $P$. mandelli. Phylogenetic analysis also grouped CBS-1 together with $P$. mandelli with 96% bootstrap support (Figure 2).

The GN III MicroPlate Database (Biolog Catalog #22730D) identifies 564 common species in accordance with classical identification methods and current taxonomic nomenclature. Unfortunately, $P$. mandelli was not present in the database. The GN MicroPlate performance characteristics indicated that strain CBS-1 was similar to $P$. fluorescens biovar C. The similarity index was 0.81 at 24 h (Table 1). $P$. mandelli is classified in the

![Figure 1](http://www.springerplus.com/content/2/1/335) Transmission electron micrograph of a thin section showing dividing $P$. mandelli CBS-1-containing granules of PHB. Magnification 2,500×.
P. fluorescens species (Anzai et al., 2000). Therefore, the BioLog GN assay result did not contradict the 16S rRNA gene sequence, and we concluded that CBS-1 is a P. mandelli strain.

**Physiological characteristics of CBS-1**

Sucrose and monosodium glutamate were the optimum carbon and nitrogen sources for culturing CBS-1 (Table 2) at a C/N ratio of 5 (Figure 3B). Strain CBS-1 grew to significant numbers between 10 and 35°C, and the optimum growth temperature was 20°C (Figure 3A). Strain CBS-1 grew between pH 6.0 and 9.0 and optimally between pH 7.0 and 7.5 (Figure 3C). Significant growth was observed in media containing 0–3% NaCl (Figure 3D).

**PHB synthesis by CBS-1**

After extracting cells with SDS and isolating PHB as a dried residue as described in Materials and methods, we conducted 1H-NMR analysis (Figure 4). PHB peaks were detected at δ = 5.2, 2.5, and 1.2 ppm, which correspond to a –CH doublet, a –CH2 multiplet, and a –CH3 doublet, respectively (Verlinden et al., 2011; De Rooy et al., 2007). The large peak at δ = 7.3 ppm represents the solvent (CD3Cl), and the peaks at δ = 0.0 ppm and δ = 0.8 ppm represent the internal standard (CH3)4Si. A

**Table 1 Biochemical characteristics of strain CBS-1 and related Pseudomonas species**

| Growth on: | Strain CBS-1 | P. fluorescens biovar C | P. fluorescens Azas |
|------------|--------------|--------------------------|---------------------|
| L-Arabinose | +            | +                        | +                   |
| α-L-Rhamnose| -            | -                        | -                   |
| Sucrose    | +            | +                        | +                   |
| Sebacic acid| -            | -                        | -                   |
| Succinic acid| -        | -                        | +                   |
| L-Asparagine| +            | +                        | +                   |
| L-Aspartic acid| +        | +                        | +                   |
| L-Histidine| +            | +                        | +                   |
| L-Leucine  | +            | +                        | +                   |
| L-proline  | +            | +                        | +                   |
| L-Threonine| +            | +                        | +                   |

+ Positive; - negative.

*a*Biolog GN assay data.

*b*Data from Jiang et al., (2008).

**Table 2 Effect of different carbon and nitrogen sources on PHB synthesis by strain CBS-1**

| Carbon source | Cell dry weight (g/L) | Nitrogen source | Cell dry weight (g/L) |
|---------------|-----------------------|-----------------|-----------------------|
| Yeast extract | 30.2 ± 3.1            | Tryptone        | 190 ± 0.9             |
| Glucose       | 9.9 ± 1.4             | Peptone         | 17.4 ± 1.8            |
| Sucrose       | 29.1 ± 3.0            | Beef extract    | 18.4 ± 1.3            |
| Fructose      | 7.8 ± 1.1             | Monosodium glutamate | 23.5 ± 1.0           |
| Glycerol      | 16.3 ± 2.9            | Urea            | 11.5 ± 0.8            |
| Ethanol       | 2.9 ± 0.2             | NH4Cl           | 13.6 ± 1.5            |
Figure 3 Effect of culture conditions on *P. mandelii* CBS-1 growth. Effects of temperature (A), C/N ratio (B), pH (C), and NaCl concentration (D) were investigated.

Figure 4 $^1$H-NMR spectra of a PHB standard and the PHB produced by *P. mandelii* CBS-1.
small peak at \( \delta = 1.6 \) ppm was attributed to minor contamination of the solvent with \( \text{H}_2\text{O} \). These results were identical to those for the PHB standard. Therefore, we concluded that the only PHA synthesized by strain CBS-1 grown in the presence of sucrose as the carbon source was PHB.

**PHB production in aerated CBS-1 cultures**

Strain CBS-1 was cultured in 150-mL sucrose-containing medium, pH 7.0, at 20°C in a 250-mL flask, which was shaken as described in Materials and methods. Cells were harvested upon reaching the stationary phase (48 h). The CDW was 29.3 g/L, and the PHB concentration reached 22.3 g/L (Figure 5). The concentrations of PHB were 9 g/L and 13 g/L when the cells were grown at 15°C and 25°C, respectively, under the same conditions. These data indicate that the yields of PHB depend critically on growth temperature.

**Discussion**

Because of the efficient and diverse biosynthetic capabilities of psychrophils at relatively low temperatures, these organisms provide an important resource for biotechnology (Moyer and Morita, 2007). Soil samples acquired at circumpolar latitudes are rich in psychrophils. For example, Jiang et al. (2008) first isolated the *P. fluorescens* strain A2a5 from Alaskan soil, and the *P. extremaustralis* sp. nov. strain 14-3\(^T\) was isolated from an ephemeral pond in Antarctica (Lopez et al., 2009). These two psychrophils accumulate large amounts of PHB at a low temperature, strain A2a5 grew at 25°C, while strain 14-3\(^T\) could grow at a temperature range from 4 to 37°C. The nucleotide sequence of the genome of strain 14-3\(^T\) has been determined (Tribelli et al., 2012).

Regions of high mountains perennially covered with snow are another abundant source of psychrophils. The mean temperature above 2000 m on Changbai Mountain is 3.3°C (Liu 1997). We report here the isolation of a *P. mandelii* strain designated CBS-1, a facultative psychrophil, from soil located at an altitude of 2183 m. Species identification was based on sequence analysis of 16S rRNA and the results of the Biolog GN assay. Strain CBS-1 grew to high density (29.3 g/L of CDW at the stationary phase) and robustly produced PHB (76.1% CDW) at 20°C. The yields of PHB produced at 15, 20, and 25°C were significantly different. The yield of PHB produced at 20°C was significantly higher than at 15°C \( (P < 0.001) \) and 25°C \( (P < 0.05) \).

Compared with a few other *Pseudomonas* strains that produce PHB at a low temperature, strain CBS-1 is more promising for industrial use. For example, strain CBS-1 uses sucrose as its best carbon source; therefore, PHB can be produced more economically than by strain 14-3\(^T\), which requires the more costly carbon source octanoate (Lopez et al., 2009). Moreover, strain CBS-1 grew to a higher maximum cell density than strain 14-3\(^T\) (Lopez et al., 2009). Jiang et al. (2008) reported that cultivation for 4 days was required to produce the maximum cell density of strain A2a5 and PHB concentration, in contrast to 48 h for strain CBS-1. Therefore, strain CBS-1 may produce higher yields of PHB at a lower cost. Therefore, strain CBS-1 may produce higher yields of PHB at a lower cost and more efficiently than strain 14-3\(^T\) and A2a5.
Competing interests
The authors declare that they have no competing interests.

Authors' contributions
LRP, JYJ and HN carried out the strains isolation, characteristics, PHB extraction and identification, and drafted the manuscript. WXJ carried out the Biolog Gram-negative assay. YJJ carried out the 16S rRNA gene amplification. GYJ and GGF participated in the cell cultivations and 16S rRNA sequence alignment. All authors read and approved the final manuscript.

Authors' information
Rongpeng Li and Yuji Jiang co-first author.

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Author details
1 Colloge of Biotechnology and Pharmaceutical Engineering, Nanjing University of Technology, Nanjing 211800, People's Republic of China.
2 Institute of Soil Science, Chinese Academy of Sciences, Nanjing 210008, People's Republic of China.
3 Jiangsu Key Laboratory for Biomass-based Energy and Enzyme Technology, Huaiian 223300, People's Republic of China.

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References
Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410
Anza Y, Kim H, Park JY, Wakabayashi H, Oyaizu H (2000) Phylogenetic affiliation of the Pseudomonads based on 16S rRNA sequence. Int J Syst Evol Microbiol 50:1563–1569
Choi J, Lee SY (1999) Factors affecting the economics of polyalkylfarnate production by bacterial fermentation. Appl Microbiol Biotechnol 51:13–21
De Rooy SL, Wahyuni ET, Wiratni SS, Ismail J (2007) Purification and characterization of poly-hydroxybutyrate (PHB) in Cupriavidus necator. Indo J Chem 7:243–248
Dowes EA, Senior PJ (1973) The role and regulation of energy reserve polymers in microorganisms. Adv Microbiol Physiol 10:135–266
Garland JL, Mills L (1991) Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. App Environ Microbiol 57:2351–2359
Hollenses PA (1985) Applications of PHB: a microbially produced biodegradable thermoplastic. Phys Technol 16:32–36
Huang R, Reusch RN (1996) Poly(3- hydroxybutyrate) is associated with specific proteins in the cytoplasm and membranes of Escherichia coli. J Biol Chem 371:22196–22202
Jiang Y, Song X, Gong L, Li P, Dai CC, Shao WL (2008) High poly (β-hydroxybutyrate) production by Pseudomonas fluorescens A265 from in expensive substrates. Enzyme Microb Technol 42:167–172
Kimura H, Yoshida Y, Doi Y (1992) Production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) by Pseudomonas acidovorans. Biotechnol Lett 14:445–450
Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5:150–163
Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley, Chichester, pp 115–175
Lee SY, Choi JI (2004) Polyalkylfarnates: biodegradable polymer. In: Demain AL, Davies JE, Atlas RM, Cohen G, Hershberger CL, Hu WS, Sherman DH, Wifson RC, David JH (eds) Manual of industrial microbiology and biotechnology, 2nd edn. American Society of Microbiology, Washington, pp 616–pp 624
Linko S, Vaferi H, Seppala J (1993) Production of poly-β-hydroxybutyrate on lactic acid by Alcaligenes eutrophus H16 in a 31 bioreactor. Enzyme Microb Technol 15:401–406
Liu Qi (1997) Structure and dynamics of the subalpine coniferous forest on Changbai mountain, China. Plant Ecol 13297–105
Lopez NI, Pettinari MJ, Stackebrandt E, Tribelli PM, Potter M, Steinbuchel A, Mendez BS (2009) Pseudomonas extremaustralis sp nov, a poly(3-hydroxybutyrate) producer isolated from an Antarctic environment. Curr Microbiol 59:514–519
Magee CM, Rodeheaver G, Edgerton MT, Edlich RF (1975) A more reliable Gram staining technique for diagnosis of surgical infections. Am J Surg 130:341–346
Meyer C, Moita R (2007) Psychrophiles and psychrophilts. Encyclopedia of Life Science. doi:10.1002/0470074007.902a0004002.pub02
Page WJ (1992) Production of poly-β-hydroxybutyrate UWM in media containing sugars and complex nitrogen sources. Appl Microbiol Biotechnol 31:171–121
Spiekermann P, Rehm BHA, Kalscheuer R, Baumester D, Steinbuchel A (1999) Assesive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. Arch Microbiol 17:73–80
Steinbuchel A, Valenin HE (1995) Diversity of bacterial polyhydroxybutyrate acids. FEMS Microbiol Lett 128:219–228
Steinuchel A, Byrom D (1991) Biomaterials: Novel materials from biological sources. Stockton, New York, pp 123–213
Tang L, Luo GY (2008) Study on nitrogen removal characteristics of an aerobic denitrifying bacterium Rhodococcus strain T7. Environ Ecol Three Gorges 1:24–27
Tribelli PM, Lustman LJR, Catone MV, Martino CD, Revale S, Mendez BS, Lopez NI (2012) Genome sequence of the polyhydroxybutyrate producer Pseudomonas extremaustralis, a highly stress-resistant Antarctic bacterium. J Bacteriology 194:2381–2382
Verlinden RA, Hill DJ, Kenward MA, Williams CD, Piotrowska-Seget Z, Radecza IK (2011) Production of polyalkylfarnates from waste frying oil by Cupriavidus necator. AMB Express 1:11–19
Yue WJ, Liu C, Zhang XF (2007) Accumulation of poly-β-hydroxybutyrate acid (PHB) by Rhodopseudomonas palustris. Environ Sci Technol 40:26–28

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