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Research article

Selection of polyhydroxybutyrate-producing bacteria and their polyhydroxybutyrate production using cassava and glycerol as carbon sources

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Abstract

Importance of the work: Polyhydroxybutyrate (PHB) produced by microorganisms has desirable properties and has been applied across various fields. However, its use in bioplastic production remains costly compared to synthetic plastics, mainly due to the expensive substrates and downstream processing. Using low cost raw materials and bacterial strains is a potential strategy to reduce production cost.

Objectives: To obtain effective PHB-producing bacteria and to utilize various substrates as carbon sources for production.

Materials and Methods: PHB-producing bacteria were obtained from cassava plants and their associated soil rhizosphere. Initially, all bacterial isolates were investigated qualitatively for PHB production using staining dyes and estimated quantitatively based on extraction using chloroform. Subsequently, various substrates with different concentrations were used to cultivate these bacteria for their growth and PHB production. The PHB extracts from the bacteria were investigated using gas chromatography-mass spectrometry. The effective PHB-producing bacteria were identified to the genus and species levels using 16S rRNA gene sequencing.

Results: The PHB-producing bacteria isolated from the plant and soil samples had stained granules inside their vegetative cells and fluorescent colonies growing on medium under ultraviolet light. Quantitatively, the bacterial isolates CAD2 and CAD9 preliminarily cultured with glucose had higher contents of PHB extracted using chloroform than the other PHB-producing isolates. Application of various substrates to the culture isolates CAD2 and CAD9 on 1.5% (weight per volume) starch and cassava pulp produced the highest mean contents of PHB (367.75 ± 7.58 and 126.94 ± 10.35 g/L/g biomass, respectively). On the other hand, supplementation of glucose increased the number of bacteria more than it did PHB production. The isolates CAD2 and CAD9 were identified as *P. aryabhattai* and *P. filamentosa*, respectively.

Main finding: Utilization of various substrates, especially cassava pulp, enhanced PHB production, including the growth of the bacteria.

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Introduction

Many types of non-degradable and single-use plastic manufactured using non-renewable raw materials (especially the petroleum group) have broad impacts on the environment. These petroleum plastics consist of polyethylene terephthalate, high, low and linear-low density polyethylene, polyvinyl chloride, polypropylene and polystyrene (Materić et al., 2020). Most plastic garbage is disposed of as landfill; this can result in the spread of plastic micro particles or nano particles into the environment and water sources (Yeo et al., 2017; Materić et al., 2020).

Bioplastics containing degradable materials can be used as substitutes to reduce the dangers from pollutants released from petroleum plastics. Bioplastics can be decomposed of using biological methods that are based on natural microorganisms such as algae, bacteria, and fungi (Vroman and Tighzert, 2009). Degradable plastic can be manufactured from agricultural materials such as starch, cellulose, chitosan and protein extracted from renewable biomass, including products of microorganisms such as polyhydroxybutyrate (PHB) (Azahari et al., 2011). PHB as a member of the polyhydroxyalkanoate (PHA) group has the properties of a thermoplastic and aliphatic polyester comprising short polymer chains of functional groups, with the ester linkage group (−COOR) and methyl functional group (CH_3) (Yeo et al., 2017). PHB produced by microorganisms has a good combination of mechanical, thermal, biological and surface properties, including crystallinity, excellent gas barrier characteristics and a polypropylene like physical structure (Sirohi et al., 2021a). Therefore, it has been applied in many fields such as the medical sector, packaging industries, nanotechnology and nanomaterial production, the agrochemical and fertilizer sector including other agricultural systems and in biomedical devices (Sirohi et al., 2021b).

To date, the cost of PHB for the production of bioplastics is greater than that of synthetic plastics due to the high cost in part of the production process that includes the cost of the substrate and downstream processing (Osman et al., 2016; Sirohi et al., 2021b). Agricultural matter and microbial products are alternatives that can be applied as sources of low-cost raw materials for producing biodegradable plastics, with the resultant biopolymer being obtained from bacteria by cultivation with several basic substrates such as agricultural materials and industrial byproducts supplemented in a culture medium (Rodriguez-Perez et al., 2018; Sirohi et al., 2021b). Cassava is a major feedstock for the starch industry and is used to manufacture many food materials, with the byproducts after starch processing including dried cassava pulp and wastewater (Balagopalan et al., 1988). Starch and cassava pulp, which are capable of being used as carbon sources, are converted into subunits of many sugars by saccharification from the enzymes in biological processes or by chemical pre-treatment (Balagopalan et al., 1988). Furthermore, the cost of PHB can be reduced by incorporating microbes with several simple materials such as rice straw, sugarcane bagasse, cassava, molasses, glycerol, CO_2 and whey (Kopp et al., 2018; Naitam et al., 2022).

Glycerol ($C_3O_3H_8$) is generated as a byproduct in the biodiesel production process. The components of glycerol are sugar alcohol with low toxicity that is a colorless, odorless and viscous liquid that can be applied as a feedstock in microbial conversion (Naik et al., 2018). Many microorganisms can synthesize valuable products by using glycerol as culture substrate such as *Saccharomyces cerevisiae*, *Yarrowia lipolytica*, *Klebsiella pneumoniae*, *Clostridium butyricum*, *C. beijerinckii* and *Escherichia coli* (Dobson et al., 2012; Naik et al., 2018). They can metabolize glycerol as a carbon source in a synthetic medium using fermentation and convert the glycerol into valuable substances (Chen and Liu, 2016).

Over 75% of Gram-positive and Gram-negative bacteria can synthesize intracellular PHB granules (Aysel et al., 2002; Sindhu et al., 2011). Such granules contain water-insoluble polymers in the cell cytoplasm that store carbon groups as reserve energy for the bacteria (Anderson and Dawes, 1990; Aysel et al., 2002). PHB is generated under conditions of excess carbon, low nitrogen and a scarcity of other growth factors (Sindhu et al., 2011). Many sugar types, such as glucose, fructose, and sucrose, supplemented in the culture medium can be utilized as substrates for producing PHB (Wu et al., 2022). Reserved energy granules that form during the growth phase can accumulate within bacteria at up to 90% of the cell dry weight (Madison and Huisman, 1999). PHB-producing bacteria have been reported such as *Azobacter* spp., *Bacillus* spp., *Pseudomonas* spp., *Alcaligenes eutropha*, *Ralstonia eutrophes*, *Rhodococcus* spp., *Staphylococcus* spp., *Rhizobium* spp. and *Micrococcus* spp., (Aysel et al., 2002). Cassava that accumulates a large amount of starch in underground tubers (Tiwari et al., 2015). Thus, associated microorganisms in the plant can consume several essential substances from the plant, especially starch, as their nutrient sources for bacterial cell activity and convert this bacterial reserved energy source (Okon et al., 2024).

Cheaper substrates can be applied to bio-synthesize PHB to increase the value of industrial and agricultural byproducts.

Degradable PHB plastic is environmental-friendly and can be synthesized using many strains of bacteria; however, the high cost of PHB, due to the costs of substrates and downstream processing, have limited the utilization of those bio-plastics (Zhang et al., 2022). The selection of raw materials and bacterial strains are options to reduce the cost of PHB production. Therefore, the current research aimed to obtain efficient PHB-producing bacteria from utilizing cassava and glycerol as carbon sources for PHB production.

Materials and Methods

Isolation of bacteria

Samples of cassava tuber and the associated soil rhizosphere were collected from crop fields (14.89100°N; 102.12559°E). The soil samples (each 25 g) were weighed and then suspended in 225 mL of 0.85% (weight per volume; w/v) sodium chloride solution followed by making ten-fold serial dilutions. Cassava tuber samples with roots and leaves were cut using a knife into small pieces (each approximately 25 g). The plant samples were sterilized by soaking in 7% (w/v) chloramine T solution for 5 min, 70% ethanol for 5 min and then washing with sterile water (Costa et al., 2012). The success of the sterilization was using nutrient agar (peptone 5.0 g/L; yeast extract 3.0 g/L; agar 15.0 g/L), and then each sterilized sample was crushed into a plant extract suspension with 225 mL of 0.85% (w/v) sodium chloride to make the serial dilution. All diluents were spread on nutrient agar and incubated at 30°C for 24−48 hr. Different colony morphologies of bacteria were selected and purified to a single colony on fresh medium.

Qualitative polyhydroxybutyrate screening

Sudan black B staining

All bacterial isolates were cultured on nutrient agar supplemented with 1% (w/v) glucose (Cánovas et al., 2021) and incubated at 30°C for 24−48 hr. After incubation, intracellular PHB granules of bacteria were detected using the following procedures. Bacterial colonies were smeared on a glass slide, followed by air drying and heat fixing. The smears were fully flooded with Sudan black B solution for 10 min, rinsed with water and then re-stained with 0.5% (w/v) Safranin O solution for 5 s. Intracellular PHB granules presenting as a black shade when observed under light microscopy using a 100× objective lens were considered as a positive result (Jape et al., 2014).

Nile red colony staining

All bacterial isolates were grown on nutrient agar supplemented with 1% (w/v) glucose and 0.5 µg/mL of Nile Red by incubating at 30°C for 24−48 hr. A positive result of PHB-producing bacteria was indicated by pink or orange fluorescent colonies under ultraviolet (UV) light on the medium (Spiekerman et al., 1999).

Quantitative polyhydroxybutyrate estimation

Preparation of bacteria

All bacteria containing intracellular granules were selected to investigate the quantitative content of PHB. Inocula of bacteria were prepared by culturing in nutrient broth and incubating at 120 revolutions per minute (rpm) and 30°C for 24−48 hr. Bacterial cells were harvested using centrifugation at 8,000 rpm for 15 min, followed by washing and re-suspension with normal saline solution. The turbidity of the bacterial cells was measured using a spectrophotometer at an optical density (OD) of 600 nm as 0.05 (10^8 colony forming units (cfu)/mL). The prepared inocula were transferred to nutrient broth supplemented with 1% (w/v) glucose and incubated at 120 rpm and 30°C for 24−48 hr (Cánovas et al., 2021).

Extraction of polyhydroxybutyrate

Bacterial cells were harvested using centrifugation at 8,000 rpm for 15 min. The cell pellets were successively washed with acetone followed by ethanol and then allowed to dry at room temperature for 20 min. To extract PHB, the cell pellets were re-suspended with 6% (w/v) sodium hypochlorite solution and incubated at 37°C for 2 hr. The suspensions were centrifuged at 8,000 rpm for 10 min to collect the lipid granules. Then, the sediments were washed with acetone followed by ethanol. The PHB was extracted using chloroform and the solvent was evaporated using boiling water (100°C). The dry sediments were re-suspended in concentrated sulfuric acid and heated with boiling water for 10 min, followed by cooling immediately. The concentration of the extracted PHB in sulfuric acid was measured using a spectrophotometer at an OD of 235 nm (Rehman et al., 2016; Wendy et al., 2022).

Effect of the carbon sources

Cultivation of bacteria

Various substrates comprising soluble starch, cassava pulp, glycerol and glucose were utilized as a carbon source to culture PHB-producing bacteria. Cassava pulp samples

were prepared by drying with hot air at 50°C for 24−48 hr. Then, the dried sample was finely ground and sieved to a powder. Various substrates were supplemented as carbon sources in nutrient broths with different concentrations at 0.5%, 1.0% or 1.5% (w/v). Bacterial inocula at 10% (v/v) were transferred to the broth and incubated at 120 rpm and 30°C for 24−48 hr. The PHB from the bacteria was extracted following the above procedure. The growth of bacteria was measured using the plate count technique on nutrient medium with incubation at 30°C for 24 hr and presented in units of log cfu per milliliter.

Analysis of polyhydroxybutyrate using gas chromatography-mass spectrometry

Dry PHB extract samples from bacteria were re-suspended with chloroform and then subjected to GC-MS including a DB-wax capillary column (60 m × 0.25 mm × 0.25 µm; 7890A; Agilent Technologies; USA) with helium as the carrier gas at 1.0 mL/min. The column temperature was set at 70°C, increased at 20°C/min up to 245°C and then maintained at this level for 5 min. The temperatures of the injection and detector were set at 250°C and 280°C, respectively (Li et al., 2022). Analysis of PHB amounts were compared to standard PHB (Sigma-Aldrich; Germany) (10−500 parts per million; ppm).

Identification of bacteria using 16S rRNA gene

The PHB producing-bacteria were prepared by culturing with nutrient agar and incubating at 37°C for 24 hr. The genomic DNA from the PHB-producing bacteria was extracted using a Genomic DNA Mini Kit (Blood/culture cell; Geneaid Biotech Ltd.; Taiwan). The full-length 16S rRNA gene was amplified using two primers: 20F (5'−GAGTTTGATCCTGGCTCAG−3') and 1500R (5'−GTTACCTTGTTACGACTT−3') (Brosius et al., 1981). In 100 µl of reaction mixture, there were 15−20 ng of DNA template, 2.0 µmoles each of the two primers, 2.5 units of *Taq* polymerase, 2.0 mM $MgCl_2$, 0.2 mM dNTP and 10 µL of 10×Taq buffer at pH 8.8 containing $(NH_4)_2SO_4$, which was comprised of 750 mM Tris-HCl, 200 mM $(NH_4)_2SO_4$ and 0.1% Tween 20. Polymerase chain reaction (PCR) was programmed in a DNA Engine Dyad® Thermal Cycler (Bio-Rad Laboratories; Singapore) with an initial step at 94°C for 3 min, 25 cycles at 94°C for 1 min, at 50°C for 1 min, at 72°C for 2 min and at 72°C for 3 min. The PCR product was investigated on 0.8% (w/v) agarose gel electrophoresis and purified using a GenepHlow™ Gel/PCR Kit (Geneaid Biotech Ltd.; Taiwan). The purified PCR product was stored at -20°C for further step. Then, sequencing of the purified PCR products was performed on an ABI Prism® 3730XL DNA Sequencer (Applied Biosystems; USA). The information on bacterial nucleotides was submitted to the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/), and compared for similarities with stored data using the BLAST tool and the 16S rRNA sequences of other closely related bacteria retrieved from the GenBank database. A phylogenetic tree diagram was constructed using the neighbor-joining method with bootstrap analysis (1,000 replications).

Results

Isolation of bacteria

In total, 21 bacterial isolates were obtained from the rhizospheric soil and plant samples based on their different colony morphologies on nutrient agar (Table 1). The colonies had the different colors (orange, white, yellow-green and cream) in circular and irregular forms. They were preliminarily classified into 11 Gram-positive and 10 Gram-negative isolates, based on their cell shapes being coccobacilli, bacilli, and cocci.

Qualitative and quantitative polyhydroxybutyrate

In total, eight bacterial isolates contained black intracellular granules stained with Sudan black B inside red-stained vegetative cells, preliminarily considered as PHB-producing bacteria. The incubation time of the bacteria was considered as one factor influencing the presence of granules within a cell. Specifically, the size of the intracellular granules of bacteria observed under a microscope increased after incubation for 48 hr, compared to 24 hr. In addition, seven bacterial isolates responded to Nile Red by showing a pink fluorescent colony on the medium under UV light. In the qualitative experiment, most of the PHB-producing bacteria responded to both dyes, except for the isolates CAS3, CAS5 and CAS12 that only reacted to one dye. Details of the PHB-producing bacteria isolated are presented in Table 2 and Fig.1. Based on the clear evidence of black granules inside cells, eight bacterial isolates were selected for the quantitative estimation of their PHB contents. In addition, mucoid and sticky characteristics of the bacterial colony were generally observed on the agar medium composed of glucose compared to the medium without glucose.

Table 1 Characteristics of bacterial colony and cell

Isolate ID	Colony morphology*	Cell morphology	Gram stain	Source
CAS1	Small, orange, smooth, convex, circular, entire	Coccobacilli	Negative	Plant
CAD2	Small, cream, smooth, convex, circular, entire	Bacilli	Negative	Plant
CAS3	Small, yellow-green, smooth, convex, circular, entire	Cocci	Positive	Plant
CAD4	Moderate, cream, smooth, convex, circular, entire	Bacilli	Positive	Plant
CAS5	Moderate, white, dry, raised, convex, circular, entire	Bacilli	Positive	Plant
CAS6	Small, orange, smooth, convex, circular, entire	Coccobacilli	Negative	Plant
CAS7	Small, yellow-green, smooth, convex, circular, entire	Coccobacilli	Negative	Plant
CAS8	Small, white, smooth, convex, circular, entire	Coccobacilli	Negative	Plant
CAD9	Moderate, white, dry, flat, irregular, undulate	Bacilli	Positive	Plant
CAS10	Small, white, dry, flat, irregular, lobate	Bacilli	Positive	Plant
CAS11	Small, cream, smooth, convex, circular, entire	Bacilli	Negative	Plant
CAS12	Small, cream, smooth, convex, punctiform, entire	Bacilli	Positive	Plant
CAS13	Small, yellow-green, smooth, convex, circular, entire	Bacilli	Positive	Plant
CAS14	Moderate, cream, smooth, convex, circular, entire	Bacilli	Positive	Plant
SOC2	Small, cream, smooth, convex, circular, entire	Bacilli	Negative	Soil
SOC3	Large, transparent cream, mucoid, convex, circular, entire	Bacilli	Positive	Soil
SOC5	Moderate, cream, smooth, convex, circular, entire	Bacilli	Positive	Soil
SOC6	Small, white, smooth, convex, circular, entire	Coccobacilli	Negative	Soil
SOC7	Large, cream, smooth, convex, circular, entire	Bacilli	Negative	Soil
SOC8	Moderate, cream, smooth, convex, circular, entire	Bacilli	Negative	Soil
SOC9	Moderate, cream, mucoid, convex, circular, entire	Bacilli	Positive	Soil

*Colony morphology: size, color, texture, surface appearance, elevation, shape and edge.

Table 2 Preliminary screening of polyhydroxybutyrate-producing bacteria based on qualitative methods

Isolate	Sudan Black B	Nile Red
CAS1	ND	ND
CAD2	PHB granules	+++
CAS3	ND	+
CAD4	PHB granules	+++
CAS5	PHB granules	ND
CAS6	ND	ND
CAS7	ND	ND
CAS8	ND	ND
CAD9	PHB granules	++
CAS10	PHB granules	+
CAS11	PHB granules	+
CAS12	PHB granules	ND
CAS13	ND	ND
CAS14	ND	ND
SOC2	ND	ND
SOC3	ND	ND
SOC5	ND	ND
SOC6	ND	ND
SOC7	ND	ND
SOC8	PHB granules	+
SOC9	ND	ND

ND : not detected.

Pink-red fluorescence under ultraviolet light level of Nile Red; + : minimum; ++ : moderate; +++ : maximum.

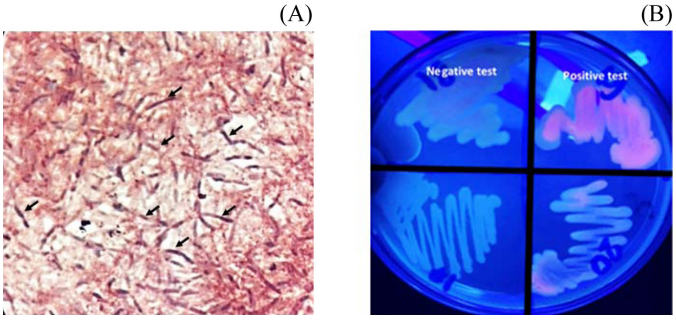


Fig. 1 Screening of polyhydroxybutyrate-producing bacteria using: (A) Sudan Black B staining showing intracellular PHB granules (indicated by arrows); (B) detection of PHB-positive colonies as pink-red fluorescence on Nile Red-supplemented medium under ultraviolet light, compared to non-fluorescent negative controls.

The PHB-producing bacteria CAD2 and CAD9, cultivated with 1% glucose as the carbon source, synthesized more PHB contents (16.96 µg/mL and 14.26 µg/mL, respectively) than the other PHB-producing bacteria (Fig. 2). The PHB extract from the bacteria was characterized as white and brown in color, with the texture of a dry sediment and powder. Thus, the PHB-producing bacterial isolates CAD2 and CAD9 were selected to estimate their ability to produce PHB and growth using various substrates as the carbon source in the culture medium.

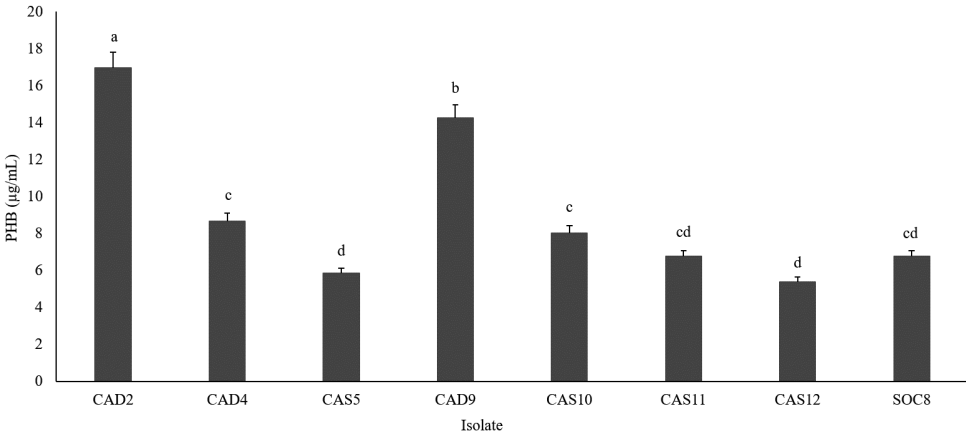


Fig. 2 Polyhydroxybutyrate (PHB) production by bacterial isolates cultured with 1% (weight per volume) glucose as the sole carbon source. Different lowercase superscripts letters indicate significant difference ($p < 0.05$) in PHB yield among isolates.

Effect of various carbon sources

Various substrates enhanced the PHB production and growth of bacteria (Figs. 3 and 4). In particular, the application of cassava pulp at concentrations of 1.0%−1.5% promoted greater production of PHB than the other substrates. Isolates CAD2 and CAD9 responded very well to starch and cassava pulp depending on the concentration of these substrates. Isolate CAD9 cultured with 0.5% and 1.0% starch for 48 hr at 30°C had a PHB concentration that was 16.01 µg/mL higher than for CAD2. Furthermore, utilization of 1.5% cassava pulp to culture isolate CAD2 for 48 hr at 30°C produced the highest PHB amount (24.72 µg/mL). Bacteria cultured with the various concentrations of starch, cassava pulp and glycerol after incubation for 24 and 48 hr produced average numbers of 7.50 log cfu/mL and 9.48 log cfu/mL, respectively. On the other hand, while the utilization of glucose as the carbon source slightly enhanced PHB production, it clearly increased the number of bacteria more than the other substrates. In particular, 1.5% glucose produced the highest average numbers of bacteria after 24 and 48 hr of incubation (8.52 log cfu/mL and 10.0 log cfu/mL, respectively).

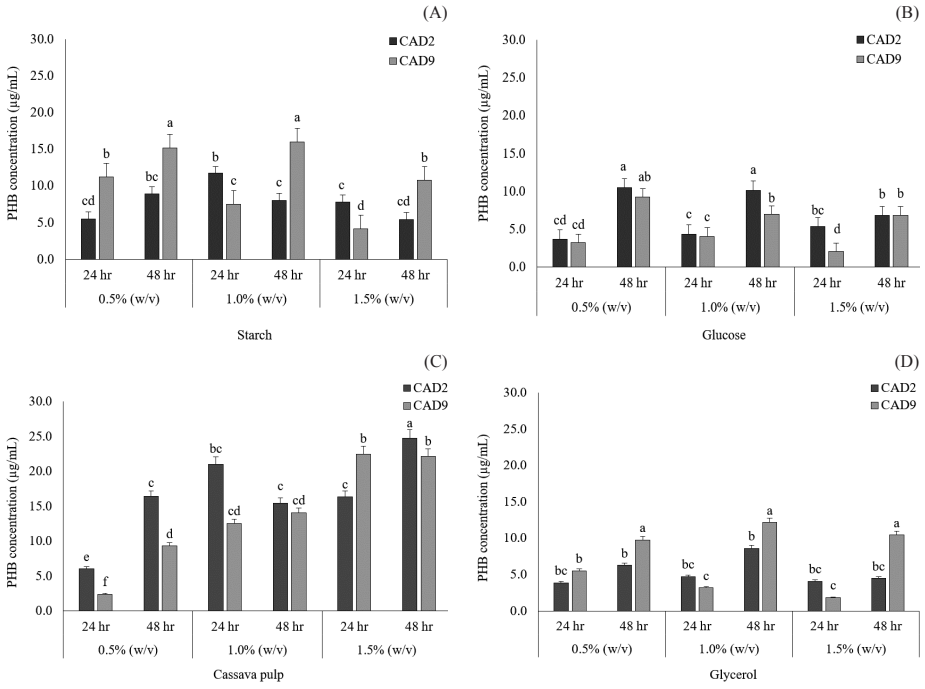


Fig. 3 Polyhydroxybutyrate (PHB) production by bacterial isolates CAD2 and CAD9 using different carbon sources and concentrations: (A) starch; (B) glucose; (C) cassava pulp; and (D) glycerol. Bars with different lowercase letters are significantly different ($p < 0.05$).

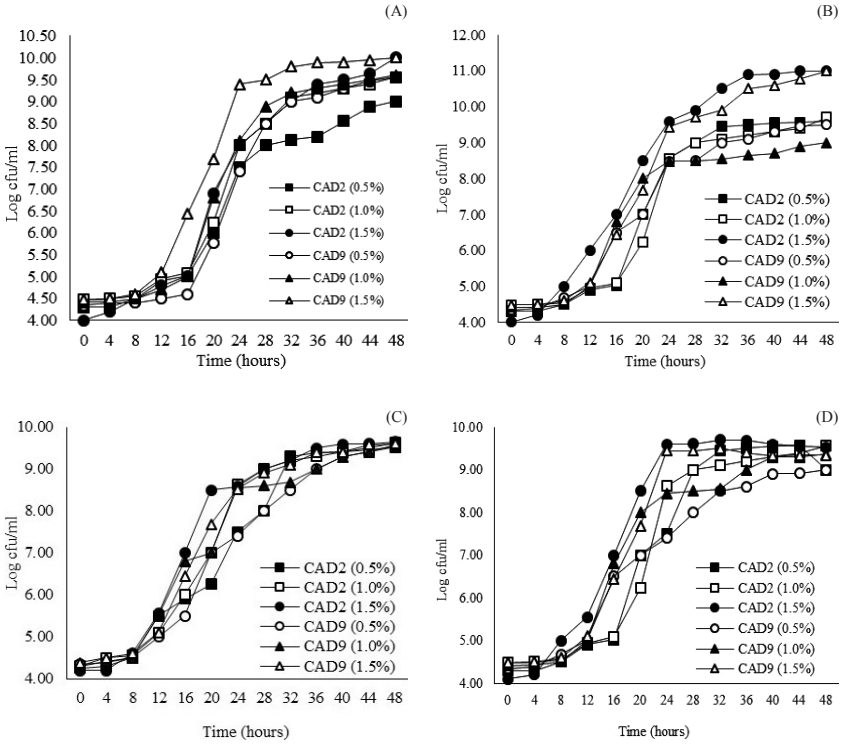
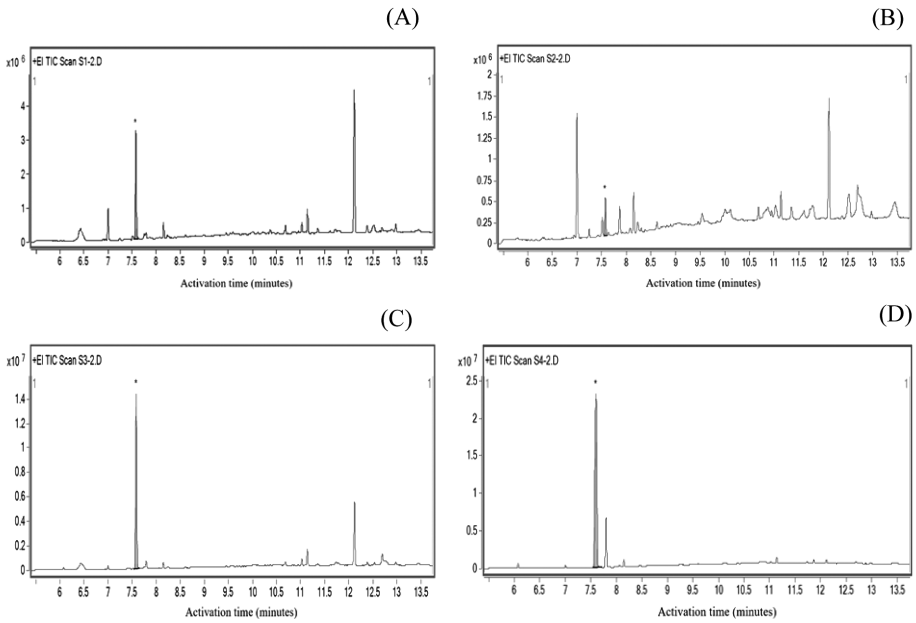


Fig. 4 Growth of polyhydroxybutyrate-producing bacterial isolates of CAD2 and CAD9 with different carbon sources and various concentrations: (A) starch; (B) glucose; (C) cassava; pulp; (D) glycerol

Analysis of polyhydroxybutyrate product using gas chromatography-mass spectrometry

The highest PHB synthesis from isolates CAD2 and CAD9 cultured based on soluble starch and cassava pulp were selected for analysis using GC-MS. The standard PHB had a retention time at 7.6 min. The results of the analysis confirmed the PHB extract from the bacteria. Isolates CAD2 and CAD9 cultured based on soluble starch produced the highest concentrations of PHB (2,554.45 ppm and 8,320.57 ppm, respectively), as shown in Fig. 5.



* Peak area of PHB

Fig. 5 Analysis of polyhydroxybutyrate (PHB) product from bacterial isolates cultivated with cassava pulp: (A) CAD2; (B) CAD9; and cultivated with starch: (C) CAD2; (D) CAD9

Identification of polyhydroxybutyrate-producing bacteria

Partial sequencing of the 16S rRNA length of the isolates CAD2 and CAD9 obtained 1,441 bp and 1,442 bp, respectively. Isolate CAD2 had the highest percentage identity (100%) with the type strain *Priestia aryabhattai* B8W22 (accession no. NR_115953), while bacterial isolate CAD9 had the highest percentage identity (99.71%) with the type strain *Priestia filamentosa* SGD−14 (accession no. KF265351). The phylogenetic analysis showed that isolates CAD2 and CAD9 were closely related to strains of *P. aryabhattai* and *P. filamentosa*, respectively (Fig. 6).

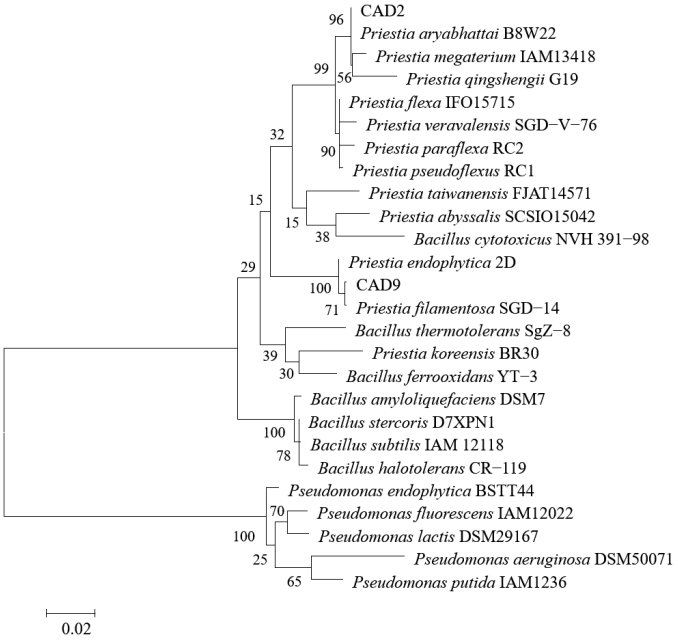


Fig. 6 Phylogenetic relationships of *Priestia aryabhattai* CAD2 and *Priestia filamentosa* CAD9 based on 16S rRNA gene sequences constructed using maximum likelihood method, where bootstrap values are based on 1,000 replicates at branch points

Discussion

Sudan Black B and Nile Red can stain fat and oil globules, including lipidic matter surrounding the cytoplasmic membrane and intracellular lipids (Jape et al., 2014). Bacterial cells absorbed Nile Red molecules during growth on culture media that displayed pink fluorescent colonies under UV light. The lipophilic fluorescent dye Nile Red has the property of nonspecific binding to hydrophobic cell structures following membrane layers or lipid-like inclusion bodies such as PHB and triacyl-glycerides (Spiekerman et al., 1999; Cánovas et al., 2021). PHB in the form of inclusion bodies can be dissolved in chloroform, but does not solubilize in water, methanol, acetone and ether, as well as being resistant to degradation with alkaline hypochlorite and can be acid-catalyzed to crotonic acid at 100–374°C (Huang and Reusch, 1996; Li and Strathmann, 2019). The growth and PHB production stage of the bacteria in the current study were similar to those reported by Rehman et al. (2016), where the stationary phase of bacterial growths produced the maximum PHB and bacterial cell mass. Over time, the bacterial numbers and PHB levels gradually decreased, especially since the nutrients were limited and accumulated metabolites, toxins and inhibitors during 57−75 hr of the experiment. After that time, the bacteria entered the decline phase with the absence of an energy supply, resulting in the release of PHB depolymerase, which hydrolyzes the polymer to water-soluble monomers or oligomeric esters (Kumaravel et al., 2010; Rehman et al., 2016). The total nitrogen content in the nutrient medium in the current experiment was 0.8%, while the carbon content was 0.5%, 1.0%, or 1.5%. The PHB-producing bacteria growing on the nutrient medium without any carbon source did not produce any black intracellular PHB granules. Thus, while excess carbon (1.0% and 1.5%) could increase the PHB production of bacteria according to the experimental results of Sindhu et al. (2011), their carbon source content was higher than that of nitrogen in their medium composition. Wendy et al. (2022) stated that glycerol affected cellular biomass and total PHA with an optimum concentration of 2%; however, increasing the concentration of glycerol up to 9% reduced the product.

The effect of the various concentrations of substrate on bacterial cells growth in culture broth did not differ; however, they did influence the accumulation of PHB in cells. Initially, the bacteria digested the macromolecules or complex structures of substrates with extracellular enzymes to make molecules available before adsorbing into their cells. Thus, the culture broths consisted of equal contents of available carbon sources, although with different types and various concentrations of substrates. Finally, the bacteria rapidly consumed the nutrients (especially glucose) to increase the number of cells as found in the exponential growth phase.

The 16S rRNA sequences of the isolates CAD2 and CAD9 have been compared to type strains of the *Priestia* genus (Gupta et al., 2020). *P. aryabhattai* and *P. filamentosa* are known as members of the family *Bacillaceae*. *P. aryabhattai* is an important strain that has been applied in many industries due to its resistance to arsenic and UV radiation,

making it an alternative to conventional, costly, metal remediation technologies (Gupta et al., 2020; Yang et al., 2024). *P. filamentosa* and *P. aryabhattai* enhanced the growth of plants under drought and nutrient deficiency and altered the root transcriptome (Khalifa and Alsowayeh, 2023). *P. aryabhattai* can be obtained from diverse sources including feces, soil, the upper atmosphere, the inner tissues of cotton plants, sea sediment and the rhizosphere of willow roots (Gupta et al., 2020). In addition, *P. aryabhattai* CAD2 and *P. filamentosa* CAD9 have been investigated for their ability to solubilize soluble starch supplemented in starch medium by exhibiting a clear zone of solubilization on medium (data not shown). Starch and cassava pulp could be degraded by α-amylase and oligo 1,6-glucosidase into sugars, such as dextrin, maltose, and glucose, which they utilized for the proliferation of cells and the precursors to generate PHB as bacterial reserve energy (Page, 1989; Kopp et al., 1999).

Conclusions

The bacterial isolates *P. aryabhattai* CAD2 and *P. filamentosa* CAD9 exhibited the potential to produce PHB using various substrates as carbon sources in the medium, especially with starch and cassava pulp supplementation. The highest PHB content was achieved with *P. aryabhattai* CAD2 cultured on 1.5% cassava pulp for 48 hr at 30°C.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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