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Polyhydroxyalkanoate Production by Actinobacterial Isolates in Lignocellulosic Hydrolysate

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Abstract: Polyhydroxyalkanoate (PHA) polymers are environmentally friendly alternatives to conventional plastics. In support of a circular bioeconomy, they can be produced by growing microbial strains in waste materials, including lignocellulosic biomass, such as Canola fines (straw). In this study, PHA and polyhydroxybutyrate (PHB) production by a selection of seven wild-type actinobacterial strains, including three strains of *Gordonia* species, were assessed. When grown in defined media and hydrolysates of Canola fines, the highest amounts of PHB were produced by *Nocardia gamkensis* CZH20^T (0.0476 mg/mL) and *Gordonia lacunae* BS2^T (0.0479 mg/mL), respectively. Six strains exhibited a substrate preference for cellobiose over glucose, xylose, and arabinose in the hydrolysates. Analysis of Fourier transform infrared spectra indicated that the strains produced co-polymers of short- and medium-chain-length PHAs. None of the core *phaABC* genes were found on defined operons in the genomes of the top PHB-producing strains (all *Gordonia* strains, *N. gamkensis* CZH20^T, and *Streptomyces* sp. strain HMC19). The *Gordonia* strains all harbored three *phaA* genes, a single *phaB* gene, and, with the exception of strain BG1.3 (with two predicted *phaC* genes), a single *phaC* gene. Predictive analyses of the proteins likely to be translated from the *phaC* genes revealed PhaC proteins of 37.7–39.2 kDa from *Gordonia* sp. strain BG1.3, *G. lacunae* BS2^T, and *N. gamkensis* CZH20^T; PhaC proteins of 106.5–107 kDa from *Gordonia* sp. strain JC51; and the second PhaC from *Gordonia* sp. strain BG1.3 and *N. gamkensis* CZH20^T, possibly representing a new class of PHA synthases.

Keywords: bioplastic; biopolymer; *Gordonia*; *Micromonospora*; polyhydroxyalkanoate synthase

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1. Introduction

Polyhydroxyalkanoates (PHAs) are non-toxic, bio-based, and biodegradable aliphatic polyesters that are environmentally friendly alternatives to conventional plastics. They are suited to a variety of applications, including food packaging materials, medical devices, cosmetics, agricultural films, and biofuels [1–5]. Microorganisms typically synthesize PHAs as carbonaceous storage polymers when the concentrations of essential nutrients, such as oxygen (O_2), and/or nitrogen (N), and/or phosphorus (P) are depleted [3,6–8]. These biopolymers are classified as either short chain length (SCL), medium chain length (MCL), or long chain length (LCL) according to their hydroxyalkanoic monomeric composition [7]. The SCL, MCL, and LCL PHAs consist of three to five carbon (C) atoms, six to fourteen C atoms, and over fifteen C atoms, respectively [2,6,7]. Polymers containing the SCL monomer, polyhydroxybutyrate (PHB), are ubiquitous in nature. The homopolymeric form of PHB is brittle, with a low impact strength, but the structural integrity can be improved by polymerization with other SCL or MCL monomers to form heteropolymers, such as poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) or 3HB and (R)-3-hydroxyhexanoate (3HHx), P(3HB-co-3HHx) [1,3,7].

The number and configuration of monomers within each PHA polymer are dependent on catalytic reactions directed by polyhydroxyalkanoate synthases (pha) encoded by

phaA, *phaB*, and *phaC* genes [3]. A range of metabolic pathways for PHA synthesis exist, utilizing either fermentable sugars or fatty acids as primary substrates [2,3,6]. Some of these pathways have been well elucidated, including pathways used by the well-studied *Cupriavidus necator* [9]. The ultimate character of PHAs can be controlled to some extent by selecting microbial species capable of producing the polymer/s of choice and utilizing the most appropriate substrates and environmental parameters to promote the desired metabolic pathways [2]. Both wild-type and genetically engineered microbial strains have been explored for PHA production in a variety of growth media, including defined media and materials, such as lignocellulosic waste and other agri-industrial residues [2,6,8,10–12].

The collective term 'actinobacteria' is often used to refer to bacteria belonging to the Class *Actinomycetes*, Phylum *Actinomycetota*. Members of this large and diverse group of bacteria include genera that harbor sizeable genomes (some in excess of 10 Mbp), which, in turn, is reflected in their great biosynthetic potential [13–16]. Actinobacteria associated with PHA synthesis include a range of *Streptomyces* [17–20] and *Rhodococcal* species [21–29]. The PHAs have been shown to provide a source of energy, as well as precursor molecules for antibiotic production by *Streptomyces* [17,19,30]. Actinobacterial PHA biosynthetic genes have also been expressed in host vectors, including *Streptomyces aureofaciens* genes in *Escherichia coli* [31,32] and *Nocardia corrallina* genes in *Rhodospirillum rubrum* [33] and Gram-negative vectors [34]. In addition to PHA production, some strains of actinobacteria are capable of degrading PHA via the action of PHA depolymerases. Strains of *Nocardiopsis aegyptia* [35], a strain of *Actinomadura* [36], and various *Streptomyces* species [37–43] have been found to be capable of depolymerizing PHB and/or PHB copolymers. An extracellular MCL PHA depolymerase from *Streptomyces exfoliatus* K10 DSMZ 41693 has been cloned and characterized [44] and predicted depolymerization genes have also been found in uncultured actinobacteria [45], including *Nocardiopsis dassonvillei* [46] and *Nocardiopsis alba* [47].

Canola fines are a source of lignocellulose that is generated year-round in South Africa, making it an ideal candidate for biorefinery applications [48]. It has been shown that high sugar yields can be achieved from Canola fines by pre-treating the biomass with low concentrations of acid (2% v/v H_2SO_4) and steam at a biomass loading of 5% (w/w), followed by enzyme hydrolysis [49]. The high sugar yields make Canola fines a promising lignocellulosic feedstock for PHA-based bioplastic production. This study focussed on comparing PHB production by a range of actinobacteria in a defined medium and the synthetic hydrolysate of Canola fines (SHCF). This study included strains of *Streptomyces* and one strain of *Nocardia*, genera previously shown to generate PHA in culture. In addition, one strain of *Micromonospora* and three strains of *Gordonia* were included in this study. An extensive literature search revealed only one previous report describing PHB production from *Gordonia*, namely, a strain of *Gordonia amarae* isolated from Patagonian soil [50], and no reports describing PHB production from *Micromonospora*.

2. Materials and Methods

2.1. Actinobacterial Strains

2.1.1. Strain Origin and Selection

Nine strains of actinobacteria (Table 1) were selected from an in-house culture collection of approximately 2000 isolates for evaluation as potential PHA producers.

Table 1. Background details of actinobacterial strains used in this study.

Genus and Species	Strain	Type and Origin	Reference
Gordonia sp.	BG1.3	Goukou river peat, Western Cape, South Africa	Unpublished
Gordonia lacunae *	BS2^T	Plettenberg bay estuarine sand, Western Cape, South Africa	[51,52]
Gordonia sp.	JC51	Deep sea sediment, Mariana Trench, Western Pacific	Unpublished
Micromonospora sp. **	BT2	Edible oil wastewater, Western Cape, South Africa	[53]
Nocardia gamkensis ***	CZH20^T	Swartberg mountain soil, Western Cape, South Africa	[53,54]
Streptomyces albidoflavus	BT3	Edible oil wastewater, Western Cape, South Africa	[53]
Streptomyces sp.	HMC19	Swartberg mountain soil, Western Cape, South Africa	Unpublished

* Culture collection strain assignments: DSM 45085^T, JCM 14873^T, NRRL B-24551^T. ** 99.27% 16S rRNA gene sequence similarity to *Micromonospora fluminis* A38. *** Culture collection strain assignments: DSM 44956^T, JCM 14299^T, NRRL B-24450^T.

The actinobacterial isolates were plated for single colonies from glycerol stocks kept at −80 °C onto International *Streptomyces* Project (ISP) #2 agar [55].

2.1.2. Staining and Microscopic Morphology

For Sudan black-stained plate cultures, dilutions of the strains were made in dH_2O [56] and 0.1 mL of each dilution was spread on 50% (v/v) ISP #2 medium agar and incubated at 30 °C until colonies > 1 mm were formed (3–6 days). The plates were flooded for 30 min with 0.02% (w/v) ethanolic Sudan black (Sigma, Darmstadt, Germany cat. no. 199664) and then excess stain was washed off with 96% ethanol [57]. The number of colonies taking up the black dye was counted on plates that contained between 20 and 100 colonies.

Separate liquid cultures were used for microscopic staining studies (Gram stain and Nile red stain), with each strain being grown for 3 days in 25% (v/v) ISP #2 liquid medium and kept at 4 °C until stained. Gram staining was performed according to a previously described method [58].

The Nile red staining and microscopy were performed at the Central Analytical Facility at the University of Stellenbosch, South Africa. The samples were vortexed vigorously and stained with 20 µL of 0.5 mg/mL Nile red (Sigma cat. no. 72485) in 100% ethanol, refrigerated at 4 °C overnight, and then sonicated for 5 min. Thereafter, 100 µL of each sample was transferred to a cell of a sterile borosilicate eight-well Nunc™ Lab-Tek™ II Chambered Coverglass (Roskilde, Denmark) and allowed to settle for 2 h. Approximately 200 µL of warm 2% (w/v) agarose (Sigma) was added to each well and allowed to solidify for approximately 15–30 min at 4 °C. Super-resolution structured illumination microscopy (SR-SIM) images were acquired with a Zeiss LSM780 with an ELYRA PS1 microscope with ZEN 2012 Version 4 software (Jena, Germany). The samples were imaged with a PlanApochromat 100×/1.46 oil objective, using a 561 nm 100 MW excitation laser. Emission was detected using a BP570-620+LP750 filter and images were captured with an Andor EM-CCD camera IXon DU 885 for SIM (exposure time of 50 ms). Z-stacks with step widths of 0.116 nm were acquired, with a 51 µm SIM grating in 3 rotations. Post-imaging processing with ZEN 2012 software was achieved with the structured illumination plugin and included noise filtering and Fourier transform sectioning. The image stacks of actinobacteria with filamentous morphologies were represented in maximum intensity projections while those with non-filamentous morphologies were represented by single-layer images to visualize internal lipid accumulation.

2.2. Growth Curves and Cultures for the Production of Polyhydroxyalkanoates

The inocula were prepared from 4–5 bacterial colonies picked from ISP #2 agar and grown in 100 mL ISP #2 broth in 250 mL flasks at 30 °C in a shaking incubator at 160 rpm for 3 days. The strains were then grown using a 5% inoculum (v/v) under the same conditions in (i) 100 mL 25% (v/v) ISP #2 broth and (ii) 100 mL SHCF. The SHCF was based on the optimized sugar composition of hydrolysates obtained from the acid extraction and enzyme

hydrolysis of Canola fines [49]. In addition to sugars, the carbon, hydrogen, nitrogen, and sulfur (CHNS) concentrations (%w/w) in the optimized SHCF were determined at the Central Analytical Facility of Stellenbosch University using an Elementar (Hamburg, Germany) Vario EL cube Elemental analyzer according to the manufacturer's instructions. Major and minor elements were quantified at the same facility using a Thermo ICap 6200 ICP-AES instrument for trace analyses while ultra-trace analyses were performed on an Agilent (Santa Clara, CA, USA) 7900 ICP-MS instrument according to the manufacturer's instructions. These results were used to formulate the inorganic fraction of the SHCF. Each liter of SHCF contained 4 g cellobiose, 6 g glucose, 10 g xylose, 2 g arabinose, 960 mg potassium carbonate (K_2CO_3), 500 mg calcium carbonate ($CaCO_3$), 240 mg magnesium sulfate ($MgSO_4$), 100 mg sodium nitrate ($NaNO_3$), 30 mg di-potassium hydrogen phosphate (K_2HPO_4), 6 mg ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), and 1 mg zinc acetate ($ZnC_4H_6O_4$).

Preliminary testing showed that the maximum biomass and PHB production of all strains took place within 5 days in 25% (v/v) ISP #2 broth, but that the strains took some time to acclimate and grow in SHCF. The PHB and biomass determination studies were therefore conducted for 5 days and 3 weeks, respectively, and results were analyzed either each respective day (Day 1–5) or week (Week 1–3). Each fermentation was conducted in duplicate. To avoid reducing the culture volume by extracting broth for daily/weekly analyses, separate flasks were inoculated for each day/week for each strain, giving a total of 10 flasks for each strain in 25% (v/v) ISP #2 broth and 6 flasks for each strain grown in SHCF. The daily/weekly growth curves were determined from 50 mL well-mixed aliquots of culture that were centrifuged for 20 min at 8000 rpm. After discarding the supernatant fluid, the pellets were re-suspended and transferred to 2 mL plastic centrifuge tubes, washed in dH_2O, re-pelleted at 12,000 rpm, and dried in an oven at 90 °C overnight. The pre-determined weights of the 2 mL centrifuge tubes were deducted from the weights of the same 2 mL centrifuge tubes containing the washed and dried pellets. The entire weight of the dried pellets from the 25% (v/v) ISP #2 broth cultures was assumed to consist of biomass but the SHCF cultures also contained varying amounts of inorganic precipitates (ppt.) from the culture medium.

2.3. Extraction of Polyhydroxyalkanoates

The PHA was extracted from centrifuged pellets of a 10 mL culture medium based on the chloroform extraction method [59]. All steps were conducted in glass tubes. Briefly, the pellets were lysed in 2.5 mL 12.5% (w/v) sodium hypochlorite (NaOCl) at 37 °C for 1 h in a heating block, then washed sequentially in 5 mL distilled water (dH_2O), 2.5 mL absolute ethanol, and 2.5 mL acetone, whereafter they were dried under ambient conditions in an extractor hood overnight. For each step, the contents were mixed by vortexing and re-pelleted by centrifugation. Finally, the PHB was extracted into 5 mL chloroform ($CHCl_3$) in a heating block (100 °C) for 10 min and dried under ambient conditions in an extractor hood overnight. Due to small sample sizes, the purity of the PHB was not measured. The multi-step chloroform extraction method is known for producing highly pure PHA (up to 99%) and is therefore used as a reference for comparison with other less cumbersome extraction methods. It was therefore assumed that the PHB was also highly pure [60,61].

2.4. Analytical Procedures

2.4.1. Determination of Polyhydroxybutyrate Concentrations

The PHB concentrations in the dried extracts were determined using a modification of a previously described method based on the absorbance of crotonic acid formed from PHB at UV 235 nm [61]. Firstly, 5 mL of conc. H_2SO_4 was added to the extracts. The tubes were then capped and heated at 100 °C in a heating block for 10 min and mixed intermittently using a vortex. The tubes and contents were allowed to cool to room temperature and mixed as previously. If absorbance readings at 235 nm were >1.0, samples were diluted in conc. H_2SO_4 until absorbance readings fell between 0.1 and 1.0 (either 1:10 or 1:100 dilutions). The PHB was quantified by comparison of the absorbance readings with those of a standard

curve prepared from different dilutions of PHB (Sigma cat. no. 363502) converted to crotonic acid in conc. H_2SO_4, as per the test samples.

2.4.2. Identification and Quantification of Sugars

The sugar utilization profiles of the strains were determined by measuring the sugars in the three-week fermentates of SHCF. Sugars were identified and quantified using high-pressure liquid chromatography (HPLC) with a Phenomenex (Torrance, CA, USA) Rezex RHM monosaccharide Hþ (8% cross-linkage) column and an Agilent Technologies 1100 series instrument (Santa Clara, CA, USA), as previously described [62].

2.4.3. Fourier Transform Infrared Spectroscopy

The PHA profiles of extracts of the strains after 2 and 4 days of growth in 25% (v/v) ISP #2 broth were assessed and compared using Fourier transform infrared spectroscopy (FTIR) using a Perkin Elmer 1000 series spectrometer (Waltham, MA, USA). The PHA biopolymers in the extracts were dissolved in $CHCl_3$ by sonicating for 5 h. About two drops from a Pasteur pipette were applied to the attenuated total reflection (ATR) element and the spectra were collected immediately after the evaporation of the solvent. The spectra were recorded between 400 and 4000 cm^{-1} at room temperature.

2.5. Genome Mining for Polyhydroxyalkalnoate-Encoding Genes

As part of previous research studies, the genomes of *N. gamkensis* CZH20^T, *G. lacunae* BS2^T, *Gordonia* sp. strain BG1.3, *Gordonia* sp. strain JC51, and *Streptomyces* sp. strain HMC19 were sequenced. For this study, the genomes were annotated using RAST (Rapid Annotation using Subsystem Technology [63] and Prokka (Prokaryotic genome annotation, Galaxy Version 1.14.6 + galaxy1) in order to identify the genes involved in PHA production [64]. The core genes involved in PHA production were targeted: *phaA*, annotated as either 3-ketoacyl-CoA thiolase or β-ketothiolase (EC 2.3.1.9); *phaB*, annotated as acetoacetyl-CoA reductase (EC 1.1.1.36); and *phaC*, annotated as polyhydroxyalkanoic acid synthase or poly(3-hydroxyalkanoate) polymerase subunit (EC 2.3.1.304). The predicted PhaC amino acid sequences were aligned using the MUSCLE web tool available from EMBL-EBI (accessed 10 September 2023) and visualized using MView 1.63. Predicted PHA synthases were aligned with and compared to the Class I-IV *PhaC* sequences of *C. necator* (Class I; AAW65074.1), *Pseudomonas aeruginosa* (Class II; QPV55394.1), *Allochromatium vinosum* (Class III; WP_012969309.1), and *Bacillus megaterium* (Class IV; AAD05260.1). PhaC protein MW was predicted using SnapGene Viewer (Version 7.0.1). All sequences identified in this study are provided in the Supplementary Materials (Figure S1).

The relevant sequence information of the most promising strains described in Sections 3 and 4 are available in Genbank (accession numbers in parenthesis):

Gordonia lacunae BS2^T genome (**NGFO00000000.1**), PhaA_1 (**WP_086534080.1**), PhaA_2 (**WP_086534118.1**), PhaA_3 (**WP_086535147.1**), PhaB (**WP_086533961.1**), and PhaC (**WP_086535406.1**);

Gordonia sp. strain BG1.3: *phaA*_1 (**OR762072**), *phaA*_2 (**OR762073**), *phaA*_3 (**OR762074**), *phaB* (**OR762075**), *phaC*_1 (**OR762076**), and *phaC*_2 (**OR762077**);

Gordonia sp. strain JC51 *phaA*_1 (**OR762067**), *phaA*_2 (**OR762068**), *phaA*_3 (**OR762069**), *phaB* (**OR762070**), and *phaC* (**OR762071**);

Nocardia gamkensis CZH20^T genome (**NZ_LWUB00000000.1**), PhaA (**WP_062970115.1**), PhaB (**WP_062970107.1**), PhaC_1 (**WP_084498899.1**), and PhaC_2 (**WP_062973753.1**);

Streptomyces sp. strain HMC19 *phaA* (**OR762078**) and *phaB* (**OR762079**).

3. Results

3.1. Staining and Microscopic Morphology of the Study Strains

All the individual colonies from each strain stained positive with Sudan black when grown on solid ISP #2 medium (Figure 1).

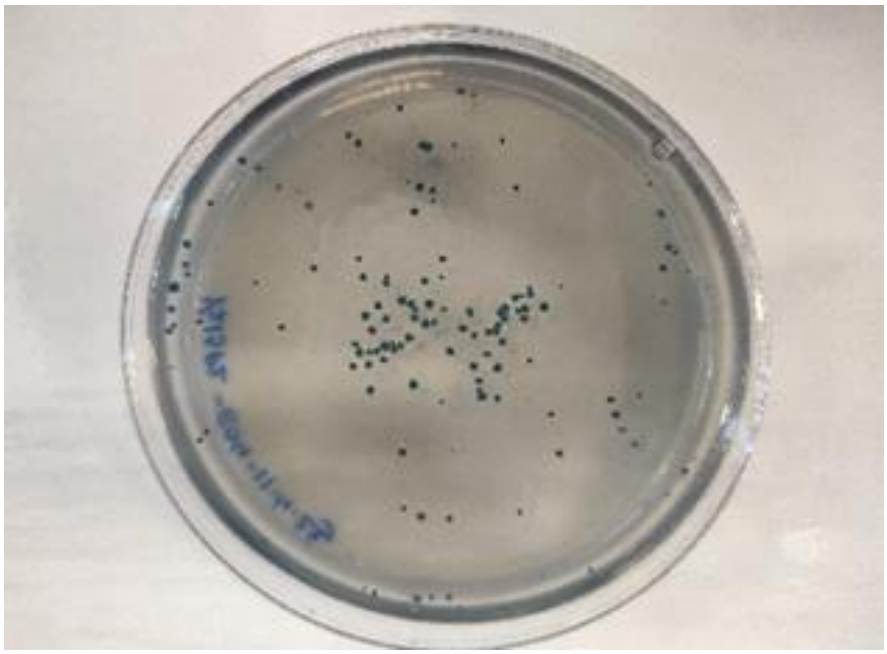

Figure 1. Colonies of *Streptomyces albidoflavus* strain BT3 stained with Sudan black.

Although the cell walls of actinobacteria are Gram-positive in nature, they may present as Gram-variable when stained. The morphologies of different genera can show considerable differences, and these may change according to the conditions under which they are cultured, including the type of growth medium, pH, temperature, time, and manner of incubation [65]. *Gordonia* species are typically short bacilli that grow confluently in a liquid medium while the other genera used in this study typically grow in filamentous forms and tend to agglomerate in 'balls' in a liquid medium. Gram stains of different study genera are shown as examples in Figure 2a–c. Intracellular PHA granules stained positive with Nile red in all the cells from each strain (Figure 2d–f).

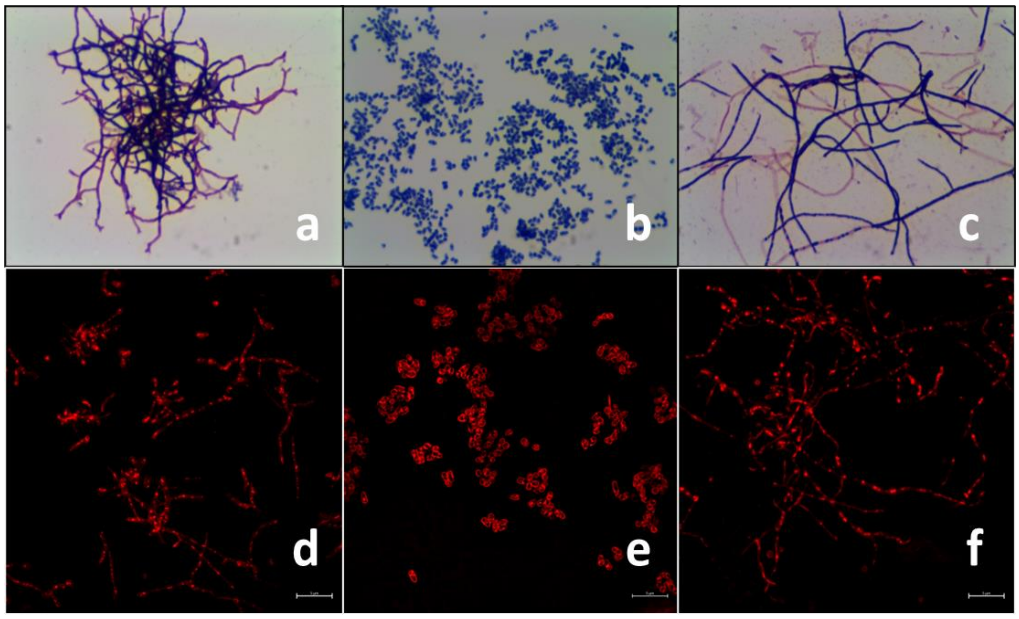

Figure 2. Gram stains, 1000× magnification (**a**–**c**) and Nile red stains (**d**–**f**) of *Micromonospora* sp. strain BT2 (**a**,**d**), *Gordonia* sp. strain JC51 (**b**,**e**), and *Streptomyces albidoflavus* BT3 (**c**,**f**) grown in liquid culture medium (ISP #2).

3.2. Growth and Polyhydroxybutyrate Production of Actinobacterial Strains

3.2.1. Cultures in 25% ISP #2 Medium

From highest (0.970 mg/mL) to lowest (0.1803 mg/mL), the biomass yields from the test strains were obtained in the order: *N. gamkensis* CZH20^T > *Gordonia* sp. strain BG1.3 > *Gordonia* sp. strain JC51 > *Streptomyces* sp. strain HMC19 > *S. albidoflavus* BT3 > *Micromonospora* sp. strain BT2 > *G. lacunae* BS2^T (Figure 3a). For PHB production, from highest (0.0476 mg/mL) to lowest (0.0124 mg/mL), the concentrations in the culture media were measured in the order: *N. gamkensis* CZH20^T > *Gordonia* sp. strain BG1.3 > *Gordonia* sp. strain JC51 > *S. albidoflavus* strain BT3 > *G. lacunae* BS2^T > *Streptomyces* strain HMC19 > *Micromonospora* strain BT2 (Figure 3b). Both the highest biomass yield and PHB concentration were measured from cultures of the *N. gamkensis* strain CZH20^T after 4 days of incubation. The biomass from this strain increased from Day 1 to Day 4 but the PHA concentration only showed a notable increase after 4 days of incubation. At this point, there was a sharp spike in the PHA concentration, which then decreased after 5 days of incubation, accompanied by a notable increase in biomass. The results suggest that nutrient limitation after 3 days caused the bacterium to produce and store copious amounts of PHB, which was then utilized as a growth substrate for continued proliferation after 4 days.

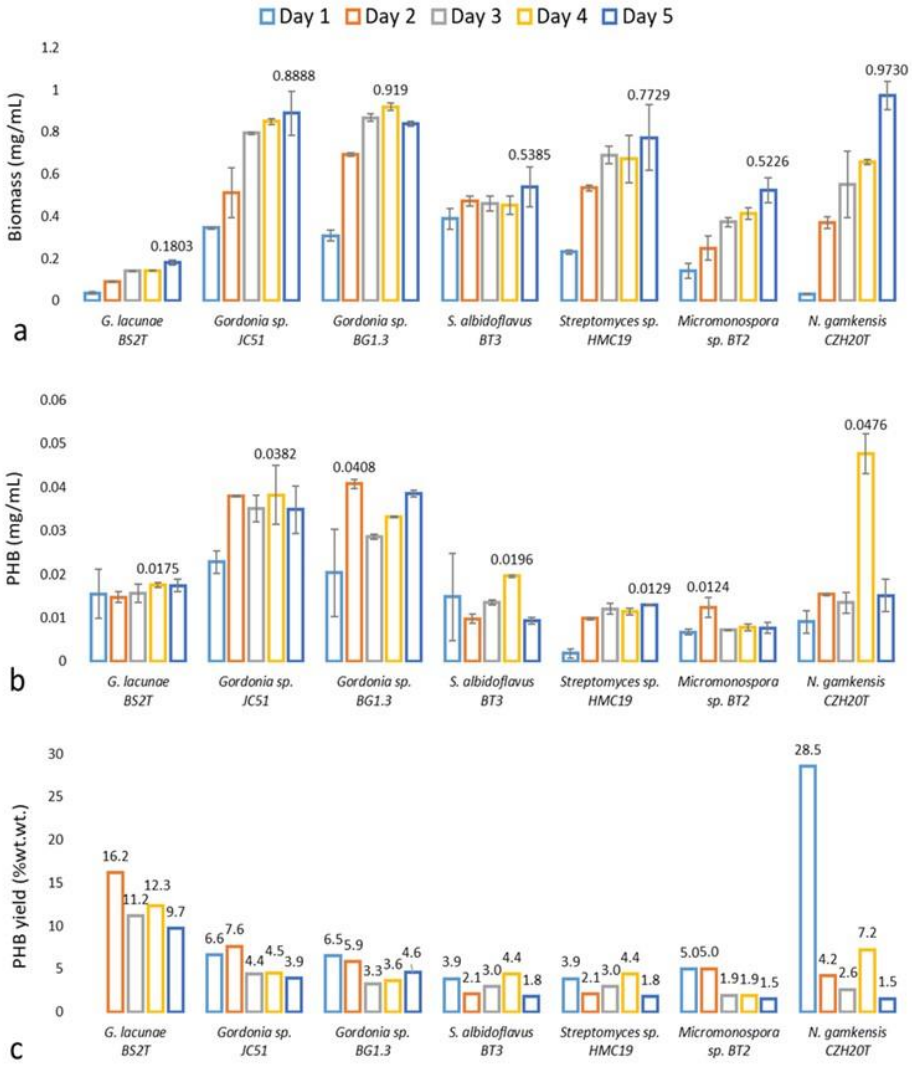

Figure 3. Biomass growth curve (**a**), polyhydroxybutyrate concentration (**b**), and yield (**c**) of actinobacterial strains grown in 25% ISP #2 medium for 5 days at 30 °C.

With the exceptions of *N. gamkensis* CZH20^T and *S. albidoflavus* BT3, the PHB concentrations in the culture media did not increase notably after 2 days. Longer incubation periods translate into higher production costs at scale so this is an important observation. The highest PHB yield (28.5% *w*/*w*) was obtained from *N. gamkensis* CZH20^T after incubation for 1 day (Figure 3c). In contrast, the biomass yield at this stage was the lowest of all strains and the PHB concentration was lower than the other strains with the exceptions of *Streptomyces* sp. strain HMC19 and *Micromonospora* sp. strain BT2.

3.2.2. Synthetic Canola Fines Hydrolysate

The trends noted when the strains were grown in 25% ISP #2 medium differed from those observed when grown in SHCF. From highest (0.6895 mg/mL) to lowest (0.1224 mg/mL), the weights of dried centrifugate from the test strains were measured in the order: *Gordonia* sp. strain BG1.3 > *G. lacunae* BS2^T > *Streptomyces* sp. strain HMC19 > *N. gamkensis* CZH20^T > *Micromonospora* sp. strain BT2 > *Gordonia* sp. strain JC51 > *S. albidoflavus* BT3 (Figure 4a). From highest (0.0479 mg/mL) to lowest (0.0025 mg/mL), the PHB concentrations in the SHCF cultures were measured in the order: *G. lacunae* BS2^T > *Gordonia* sp. strain BG1.3 > *Micromonospora* sp. strain BT2 > *N. gamkensis* CZH20^T > *Streptomyces* sp. strain HMC19 > *Gordonia* sp. strain JC51 > *S. albidoflavus* BT3 (Figure 4b).

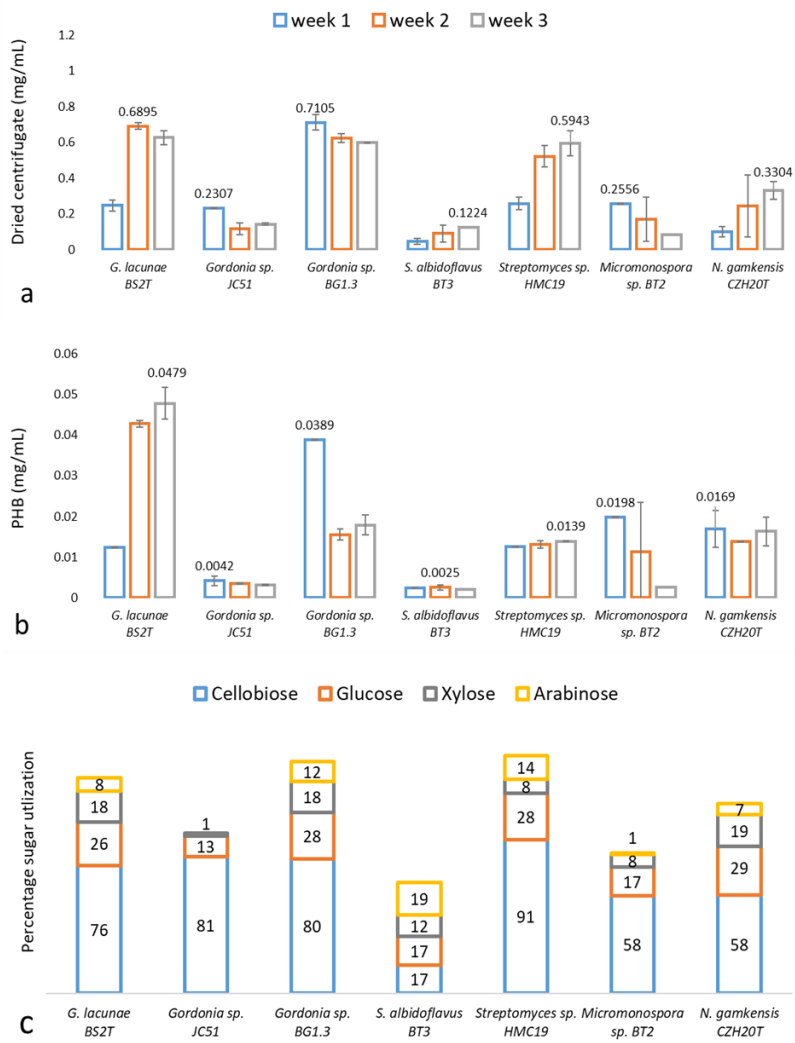

Figure 4. Biomass growth curve (**a**), polyhydroxybutyrate concentration (**b**), and sugar utilization profile (**c**) of actinobacterial strains grown in the synthetic hydrolysate of Canola fines for 3 weeks at 30 °C.

Little discernible macroscopic growth was visible in the cultures of *Gordonia* sp. strain JC51 and *S. albidoflavus* BT3, although the culture medium was slightly cloudy due to the presence of non-solubilized inorganics. It was assumed that a high portion of the dried centrifugate in the 'low biomass' cultures consisted of inorganics and that the amount that was either dissolved or ppt. in each culture varied according to the impact of microbial growth on the chemistry of the media. After 2 weeks of incubation in SHCF, the growth of *Micromonospora* sp. strain BT2 was inconsistent and, after 3 weeks, one of the duplicates, as well as a duplicate culture of *Streptomyces* strain BT3, were contaminated and excluded from this study. This had no bearing on the interpretation of the results, which were clear. Apart from *S. albidoflavus* BT3, the strains showed a substrate preference for cellobiose.

Of interest was the fact that *Gordonia* sp. strain JC51, which showed potential when grown in 25% ISP #2 medium (biomass 0.888 mg/mL; PHB 0.0382 mg/mL), did not grow well and produced very low amounts of PHB in SHCF (0.2307 mg/mL dried centrifugate; PHB 0.0042 mg/mL). Conversely, the *G. lacunae* BS2^T that did not grow well in and produced low amounts of PHB in 25% ISP #2 medium (biomass 0.1803 mg/mL, PHB 0.0175 mg/mL) grew well and produced the highest amount of PHB of all strains in SHCF (0.6895 mg/mL dried centrifugate; PHB 0.0479 mg/L). However, the maximum growth and PHB production took longer with *G. lacunae* BS2^T (2 and 3 weeks, respectively) than with *Gordonia* sp. strain BG1.3 (1 week for both maximum growth and PHB production). Although *Gordonia* sp. strain JC51 utilized cellobiose in similar amounts to the other strains of *Gordonia*, unlike the other *Gordonia* strains, it did not exhibit the ability to utilize arabinose and only utilized 1% of the xylose in the SHCF (Figure 4c).

3.3. Qualitative Analysis of Polyhydroxyalkanoates Using Fourier Infrared Spectroscopy

The functional groups of the extracted PHA polymers were confirmed using FTIR. There was a visible PHA marker ester carbonyl (C=O) band at 1719–1740 cm^{-1} in the samples produced by different strains, which showed that there was PHA accumulation in the samples. According to spectra of PHA obtained from *Gordonia* sp. strain JC51 (Figure 5a), the transmittance bands located at 1738 and 1740 cm^{-1} are attributed to the stretching vibration of the ester C=O group of MCL-PHA. The analysis of the other accompanying peaks at 2973, 2930, 2851, 1164, 1023, and 959 cm^{-1} shows that this strain was a SCL-MCL-PHA producer [66–68]. The FTIR analysis of PHA spectra produced from *Gordonia* sp. strain BG1.3 (Figure 5b), *G. lacunae* BS2^T (Figure 5c), *N. gamkensis* CZH20^T (Figure 5d), and *S. albidoflavus* BT3 (Figure 5e) showed similar peaks to those identified for *Gordonia* sp. strain JC51 strain, indicating the production of SCL-MCL-PHA. The prominent bands at 2966, 2973, 2972, 2965, 2930, 2925, 2923, 2840, 2851, and 2864 cm^{-1} resemble the C–H stretching vibrations of the methyl (CH_3) and methylene (CH_2) groups. The high intensity of the absorption peaks in this region indicates the presence of MCL-PHA [69]. Additionally, the peaks between 1164–1154, 1225–1215 and 1084–1023, and 959–971 cm^{-1} can be attributed to the presence of SCL-PHA in the polymer [66,67,70,71]. This further demonstrates that the PHA produced by the strains were SCL-MCL co-polymers.

The PHA spectra for *N. gamkensis* CZH20^T remained unaltered between Day 2 and Day 4. However, those from *Gordonia* sp. strain JC51 and *Gordonia lacunae* BS2^T strains showed a slight increase in the wavenumber of the absorbance of the carbonyl band between Day 2 and Day 4 as a result of the polymer becoming amorphous. For *S. albidoflavus* BT3, the characteristic band C=O was observed at 1740 cm^{-1} and shifted to 1724 cm^{-1} by Day 4 due to a spectral change from amorphous to crystalline [72]. It has been reported that an increase in the wavenumber of absorbance in the amorphous structure can be associated with the absence of an ordered structure and reduced hydrogen-bonding effects [73,74]. The intensity of the carbonyl marker peak increased by Day 4 due to the increased quantity of PHA. The existence of the other transmissions in the region between 1650 and1600 cm^{-1}, as observed in the biopolymer spectra, could be attributed to the interference of residual bacterial intracellular components during the extraction process or to the presence of amino acids [75–77]. The C–O stretching absorption bands in the polymer were recorded between

1215 and 1230 cm^{-1} [78]. The broad band in the biopolymers in the 3310–3422 cm^{-1} region depicted the presence of a hydroxyl group (OH) [76]. Furthermore, the polymers showed other accompanying peaks corresponding to the C–H bond in CH_2 and CH_3 groups at the band regions around 1457–1474 cm^{-1} and 1372–1390 cm^{-1}, respectively [78,79].

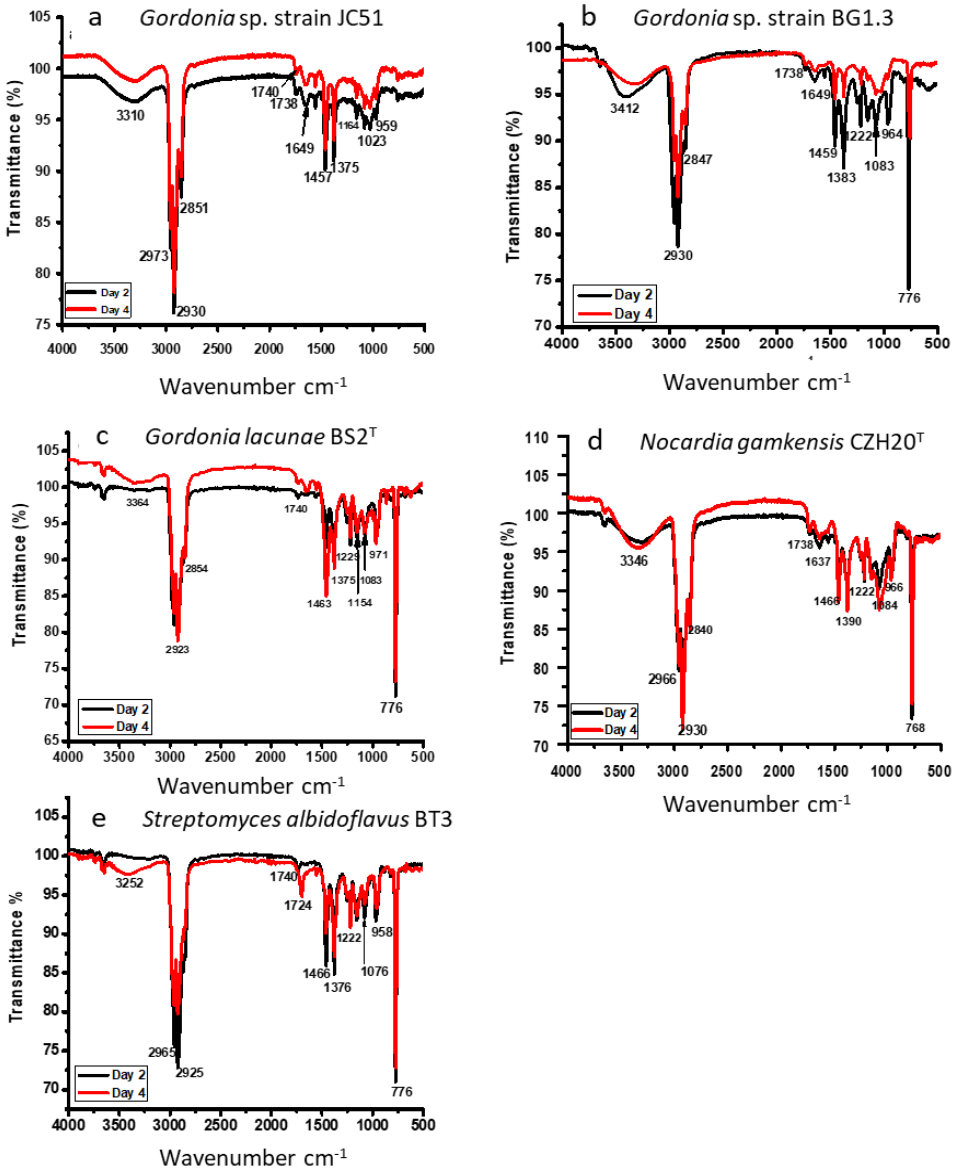

Figure 5. Fourier transform infrared spectra of polyhydroxyalkanoate biopolymers produced from *Gordonia* sp. strain JC51 (**a**), *Gordonia* sp. strain BG1.3 (**b**), *Gordonia lacunae* BS2^T (**c**), *Nocardia gamkensis* CZH20^T (**d**), and *Streptomyces albidoflavus* BT3 (**e**).

Although the majority of the strains showed positive results for the PHA marker peak, no band was observed in extracts from *Micromonospora* sp. strain BT2 or *Streptomyces* sp. strain HMC19 (Supplementary Materials, Figure S1). It was assumed that the PHA could not be detected by FTIR because of the low concentrations in these samples (Figure 3b). Based on the results, it was concluded that the FTIR method was able to confirm PHA production and differentiate between SCL-PHA, MCL-PHA, and SCL-MCL-PHA co-polymers.

3.4. Analysis of the Genomes of the Top Polyhydroxybutryrate-Producing Actinobacterial Strains

In the majority of non-actinobacterial PHA producers, the core genes (*pha*ABC) involved in PHA production are clustered in defined operons [80,81]. Analysis of the genomes

of the three *Gordonia* strains, *N. gamkensis* CZH20^T, and *Streptomyces* sp. strain HMC19 revealed that none of the core genes were located in defined operons. The *Gordonia* strains all had three *phaA* genes and a single *phaB* gene. With the exception of strain BG1.3 (with two predicted *phaC* genes), the *Gordonia* strains harbored a single *phaC* gene. Similarly, the genome of *N. lacunae* CZH20^T was predicted to contain two *phaC* genes but only single *phaA* and *phaB* genes. Amino acid sequence alignment with known PHA synthases representing the four classes highlighted the presence of the lipase box (Figure 6). The presence of multiple *phaC* genes has also recently been reported for a rare actinobacterium, *Aquabacterium* sp. A7-Y, which is not surprising since this core enzyme is considered essential for PHA production [82]. As previously reported for streptomycete PHA synthases, the conserved cysteine in the active site is replaced by a serine for the PHA synthases analyzed in this study [83]. In addition, the PhaC from *G. lacunae* BS2^T lacked the initial conserved glycine; this may be due to a sequencing error or assembly error and can only be confirmed via cloning and resequencing. The conserved aspartic acid (D_{452}) and histidine (H_{480}), which, in conjunction with the cysteine/serine (C/S_{296}) in the lipase box, form the catalytic triad, were present in all of the sequences analyzed (Supplementary Materials, Figure S2).

G Y S Q G G	*Gordonia* sp. strain JC51
G Y S Q G G	*Gordonia* sp. strain BG1.3
A W S I G G	*Gordonia lacunae* BS2^T
G W S L G G	*Nocardia gamkensis* CZH20^T
G Y C M G G	*Bacillus megaterium* Class IV PhaC
G I C Q G G	*Allochromatium vinosum* Class III PhaC
G A C S G G	*Pseudomonas aeruginosa* Class II PhaC
G F C V G G	*Cupriavidus necator* Class I PhaC

Figure 6. Identification of the proposed lipase box (GX[S/C]XG) typically associated with PHA synthases [81].

Class I and II PHA synthases (PhaC) are typically 61–73 kDa in size while, in Class III and IV, PhaC is approximately 40 kDa in size [81]. Predictive analyses of the proteins likely to be translated from the *phaC* genes in the test strains revealed PhaC proteins of 37.7–39.2 kDa from *Gordonia* sp. strain BG1.3, *G. lacunae* BS2^T and *N. gamkensis* CZH20^T, and PhaC proteins of 106.5–107 kDa from *Gordonia* sp. strain JC51 and the second PhaC from *Gordonia* sp. strain BG1.3 and *N. gamkensis* CZH20^T, possibly representing a new class of PHA synthases.

A *phaC* gene could not be identified in *Streptomyces* sp. strain HMC19. This is similar to what has been reported for *Streptomyces coelicolor* A3(2), where it was shown that the strain has the ability to produce PHA, but lacked a specific gene annotated as a PHA synthase/PhaC. A gene annotated as a hypothetical protein has been identified in the genome of *S. coelicolor* exhibiting some sequence similarity to the *phaC* gene of *Streptomyces aureofaciens* (AY032926.1) [80]. Mutation and complementation studies confirmed the role of the gene as a PHA synthase. A similar study would need to be performed on strain HMC19 in order to identify the gene encoding for its PHA synthase. Interestingly, annotation by Prokka identified the putative *phaA* as *fadA_3* and *phaB* as *fabG_4*, genes involved in triacylglycerol and fatty acid production [84,85].

4. Discussion

Actinobacteria have been screened for PHA production with microscopic or culture-dependent staining techniques; for example, actinobacterial isolates from soil samples in Brazil were screened for PHA production using Sudan black and Nile red dyes on colonies grown on N-deficient medium with glucose as the sole C source [86]. Nile red

has also been used to quantify the PHA yield in non-actinobacterial strains by measuring dye uptake using scanning electron microscopy [87]. In this study, the Sudan black plate culture staining results suggested that PHA was produced by all the cells within each actinobacterial strain population as each colony arises from the proliferation of a single cell. However, actinobacteria are known to accumulate intracellular triacylglycerols that can stain positive with Sudan black and Nile red [50,88] and, under microscopy, it could be seen that the cell walls were stained with Nile red. It was therefore concluded that, although dye uptake is a useful screening tool, it is not a suitable method for quantification of PHA in actinobacteria. This is supported by the results obtained where only 11 of 53 actinobacterial isolates identified as potential PHA producers using the Sudan black staining method were able to produce PHB [89].

Only a few strains of non-*Streptomyces* or rhodococcal actinobacteria have been definitively identified as PHA producers in culture. These include putative PHB accumulation by a strain of *Streptacidiphilus* [86]; PHB and polyhydroxyvalerate (PHV) accumulation using a variety of hydrocarbon substrates by *Nocardia asteroides*, *Nocardia globerula*, *Nocardia restricta*, *Dietzia maris*, and *Gordonia amarae* isolated from soil samples from east Patagonia [50]; *Kineosphaera limosa* gen. nov., sp. nov. isolated from activated sludge and capable of "accumulating significant amounts of PHA" [90]; accumulation of PHB and PHV by *Nocardia corallina* using glucose as a substrate [22]; PHB accumulation by *Nocardia asteroids* [91]; PHA accumulation by *Microlunatus phosphovorus*, with the highest production being measured when grown in a solution containing 4 g/L glucose and cultured under aerobic–anaerobic cycles [92]; and PHV synthesis by *Rubrobacter xylanophilus* and *Rubrobacter spartanus* grown in mineral salt medium under aerobic conditions and a variety of different carbohydrate substrates [93]. Three studies also screened actinobacteria from different origins for PHA production, but many isolates were not identified or were unidentifiable using the method/s employed [86,89,94].

In this study, from an overall perspective of stability, growth rate, and PHB accumulation, the *Gordonia* strains were the most promising candidates for PHB production. *Gordonia* are robust bacteria that have been used in a variety of industrial applications and have also been used as recombinant vectors for PHA synthesis. Arenskötte and co-workers [95] first constructed a recombinant strain of *Gordonia polyisoprenivorans* VH2 capable of PHA synthesis from alkanes. Later, recombinant *G. polyisoprenivorans* VH2 harboring plasmid pAK68 containing *phaCAB* from *Ralstonia eutropha* and plasmid pAK71 containing *phaC1* from *P. aeruginosa* were used to produce PHA under N-starved conditions [96]. To the best of our knowledge, this is only the second report describing PHB production by *Gordonia* and the first describing PHB production by *Micromonospora*. Accumulation of PHB and PHV by *Gordonia amarae* has previously been reported [50].

Apart from *S. albidoflavus* BT3, the test strains showed a substrate preference for cellobiose, although the glucose utilization may have been masked due to the degradation of cellobiose to glucose by the action of β-glucosidases [97]. The substrate preference for cellobiose has been previously described in *Streptomyces* [97,98] and it has been suggested that organisms that show higher assimilation of cellobiose over glucose are preferential candidates for lignocellulosic biorefinery applications [97]. The measured PHB concentrations in the samples were notably lower than those previously obtained from a strain of *Streptomyces* grown in various powdered agricultural wastes added to a basal salt medium: 0.89 mg/mL (cane molasses), 0.85 mg/mL (rice bran), 4.35 mg/mL (oil cake), 0.79 mg/mL (wheat bran), 0.82 mg/mL (paddy straw) [94]. Although the crotonic acid spectrophotometric method is used ubiquitously for PHB quantification, there is a possibility that it underestimated the concentration of PHB in this study, despite extensive method development. High PHB production is associated with a dark brown color in the acidified extracts, which did not appear to translate into high PHB concentrations. To obtain a straight-line standard graph, very low concentrations of PHB were required (1.0–6.0 μg/mL) and the samples were diluted in conc. H_2SO_4 to fall within this range. Secondly, the crotonic acid method only measures PHB and PHV and other forms of PHA may have been more abundant in the

cultures. Indeed, high PHV to PHB yield ratios have been measured in *Gordonia amarae* (3.50), *Nocardia globerula* (10.1), *Nocardia asteroides* (19.0), *Nocardia restricta* (11.5), *Dietzia maris* (5.0), and strains of *Rhodococcus* (2.2–13.3, n = 3) *(50)*. In *G. amarae*, PHV and PHB constituted 78% and 22% of the PHA, respectively, giving a PHB yield of 5.7% of the dry cell mass. This is similar to the yields obtained for the *Gordonia* spp. JC51 and BG1.3 and notably lower than the 9.7–16.2% obtained for *G. lacunae* BS2^T (Figure 3). The results are somewhat anomalous because the highest biomass yield but the lowest PHB concentration of the three strains of *Gordonia* were obtained from *G. lacunae* BS2^T. Although the production of PHA/PHB is invariably reported as yield (%w/w) [83] and this parameter is important from an industrial perspective, the results of this study indicate that the yield may be misleading when reported in isolation. It is therefore important to determine and report all three parameters (PHA/PHB concentration, biomass concentration, and PHA/PHB yield) when assessing microbial species for PHA/PHB production potential.

Actinobacterial PHA biosynthetic genes (*pha*ABC) have already been expressed in host vectors, including *Streptomyces aureofaciens* genes in *Escherichia coli* [31,32] and *Nocardia corrallina* genes in *Rhodospirillum rubrum* [33] and Gram-negative vectors [34]. While the results of a metagenomic study of microbial populations from a sequencing batch reactor treating landfill leachate suggested that most actinobacteria lack the full set of genes necessary for PHB synthesis [99], it is possible that these biosynthetic genes have been overlooked because actinobacteria do not share sufficient sequence homologies with those of other bacteria, as demonstrated with *Streptomyces coelicolor* and *Streptomyces aureofaciens* [80]. Indeed, Martinez and co-workers [44] cloned and characterized a novel extracellular MCL PHA depolymerase ($PhaZ_{Sex2}$) from *Streptomyces exfoliatus* K10 DSMZ 41693.

Future research will prioritize optimizing PHA production in SHCF and other agri-industrial waste hydrolysates by *Gordonia* strains. This will involve qualitative and quantitative determination of PHB and PHV production using high-performance liquid chromatography and/or gas chromatography, with specific method development based on the existing literature. Conditions such as O_2 concentration, C:N ratio, and pH will be fine-tuned to enhance PHA production. A notable example is the increase in yield of *Isoptericola variabilis* (PPLAT 012) from 12% to 46% (w/w) in Deutsche Sammlung von Microorganismen und Zellkulturen (DSMZ) medium supplemented with 12% (w/v) glucose and 9% (w/v) potassium nitrate (KNO3) at a pH of 7.0 [94].

Based on optimized conditions obtained from flask experiments, future use of bioreactors provides opportunities for increasing process control and yields and enhancing efficiencies via improved substrate utilization and downstream processing [26,27,33]. Knowledge of the genomes and predicted enzymes described in the manuscript can be utilized in future studies. The novelty shown in the core genes suggests that the PHAs may have unique characteristics [100]. Ultimately, commercial PHA production offers environmental advantages [101] but challenges, such as unstable PHA quality and market unpredictability, need to be overcome [102].

5. Conclusions

This study examines how Canola fines can be used to produce PHA, theoretically providing both environmental and economic benefits in support of a circular economy. By utilizing agricultural wastes, such as Canola fines, the reliance on fossil fuels for plastic production is reduced, which results in lower greenhouse gas emissions and aids in combating climate change. In addition, new income opportunities for farmers and agribusinesses are created, fostering rural development and agricultural sustainability.

To the best of our knowledge, this is the first time that PHA/PHB production by *Gordonia lacunae*, or any strain of *Micromonospora* species, has been described. Production of PHB by three *Gordonia* strains compared favorably or exceeded production by other strains of actinobacteria used in this study. The genomes of the *Gordonia* strains contained one to three copies of all the genes needed for PHA production (*phaA*, *phB*, *phaC*), but none were found on defined operons in the genome. *Gordonia* are robust, fast-growing members of

actinobacteria. Further studies will be conducted in the quest to take the production of PHA from *Gordonia* species from lignocellulosic waste to successively higher technological levels.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr12061112/s1, Figure S1: Fourier transmission infrared spectra of PHA biopolymers produced by: (a) *Micromonospora* sp. strain BT2, and *Streptomyces* sp. strain HMC19, Figure S2: Analysed sequences.

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