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Isolation and Identification of a Bacterial Strain Producing Poly (3-hydroxybutyrate-co-3-hydroxyvalerate) from Municipal Landfill Soil

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ABSTRACT

Conventional plastics have been a significant source of the environmental pollution, prompting considerable research into the development of biodegradable plastics using biological methods. Poly (3-hydroxybutyrate-co-3-hydroxyvalerate) has garnered particular attention due to its unique properties, including high flexibility and resistance to organic solvents. It has been demonstrated that certain microorganisms possess the intracellular capability to synthesize this biopolymer from organic waste. This study investigates bacteria that have been isolated from the Kermanshah municipal waste landfill. Among the isolates, a strain capable of synthesizing biopolymers, exhibiting high similarity to the genus Stenotrophomonas geniculata strain Flmat 1, was identified via 16S rRNA gene sequencing. In order to verify the production of the biopolymer, FTIR and ^{1}H-NMR analyses were employed. The results have shown that the isolated bacteria is capable of producing PHBV from unrelated carbon sources from waste food and producing 2.835 g/L biopolymer with the yield of 0.52 g PHBV per gram of CDW using waste food, at the pH of 9, temperature of 33°C, nitrogen concentration of 13.25 g/L and glucose concentration of 27.71 g/L.

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1. Introduction

Petroleum-derived plastics are indispensable for modern life, enhancing comfort and efficiency. They are attractive due to their diverse qualities, such as being lightweight- which facilitates its transportation- durable, and inexpensive. Synthetic plastics are integral to many sectors, including food packaging, household appliances, industrial tools, furniture, and medical devices. However,

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escalating the production and consumption of petroleum-based plastics has led to the massive accumulation of recalcitrant waste, causing severe environmental degradation. Most synthetic polymers are stable and non-biodegradable, and their production depletes fossil fuel reserves. To address these challenges, researchers are working to produce biodegradable plastics from renewable resources [1-3].

Biodegradable polymers have physicochemical properties similar to those of conventional plastics, but they are environmentally friendly, derived from renewable resources, and biocompatible [1]. These materials can be fully decomposed by soil, marine, and sewage microorganisms, and represent a suitable alternative to petroleum-based polymers [3]. Biopolymers are generally categorized into three groups: Starch-based biodegradable plastics, which serve as fillers and cross-linking agents blended with synthetic plastics (e.g., starch-polyethylene); microbial-produced polymers, such as polyhydroxyalkanoates (PHAs); and chemically synthesized renewable polymers, including polyglycolic acid, polylactic acid, polyvinyl alcohol, and polyethylene oxide [1]. Polyhydroxyalkanoates (PHAs) are intracellular polyesters in which various microorganisms, including bacteria, produced them as aggregate into granules to store carbon and energy under conditions of excess carbon and limited nutrients (e.g., phosphorus, oxygen, and nitrogen) [4]. They are reusable, recyclable, compostable, and they decompose without releasing toxic byproducts. However, the high production cost of biopolymers relative to that of synthetic plastics is a major barrier to their commercialization.

Consequently, significant research has focused on isolating novel microbial strains that utilize low-cost substrates and optimizing extraction methods to reduce costs [5-7]. Inherently degradable plastics act as organic waste after use. Research on PHAs has demonstrated their biodegradability, biocompatibility, thermoplasticity, and cost-effective production. Among the studied PHA polymer family, poly(3-Hydroxybutyrate-co-3-Hydroxyvalerate) (PHBV) is a superior member. It consists of two parts i.e., HB and HV that give superior characteristics to this polymer, for example, strength is caused by HB and flexibility by HV. PHBVs exhibit a number of favourable properties which make them particularly well suited for biomedical applications. These properties include non-toxicity, biocompatibility, thermoplasticity, and resistance to various organic solvents and ultraviolet radiation. Furthermore, the isotactic nature of the polymer makes it insoluble in water. Finally, the material is attractive due to its versatility. It can be synthesized by different bacteria in an specific nutrient environment. It is also categorized by its demonstrated biodegradability and biocompatibility with an assortment of cell types [8]. The production costs of these biopolymers depend upon several factors, including the efficiency of the microbial strain, as well as the costs of obtaining the substrate, operating parameters of the fermentation process (e.g., temperature, aeration), and downstream processing steps. In order to address the high costs of pure carbon sources (e.g., glucose, sucrose), there has been an increasing use of recombinant microorganisms or wild strains capable of utilizing inexpensive carbon sources [1, 9].

This study investigates screening and identifying a new bacteria isolated from the Kermanshah municipal landfill and examines its capability to produce PHBV from waste substrates. The strain, capable of synthesizing biopolymer and producingbiopolymer was identified via 16S rRNA gene sequencing and characterized using various analytical techniques.

In the present study the *Stenotrophomonas geniculata* Flmat 1 species was introduced for the first time as a PHBV-producing species that has the capacity to use simple carbon sources that are structurally unrelated to produce PHBV without adding costly precursors. It does so with relatively high yield and high content of 3HV. Moreover, in view of the elevated costs of 3HV precursors, the use of wild bacterial species that are able to produce the hydroxy valerate fraction from unrelated carbon sources (without 3HV precursors) may also be recognised as a beneficial strategy for cost reduction.

2. Materials and methods

2.1. Chemicals

Peptone from casein, yeast extract, agar–agar, and biochemistry reagents, with analytical grade, were purchased from Merck KGaA, (Darmstadt, Germany). Sodium hydroxide, Potassium dihydrogen phosphate, di-Potassium hydrogen phosphate anhydrous, Magnesium sulphate heptahydrate, Sodium dihydrogen phosphate dihydrate, and Hydrochloric acid 37%, with analytical grade, Pancreatin (protease, amylase, lipase) were obtained from Merck KGaA, (Darmstadt, Germany). Gram Stain Kit (including Crystal Violet reagent, Iodine solution, Decolorizer and Safranin), Glucose Anhydrous, Sodium chloride, Chloroform and Methanol (with analytical grade) were supplied by a local market in Iran.

2.2. Isolation of microbial species

Sampling was executed from the soil in direct contact with municipal waste. A few centimeters deep of the surface soil was removed, and soil samples were collected from the deeper layers. Approximately 1 g of soil was dissolved in 100 mL of distilled water and filtered to remove particulates. A serial dilution was performed, and the sample was inoculated onto a solid culture medium consisting of ($g \cdot L^{-1}$): glucose (30), pepton (8.5), yeast extract (4), KH_2PO_4 (0.6), $MgSO_4.7H_2O$ (1), K_2HPO_4 (2), Na_2HPO_4 (2), and Agar (15), at an initial pH of 7.0. The plates were then subjected to incubation until the manifestation of colonies. The isolation of distinct colonies was based on their morphological characteristics, including size, color, opacity, and texture. Typically, Gram-negative colonies manifested as diminutive and convex, while Gram-positive colonies exhibited substantial size, opacity, and wrinkled surfaces.

2.3. Cultivation of pure strains in the liquid culture medium

The liquid culture medium, containing the nutrients essential for the growth and reproduction of microorganisms (carbon source, nitrogen, yeast extract, and various mineral salts), with a composition identical to that of the solid medium but without agar to prevent gelation, was prepared. Following the sterilization and cooling processes, the bacterial inoculum was added to the medium and subsequently placed in a shaker at 150 rpm and 30 °C.

2.4. Screening biopolymer-producing strains

The isolates were then subjected to a screening procedure that involved the use of lipophilic staining to detect the presence of intracellular lipid inclusion bodies. A bacterial sample was subjected to heat fixation and staining in a sequential manner with Crystal Violet (1 minute), Iodine (1 minute), and Safranin (30 seconds). Samples were examined under an optical microscope. The selected strains were Gram-negative, appearing within the purple-pink spectrum.

2.5. Molecular identification

The 16S rRNA genomic region was amplified via PCR. The sequencing was carried out by Topaz Gene Co., Tehran, Iran, and the resulting sequences were subsequently compared against the NCBI database using the BLAST software to identify the strain.

2.6. Measurement of the cell growth

The assessment of the cell growth was conducted by measuring the dry weight (CDW) and optical density (OD) of cells. For CDW, a known volume of the culture was subjected to centrifugation, the upper layer was then discarded, and the pellet was thoroughly washed with distilled water prior to being dried to a constant weight. The optical density (OD) was measured at 600 nanometers (OD_{600}) using a UV2150 UNICO spectrophotometer with distilled water as the blank.

2.7. Preparation of the food waste substrate

The composition of the food waste primarily consists of rice, beans, smaller quantities of chicken bones, meat, and vegetables. Prior to the initiation of the treatment, the meat and vegetables were removed. The collected food waste was mixed, dried and powdered with a kitchen blender.

Different pretreatment methods were assessed to optimize the yield of fermentable sugars.

- Acidic Hydrolysis: The waste was subjected to a treatment with HCl (0.5%–2%) for a duration of 24 hours. Thereafter, it underwent a drying process at a temperature of 45°C, and was subsequently resuspended in water.
- Alkaline Hydrolysis: Waste samples were treated with NaOH (0.5%–2%) for 12 h at 37 °C. Thereafter, it underwent a drying process at a temperature of 45 °C, and was subsequently resuspended in water.
- Acidic-Alkaline (or Alkaline-Acidic) Treatments: Acid-treated residues were subsequently subjected to an alkaline treatment, and vice versa.
- Enzymatic Hydrolysis: the enzymatic hydrolysis of the food waste was in accordance with the method described by Hathi et al. [10]. Reducing the sugar content was quantified using the 3,5-dinitrosalicylic acid (DNS) method [11]. The enzymatic hydrolysis was found to yield the highest reduction of the sugar content and was thus selected for subsequent experiments.

To validate the results, experiments were performed at least three times.

2.8. Production and extraction of biopolymers

The selected strain was cultivated in the liquid medium. The bacteria were picked up from Petri dishes and inoculated into a 250-mL Erlenmeyer flask containing 100 mL of sterilized fermentation broth. NaOH and HCl were used to adjust the initial pH of the media. The flasks were kept in a shaker incubator at 30 °C. Subsequent to achieving an inoculum cell concentration of approximately 1 g/L in the culture media, the cells were transferred to the primary fermentation media, which contained food waste hydrolyzate in lieu of glucose. Subsequently, the flasks were maintained in a shaker incubator for a period of five days at a rate of 200 rpm and 30 °C. Subsequently, the cell was subjected to a centrifugal process at a speed of 10,000 rpm for a duration of 20 minutes, and the supernatant was removed. Subsequently, the cell biomass was subjected to digestion with an equal volume of 3% (v/v) sodium chloride aqueous solution at 50°C for a duration of one hour. Subsequent to this, the resulting solution underwent a centrifugation process and then was thoroughly washed with distilled water. The harvested biomass was then mixed with a

chloroform-methanol solution (2:1 v/v). The induction of phase separation was achieved by the addition of distilled water. The lower phase, containing the biopolymer dissolved in chloroform, was subsequently recovered using a separatory funnel via vacuum drying.

2.9. Characterization of biopolymers

The extracted polymer was characterized by Fourier Transform Infrared Spectroscopy (FTIR) (KBr pellet method), and Proton Nuclear Magnetic Resonance (^{1}H-NMR) in deuterated chloroform ($CDCl_3$).

3. Results and discussion

A total of ten bacterial strains were isolated from the Kermanshah Waste Recycling and Organic Fertilizer Production Company's municipal landfill. These strains were then examined for their capacity to produce biopolymers. Two bacterial strains (out of 10 strains) that exhibited the highest production of biopolymers were selected for further study (Figure 1).

Figure 1. Pure extracted bacterial strain.

In order to investigate growth rates, these two bacteria were cultured in a liquid medium of which the composition has been reported previously. The turbidity of the media was measured at various hours. Based on the results, the yellow bacterium showed the highest growth (Figure 2) and biopolymer production yield; thus, it was selected for further experiments.

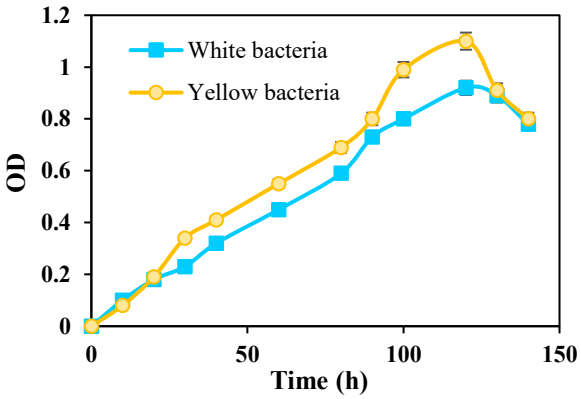

Figure 2. Growing curve of yellow and white bacteria.

As illustrated in Figure 3, the bacterial type is identified through the microscopic analysis, indicating a Gram-negative classification based on the coloration characteristics observed. The resulting phylogenetic tree and percentage of similarity of the bacterium are displayed in Figures 4 and 5 respectively. The results indicate a 98.27% compatibility between the strain and *Stenotrophomonas geniculata* strain Flmat 1 (with accession number NZ_CP140571.1). This strain produces 2.835 g/L biopolymer with the yield of 0.52 g per gram of CDW.

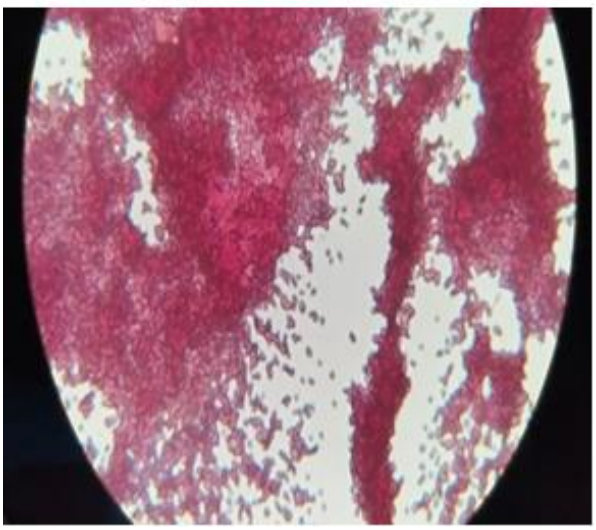

Figure 3. Gram-negative bacteria under the microscope.

To examine the capability of bacteria to use food waste, a fed-batch adaptation strategy was employed to substitute glucose with food

waste. Based on the results of this study, the yellow bacterium (on solid medium) and food waste with enzymatic pre-treatment were selected as the basis for further research.

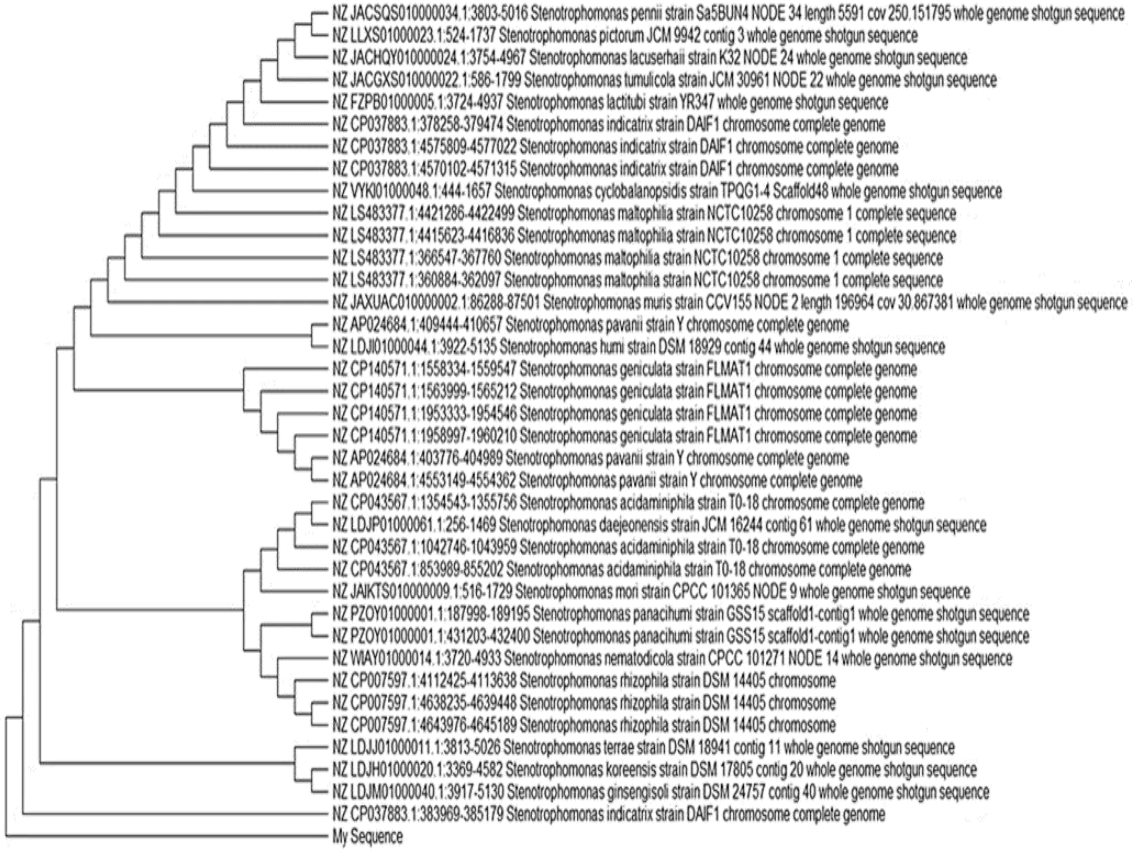

Figure 4. Phylogenetic tree of bacteria strain.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Stenotrophomonas geniculata strain FLMAT1 chromosome, complete genome	Stenotrophom...	2111	8447	95%	0.0	98.27%	4486066	NZ_CP140571.1
Stenotrophomonas pavanii strain Y chromosome, complete genome	Stenotrophom...	2106	6291	95%	0.0	98.19%	4615174	NZ_AP024684.1
Stenotrophomonas muris strain CCV155 NODE_2_length_196964_cov_30.867381, whole genom...	Stenotrophom...	2095	2095	95%	0.0	98.02%	196964	NZ_JAXUAC010000002.1
[Pseudomonas] hibiscicola ATCC 19867 G370DRAFT_scaffold00013.13, whole genome shotgun ...	[Pseudomonas...	2095	2095	95%	0.0	98.02%	1419	NZ_KB907510.1
Stenotrophomonas maltophilia strain NCTC10258 chromosome 1, complete sequence	Stenotrophom...	2095	8380	95%	0.0	98.02%	4481118	NZ_LS483377.1
Stenotrophomonas cyclobalanopsidis strain TPQG1-4 Scaffold48, whole genome shotgun sequence	Stenotrophom...	2073	2073	95%	0.0	97.69%	5377	NZ_VYKI01000048.1
Stenotrophomonas indicatrix strain DAIF1 chromosome, complete genome	Stenotrophom...	2061	8188	95%	0.0	97.53%	4639375	NZ_CP037883.1
Stenotrophomonas lactitubi strain YR347, whole genome shotgun sequence	Stenotrophom...	2061	2061	95%	0.0	97.53%	5399	NZ_FZPB01000005.1
Stenotrophomonas tumulicola strain JCM 30961 NODE_22, whole genome shotgun sequence	Stenotrophom...	2039	2039	95%	0.0	97.20%	5554	NZ_JACGXS010000022.1
Stenotrophomonas lacuserhaii strain K32 NODE_24, whole genome shotgun sequence	Stenotrophom...	2017	2017	95%	0.0	96.87%	5411	NZ_JACHQY010000024.1
Stenotrophomonas pennii strain Sa5BUN4 NODE_34_length_5591_cov_250.151795, whole geno...	Stenotrophom...	2017	2017	95%	0.0	96.87%	5591	NZ_JACSQS010000034.1
Stenotrophomonas nematodicola strain CPCC 101271 NODE_14, whole genome shotgun sequence	Stenotrophom...	2001	2001	95%	0.0	96.62%	5432	NZ_WIAY01000014.1
Stenotrophomonas rhizophila strain DSM 14405 chromosome	Stenotrophom...	1989	5969	95%	0.0	96.46%	4648976	NZ_CP007597.1
Stenotrophomonas pictorum JCM 9942 contig_3, whole genome shotgun sequence	Stenotrophom...	1984	1984	95%	0.0	96.38%	5629	NZ_LLXS01000023.1
Stenotrophomonas mori strain CPCC 101365 NODE_9, whole genome shotgun sequence	Stenotrophom...	1967	1967	95%	0.0	96.13%	5472	NZ_JAIKTS010000009.1
Stenotrophomonas humi strain DSM 18929 contig_44, whole genome shotgun sequence	Stenotrophom...	1967	1967	95%	0.0	96.13%	5644	NZ_LDJI01000044.1

Figure 5. Bacterial similarity percentage using BLAST and the NCBI database.

The glucose equivalent in the waste was determined using the DNS method. The food waste hydrolysate contains 0.82 g of glucose/g of the food waste, 0.02 g of fructose, and 0.04 g of sucrose per g of the food waste, respectively. 100 milliliters of the liquid culture solution were prepared with a replacement of the glucose in the previous medium with food waste. Initially, 20% of the glucose was replaced with food waste (normalized by glucose equivalent), and peptone/yeast extract concentrations were optimized to 4.5 and 2 g/L, respectively. The remaining material quantities were similar to those of previously media. The selected "yellow" isolate was incubated at 180 rpm and 31°C for 72 h. Subsequently, 10 milliliters of this solution were removed; the remainder was used for polymer extraction. The 10-mL aliquot was utilized for culturing on solid medium and in liquid medium. Subsequently, serial sub-culturing was performed: 5 mL of liquid culture plus colonies from solid media were inoculated into fresh broth to assess synergistic effects on yield. This process was repeated, incrementally increasing the food waste proportion, until complete substitution was achieved. This co-inoculation method yielded the highest production of biopolymers. The yields in Figure 6 illustrates the effect of pre-treatment on the production yield of biopolymers. According to the results of glucose measurement combined with the production yield of biopolymers, the enzymatic pretreatment provided the best result compared to other environments.

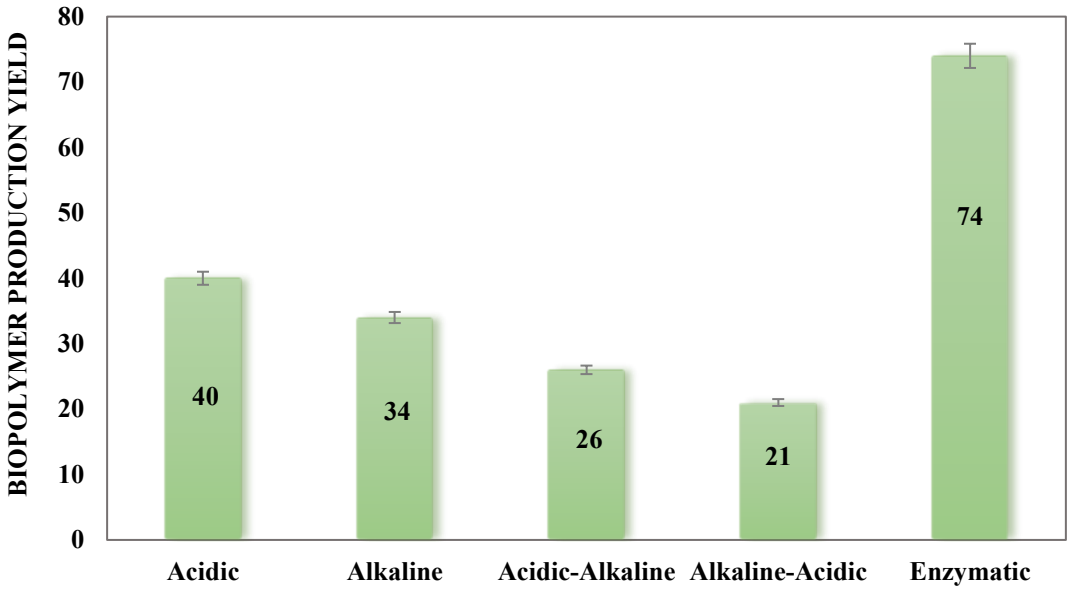

Figure 6. Investigating the effect of the pretreatment of the food waste on the biopolymer production efficiency.

For further analysis, the biopolymer was extracted and a plastic film was formed at the bottom of the container (Figure 7). Based on the FTIR spectrum (Figure 8), the peak at 1743 cm^{-1} corresponds to the C=O ester carbonyl stretching vibrations of the polymer chains. Meanwhile, C−O ester stretching vibrations appear at 1161 and 1242 cm^{-1}. At the ends of the polymer chains, alcoholic OH and acidic COOH groups are usually present. Therefore, carboxylic acid OH stretching vibrations appear as a broad peak in the 2400-3274 cm^{-1} region, and carboxylic acid C=O stretching vibrations are distinct around 1715 cm^{-1}.

Additionally, alcoholic OH stretching vibrations appear as a strong peak at 3436 cm^{-1}. Alkane C−H stretching vibrations for CH_3 and CH_2 groups appear at peaks 2854, 2923, and 2954 cm^{-1}, with bending vibrations bonds in the 1377-1554 cm^{-1} region. Alcoholic C−O stretching vibrations are evident at 1026 and 1080 cm^{-1} [12].

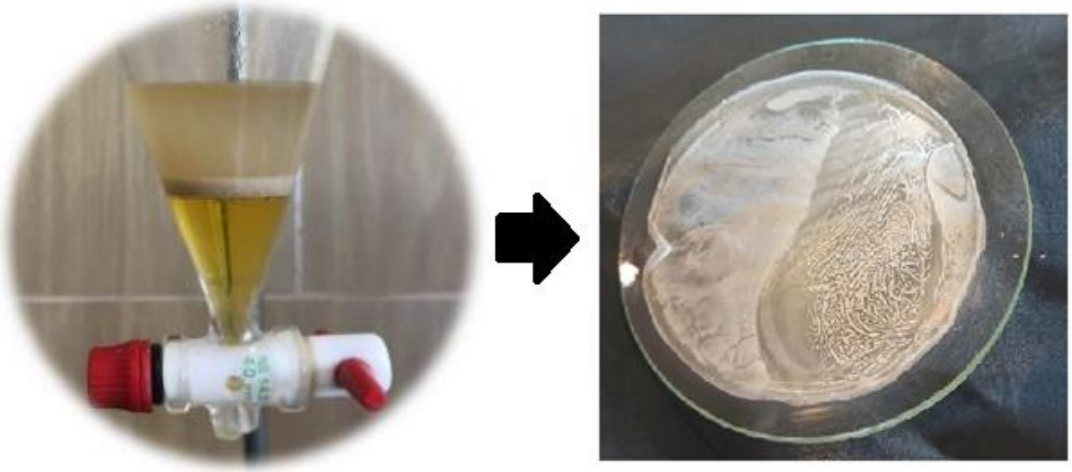

Figure 7. Extracted biopolymer.

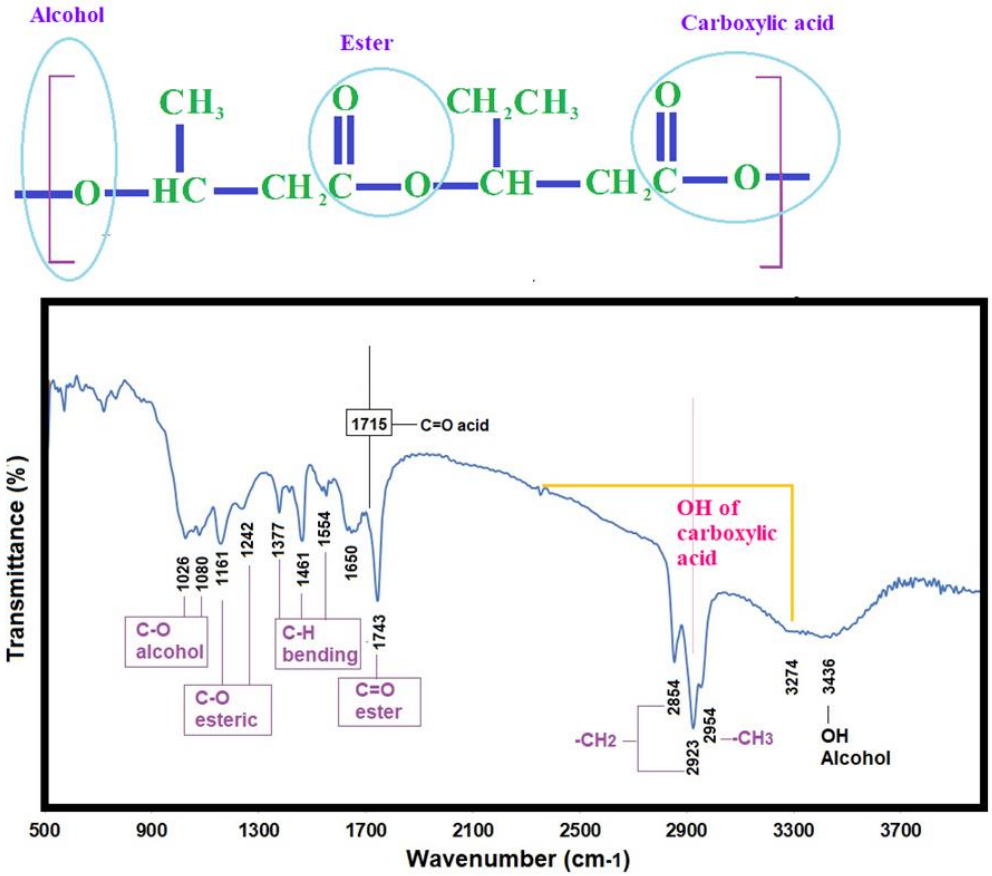

Figure 8. FTIR analysis of the synthesized biopolymer.

Based on the ^{1}HNMR analysis, the peaks corresponding to each segment of the synthesized copolymer are shown in Figure 9. The peaks obtained from this analysis

confirmed that the biopolymer is of the PHBV copolymer type [12]. The peaks corresponding to hydroxyvalerate (HV) and hydroxybutyrate (HB) monomers are shown in the table within the figure.

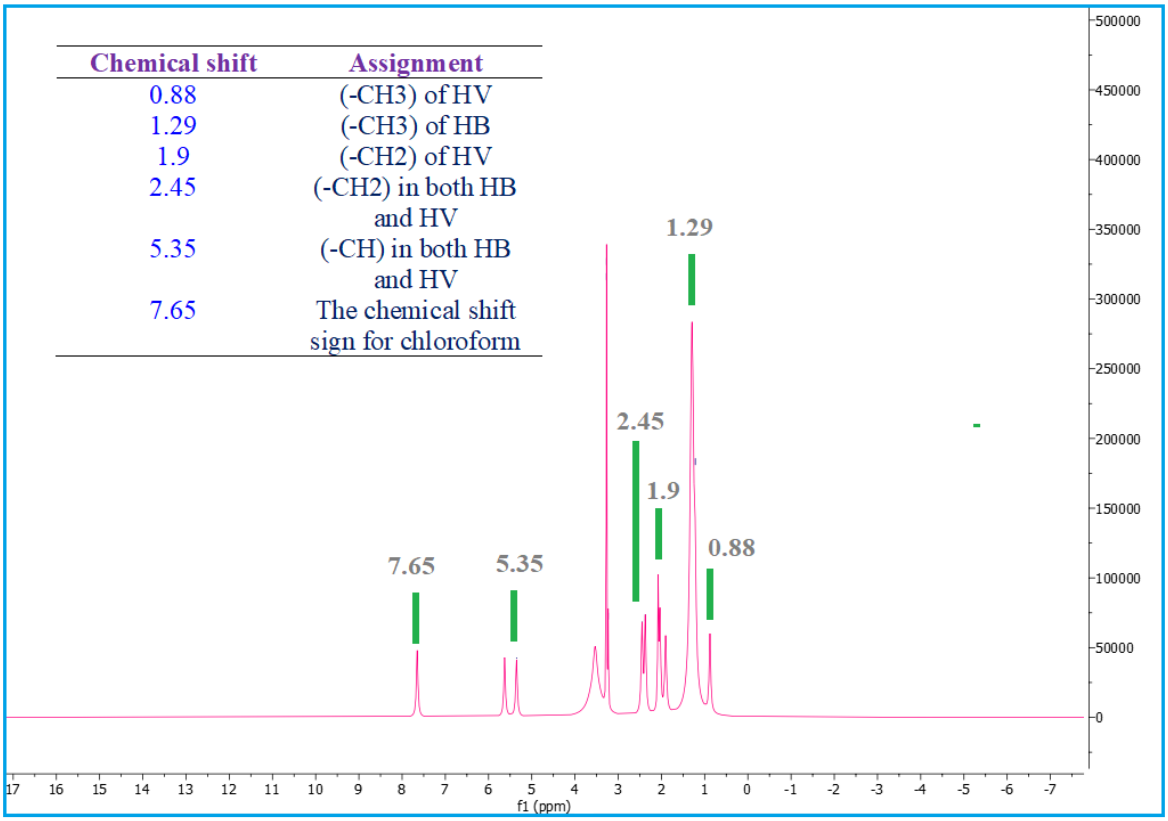

Figure 9. [1]HNMR analysis of the synthesized biopolymer.

PHBV is a short-chain thermoplastic PHA copolymer, which includes 3HV monomers within a P(3HB) structure. The alteration of crystallinity in P(3HB) can be triggered through the incorporation of 3HV monomers into its backbone. This incorporation causes a decrease in melting temperature and an increase in its biodegradability.

The glass transition (T_g) and crystallization (T_c) temperatures were determined as -16 and 89 °C respectively, as illustrated in Figure 10. Two biopolymer melting temperatures were obtained at 123.41 °C (ΔH= 0.68 W/g) and 134.24 °C (ΔH=0.68 W/g). This observation indicates the presence of two monomer groups within the PHBV structure, thereby demonstrating that the produced biopolymer consists of multiple segments.

The valerate molar ratio in PHBV was determined by calculating the area ratio of the methyl group peak of valerate at 0.88 ppm to the sum of the two methylene peaks at 2.45 ppm in NMR spectra [13]. The present study has demonstrated that the ratio of HV is approximately 42%, which is greater than other reported percentage of PHBV-producing wild microorganisms. This ratio has been documented as 19.92% by the mixed culture of granular sludge using apple fruit residues [13], 16% by *Ralstonia eutropha* cocultured with *Bacillus amyloliquefaciens* and *Bacillus subtilis* using sucrose [14], and 10% by *Haloferax mediterranei* from the hydrolysed rapeseed meal [15] respectively.

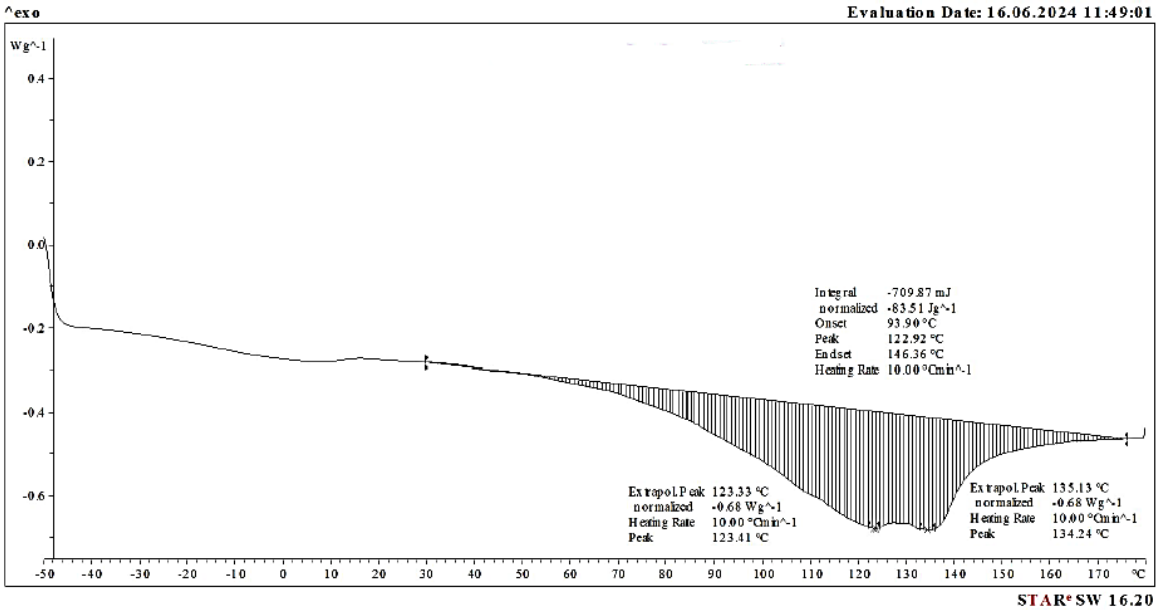

Figure 10. Differential scanning calorimeter (DSC) analysis of the PHBV produced by the Stenotrophomonas geniculata strain Flmat 1 strain

There are a few strains that can naturally produce PHBV from unrelated and single carbon sources such as simple sugars. These strains are capable of producing PHBV through both a native PHA synthesis pathway and the intrinsically sufficient propionyl-CoA generation. The predominant 3HV produced through these strains is derived from propionyl-CoA, which is generated in the methylmalonyl-CoA pathway [16, 17].

The microbial synthesis of P(3HB), the predominant form of PHA detected in bacterial species capable of producing PHAs, commences via the action of β-ketothiolase (PhaA or BktB), which facilitates the condensation of two acetyl-CoA molecules, thereby producing acetoacetyl-CoA. Subsequent to this, 3-hydroxybutyryl-CoA (3HB-CoA) is generated through the reduction process catalyzed by acetoacetyl-CoA reductase (PhaB). Subsequently, PHA synthase (PhaC) polymerizes 3HB-CoA to create P(3HB). It should be noted that the production pathway for PHBV is comparable to that of P(3HB). A key distinction, however, lies in the requirement for the generation of the 3HV monomer, which is initiated by the condensation of propionyl-CoA and acetyl-CoA. This process leads to the formation of 3-ketovaleryl-CoA, a crucial intermediate, through the action of β-ketothiolase. The conversion process is catalyzed by acetoacetyl-CoA reductase, resulting in the formation of 3HV-CoA. This compound, in conjunction with 3HB-CoA, serves as a substrate for PhaC. The acetyl-CoA levels are maintained relatively high by the modulation of flux through the central metabolic process. Conversely, the most of microorganisms maintain relatively low intracellular levels of propionyl-CoA. Consequently, the most critical metabolic step in synthesizing sufficient 3HV-CoA for the production of PHBV is ensuring the availability of the intracellular propionyl-CoA pool [16].

To date, researchers have identified five major pathways for the generation of propionyl-CoA in microorganisms, which are: the utilization of fatty acids and alcohols as secondary carbon sources, the 2-ketobutyrate pathway, the methylmalonyl-CoA pathway, which employs succinyl-CoA from the tricarboxylic (TCA)

cycle, the ethylmalonyl-CoA pathway, which utilizes 3HB-CoA, and the 3-hydroxypropionate (3HP) pathway.

To investigate the rate and type of the degradation of the polymeric films obtained in this research, a film layer was buried under soil for 3 weeks, and the degradation quality was examined at the end of each week. The degradation of this film began at the edges, and cracking was observed in its structure. Figure 11 shows the degradation of the polymeric film after approximately 21 days. The weight reduction of about 11% was observed after this time.

Figure 11. Degradation of the polymer film after three weeks.

4. Conclusions

This study demonstrates that the *Stenotrophomonas geniculata* Flmat 1, isolated from Municipal Landfill Soil, is capable of producing PHBV without precursor supplementation, marking the first report of this strain for the synthesis of PHBV. The biopolymer was successfully characterized via FTIR and ^{1}H-NMR. The results suggest that utilizing food waste as a carbon source and locally isolated bacteria offer a sustainable and promising pathway for eco-friendly bioplastic production.

Author Contributions

Hanieh Karimnezhad: Conceptualization, methodology, visualization, validation, formal analysis, writing—original draft preparation, writing—review and editing.

Farshad Rahimpour: Conceptualization, methodology, validation, formal analysis, resources, data curation, writing—original draft preparation, writing—review and editing, supervision, project administration, funding acquisition.

All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare that there is no competing interest that is relevant to the content of this article.

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