Polyhydroxyalkanoates Production from Fruit Waste Using *Bacillus* Strain from Wastewater Sludge

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Polyhydroxyalkanoates (PHAs) are biodegradable polymers produced through microbial fermentation. However, the high costs associated with traditional feedstocks and fermentation techniques limit their economic feasibility. In this study, PHA-producing strains were screened from sludge samples collected at a wastewater treatment plant in Hsinchu, Taiwan. Nile red fluorescence staining and polymerase chain reaction (PCR) were used to detect the polyhydroxyalkanoate synthase (phaC) gene fragment, leading to the selection of a high-yield PHA-producing *Bacillus* strain for further investigation. This strain can utilize various inexpensive substrates and exhibits rapid growth, enabling efficient polyhydroxybutyrate (PHB) production without the need for sterilization or costly pretreatment processes. When fruit waste was used as the substrate, the PHB content reached 17.94%, and the PHA production yield reached 2.12 g/L. These results demonstrate the feasibility of non-sterilized fermentation using low-cost waste materials, significantly reducing the overall production costs of PHAs and providing a promising strategy for economically efficient PHB production.

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INTRODUCTION

As climate change and the depletion of petrochemical resources continue, coupled with the rising awareness of global consumers towards environmental protection, the world has successively initiated plastic restriction policies and increased the demand for bioplastics. These efforts aim to improve the economics of biodegradable plastics and reduce dependence on petrochemical resources. Among the various types of bioplastics, polyhydroxyalkanoates (PHAs) are considered promising alternatives to common petrochemical-derived plastics. PHAs are biopolymers synthesized and stored within cells by bacteria using environmental carbon sources, with their most prominent feature being their exceptional biodegradability. In fact, PHAs can be rapidly and completely degraded by microorganisms in various natural environments, including soil, freshwater, and marine settings (Lee *et al.* 1996). In contrast, another common bioplastic—polylactic acid (PLA)—typically degrades effectively only under specific conditions such as controlled temperature and humidity. Therefore, PHAs are regarded as highly promising materials for addressing the increasingly severe problem of plastic pollution. To date, more than 90 bacterial species that produce PHAs and approximately 150 different monomers of PHA have been identified (Zinn *et al*. 2011).

Based on the length of the monomer side-chain R group, PHAs can be classified into short-chain length PHAs (scl-PHAs) and medium-chain length PHAs (mcl-PHAs). scl-PHAs refer to those in which the number of carbon atoms in the hydroxyl acid functional group of the monomer is between 3 and 5, while mcl-PHAs refer to those in which the number of carbon atoms in the hydroxyl acid functional group exceeds 6 (Obruca *et al*. 2015). The most common scl-PHA is poly-3-hydroxybutyrate [P(3HB)]. Due to its high crystallinity, low thermal stability, and relatively hard and brittle material properties, the development of P(3HB) for processing applications is often limited (Li *et al*. 2016). In contrast, mcl-PHAs, such as poly-3-hydroxyhexanoate [P(3HHx)], possess elasticity and higher tensile strength, and thus are widely used in various processing fields (Guo *et al*. 2022). As a potential alternative, PHAs exhibit extensive properties such as biocompatibility and biodegradability, leading to a wide range of applications in industry and agriculture (Rodriguez-Perez *et al*. 2018).

Currently, PHAs are mainly synthesized through biological methods using microbial fermentation with sugar-based feedstocks. However, the fermentation process requires energy-consuming procedures such as cultivation of dominant species, carbon source supply, sterilization, extraction, and purification, which result in excessive production costs and limit their application. One of the major factors contributing to these high costs is the use of expensive carbon substrates to cultivate microorganisms, making PHAs less competitive. Currently, the market price of PHAs ranges from $2.4 to $5.5 per kilogram, while petrochemical plastics are approximately $1.2 per kilogram (Crutchik *et al*. 2020). The raw material costs account for 30 to 50% of the total production cost of the final product (Choi and Lee 1997; Koller *et al*. 2008). Therefore, to reduce fermentation costs, the use of inexpensive and waste materials as carbon sources may represent a viable solution. Pereira *et al.* (2021) used the *Chlororaphis* subsp. *aurantiaca* strain to produce mcl-PHA using apple pomace waste as a carbon source. Another study used pomegranate peel waste as a carbon source to produce PHA (Rayasam *et al*. 2020). The study by Suwannasing *et al*. (2015) showed that the medium containing pineapple waste achieved the highest PHA concentration (1.86 g/L) and productivity (0.077 g/L·h). Additionally, oil waste has demonstrated promising results as a substrate. Song *et al*. (2008) used waste vegetable oil as a carbon source and found that the strain cultured with this oil was capable of accumulating up to 23.5% (w/w) of mcl-PHA.

Many studies have explored the application of non-sterile fermentation techniques to further reduce the production cost of PHA and enhance industrial feasibility. Non-sterile fermentation can reduce energy consumption and equipment costs associated with sterilization steps, simplifying the production process. For example, a study by Atarés *et al.* (2024) pointed out that the halophilic archaeon *Haloferax mediterranei* can use seawater as a growth medium to produce PHA under non-sterile conditions. PHA extraction can be simplified by treating the cells with pure water, further reducing production costs. Kourmentza *et al.* (2017) found that using mixed microbial cultures (MMC) to ferment organic waste under non-sterile conditions can effectively accumulate PHA, demonstrating good yield and conversion efficiency. These findings suggest that combining low-cost carbon sources with non-sterile techniques can improve the commercial competitiveness of PHA and promote its large-scale production.

This study aimed to utilize inexpensive, carbon-rich fruit waste residues and transform them into raw materials with high potential for PHA production. By employing a non-sterile fermentation process with *Bacillus* strains, PHA was produced from fruit residues, thereby reducing production costs and simplifying the process. This approach not

only enables efficient PHA production but also contributes to converting agricultural waste into high-value products, offering new perspectives for sustainable development. Additionally, by adjusting nitrogen source concentrations, optimal PHA concentration was achieved, laying the foundation for future large-scale industrial applications. Overall, the outcomes of this study have the potential to achieve competitive PHA production costs and benefit the industry by addressing environmental issues associated with fruit waste residues.

EXPERIMENTAL

Experimental Bacterial Strain

Bacillus strains used in this study were isolated from sludge samples collected at a wastewater treatment plant located in Hsinchu, Taiwan. The procedure was described previously in Chou *et al*. (2023). The collected bacterial isolates were initially screened using Nile red. To prepare Nile red-containing R2A medium, Nile red dye powder (Product Number: 72485, SIGMA-Aldrich, St. Louis, USA) was first dissolved in DMSO to create a stock solution with a concentration of 0.25 mg/mL. The stock solution was then sterile-filtered using a 0.22 μm membrane filter and stored protected from light. Subsequently, the Nile red stock solution was added to R2A agar medium containing 2.5% glucose after autoclaving and cooling to approximately 50 °C, to achieve a final Nile red concentration of 0.5 μg/mL.

The sludge samples were serially diluted using PBS and inoculated onto the Nile red-containing R2A medium. The plates were incubated at 30 °C for 2 to 7 days until single colonies were observed. Colonies exhibiting strong fluorescence under UV light were taken as positive for PHAs production. The potential isolates were chosen for further detection of polyhydroxyalkanoate synthase (*phaC*) gene fragments using Polymerase Chain Reaction (PCR). PCR was performed to screen for PHAs-producing strains using primer pairs 5'-CCGCCSTGGATCAACAAGT-3' and 5'-GTGCCGCCGAYGCA GTAGCC-3'. Each PCR reaction mixture consisted of 5 μL of bacterial culture, 1 μL of forward and reverse primers, 12.5 μL of PCR master mix, and 5.5 μL of sterile water.

The PCR conditions included an initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 60 °C for 45 seconds, and 72 °C for 1 min. A final extension was carried out at 72 °C for 10 min. The resulting PCR products were analyzed using agarose gel electrophoresis and visualized by staining with ethidium bromide.

PHAs Production in 250 mL Shaker Flasks Using Fruit Waste

PHA production was conducted using 250-mL shaker flasks with working volumes of 100 mL. Fruit waste, including discarded oranges and tangerines, was collected from the market. The collected fruit waste was homogenized into pulp using a blender, ensuring that the diameter of fruit particles in the pulp was approximately less than 5 mm. The homogenized fruit pulp was then filtered through a sieve with mesh size of 1 mm^2 and subsequently centrifuged at 4000 rpm for 20 min to obtain the supernatant. The pH of the supernatant was adjusted to pH 6 to 7 and used as the fermentation substrate. To the fruit juice, appropriate amounts of yeast peptone (YP) were added, followed by inoculation with the bacterial strain for fermentation.

Fermentation was conducted at 36 °C with agitation at 250 rpm for 72 h. Fruit juice served as the carbon source, while YP served as the nitrogen source, with yeast extract and peptone mixed in a ratio of 3:1 by weight.

1000L Bioreactor Production of PHA

PHA production was conducted using a 1000 L bioreactor with a working volume of 400 liters. The culture temperature for *Bacillus* was set at 36 °C. The pH of the culture medium was adjusted to 6.5 ± 0.2 using 5N NaOH and 10% H_2SO_4, and the vessel pressure was set to 0.4 kg/cm^2. The initial carbon-to-nitrogen ratio (C/N) was 4, with the airflow rate adjusted to 2.5 to 10 L/min and agitation speed set to 60 rpm. Fermentation was carried out for 72 h. Glucose was used as the carbon source at a concentration of 100 g/L, and YP was used as the nitrogen source.

Analysis Methods

To monitor cell growth, the samples were appropriately diluted with deionized water, and the absorbance at 600 nm was measured using a U-3000 spectrophotometer (HITACHI, Tokyo, Japan).

To measure the cell dry weight (CDW), 1 mL of culture medium was centrifuged at 4,000 rpm for 10 min at 25 °C, and the supernatant was removed. This centrifugation step was repeated five times. The resulting pellet was resuspended in 1 mL of deionized water and centrifuged under the same conditions, with the resuspension step performed twice to obtain the final pellet. The pellet was then dried in an oven at 60 °C until a constant weight was achieved, and the dry weight of the biomass was measured.

For PHB analysis, 20 mg of freeze-dried biomass was placed in a glass vial, and 2 mL of acidified methanol (3% v/v sulfuric acid, 2.5 g/L methyl benzoate) and 2 mL of chloroform were added. Methyl benzoate served as the internal standard. The vial was sealed and heated in a water bath at 100 °C for 4 h. After cooling, 4 mL of distilled water was added to the vial, and the sample was vortexed. A 1 µL aliquot of the chloroform phase was then injected into a gas chromatograph (Agilent 6890 ; Agilent, Santa Clara, CA, USA) equipped with a Agilent capillary column (J&W DB-WAX Ultra Inert, 30 m, 0.25 mm, 0.25 µm ; Agilent, Santa Clara, CA, USA) and a flame ionization detector (FID). Helium was used as the carrier gas, and the injection port, detector, and oven temperatures were set at 240 °C. The GC oven temperature was initially held at 80 °C for 2 min, then increased at a rate of 10 °C per min until reaching 240 °C, and held for 1 min. The split ratio was set at 10:1. Benzoic acid was used as the internal standard, and 3-hydroxybutyric acid (Sigma-Aldrich, Italy) was used as the external standard.

To measure sugar concentrations, liquid samples were filtered through a 0.45 µm filter and injected into a high-performance liquid chromatography (HPLC) (Agilent 1200; Agilent, Santa Clara, CA, USA) system equipped with a refractive index detector at 45 °C. Separation was achieved using a Coregel-87H3 column (Transgenomic, San Jose, CA, USA) with 8 mM H_2SO_4 as the eluent at a flow rate of 1 mL/min and a column temperature of 65 °C.

For structural analysis of PHB, ^{1}H NMR spectra and ^{13}C NMR spectra were obtained. Specifically, 10 mg of the sample were dissolved in 1 mL of chloroform-d (product 434876, Sigma-Aldrich, St. Louis, USA) and analyzed using a Bruker AV-400 NMR spectrometer, operating at 400 MHz.

RESULTS AND DISCUSSION

The Growth and PHAs Production from Fruit Waste

Figure 1 shows the growth profile and PHAs production by *Bacillus* sp. in media containing fruit waste as the carbon source with 25 g/L YP. The results indicate that fruit waste is a promising substrate for *Bacillus* sp. growth, achieving a maximum OD600 of 53.2 at 24 h of incubation. However, between 24 and 48 h, a phase decrease was observed, with OD600 values dropping to 23.8 and PHAs concentration reaching 3.23 g/L at 48 h.

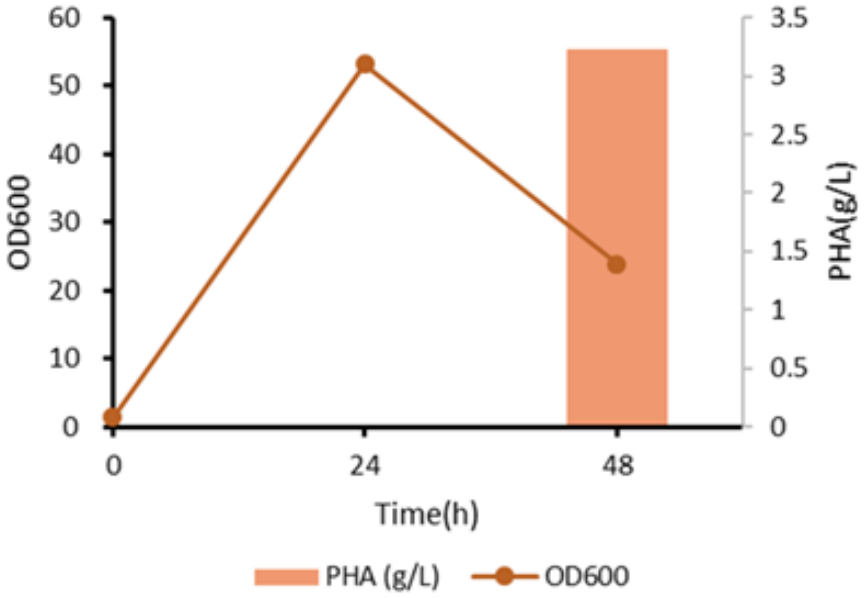

Fig. 1. Growth (OD600) and PHAs production by *Bacillus* sp for 48h in flasks with fruit waste

Effect of YP Concentration for the Production of PHAs

As shown in Fig. 1, *Bacillus* sp. exhibited rapid cell growth in a medium supplemented with YP. To further reduce fermentation costs, the feasibility of producing PHAs through non-sterilized fermentation using different concentrations of YP was analyzed. In this study, YP was used as a nitrogen source for PHAs production. The influence of various YP concentrations (0 to 25 g/L) on cell growth was demonstrated by measuring optical density (OD) values at different time points. Figure 2 illustrates the growth profile presented an exponential growth phase between 0 and 24 h. A high growth rate was recorded at 25 g/L, with OD600 reaching a value of 53.8, suggesting that *Bacillus* sp. exhibited a relatively high growth rate at YP concentration of 25 g/L. Data for PHAs production with different YP concentrations are presented in Table 1. Samples were analyzed after 48 h of cultivation. The results showed that YP concentration significantly influenced PHAs accumulation in the strain. When the nitrogen source concentration was increased to 25 g/L, the PHA concentration and PHA content were 1.29 g/L and 17.9%, respectively. This indicates that a higher nitrogen content was favorable for PHA accumulation under non-sterilized fermentation conditions, thereby reducing energy consumption in the PHA production process from fruit waste fermentation.

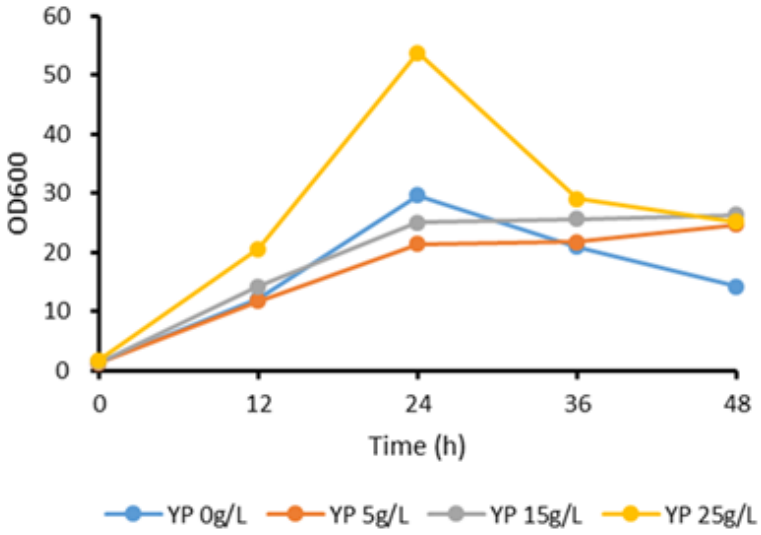

Fig. 2. Effect of YP concentration as a nitrogen source on growth (OD600) of *Bacillus* sp. for 48h in flasks with fruit waste

Table 1. Effect of YP Concentration on PHAs Production after 48 h of Cultivation

48h	CDW (g/L)	PHA (g/L)	PHA content (%)
0 g/L	6.68	0.07	1.06
5 g/L	6.84	0.43	6.26
15 g/L	13.30	2.12	15.96
25 g/L	7.20	1.29	17.94

Production of PHAs by *Bacillus* sp. in a 1000 L Bioreactor

To confirm the results observed at flask scale, the production of PHAs by *Bacillus* sp. was investigated using batch fermentation in a 1000 L bioreactor. Figure 3 presents the time profile of up-scaled PHAs production using glucose as the carbon substrate.

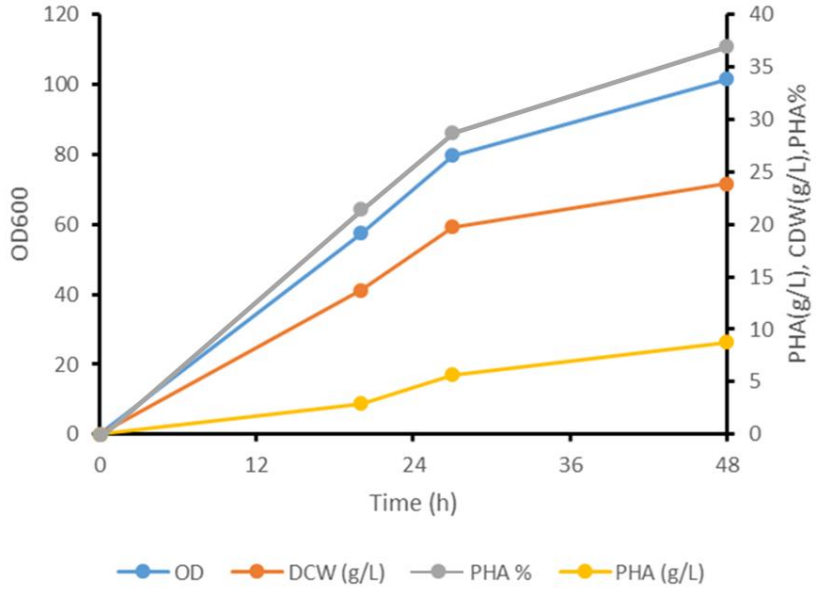

Fig. 3. Kinetics of PHAs production in 1000L bioreactor

After 48 h of fermentation, a high cell dry weight (CDW) of 23.84 g/L and a PHAs concentration of 8.79 g/L were observed. These results indicate that *Bacillus* sp. is effective for PHAs production and that the process is scalable for industrial applications.

Structure Determination of the PHB Extract from *Bacillus* sp.

The molecular structure of the polymer extracted from *Bacillus* sp. was analyzed by ^{1}H and ^{13}C NMR spectroscopy (Fig. 4). Figures 4a and 4b show the ^{1}H NMR and ^{13}C NMR spectra of the copolymers, respectively. The peak signal at 1.2 to 1.4 ppm corresponds to the -CH_3 protons, and multiplet peaks observed from 2.4 to 2.6 ppm are assigned to the presence of -CH_2 protons. The peaks at 5.2 to 5.4 ppm might be the resonance absorption of the –CH proton. The four significant signals at approximately 20, 41, 68, and 169 ppm, corresponding to -CH3, -CH2-, -CH-, and -CO- carbon moieties, respectively, belong to the PHB molecule. Results from ^{1}H and ^{13}C NMR spectroscopy indicated that the PHA polymers produced by *Bacillus* sp. were PHB polymers.

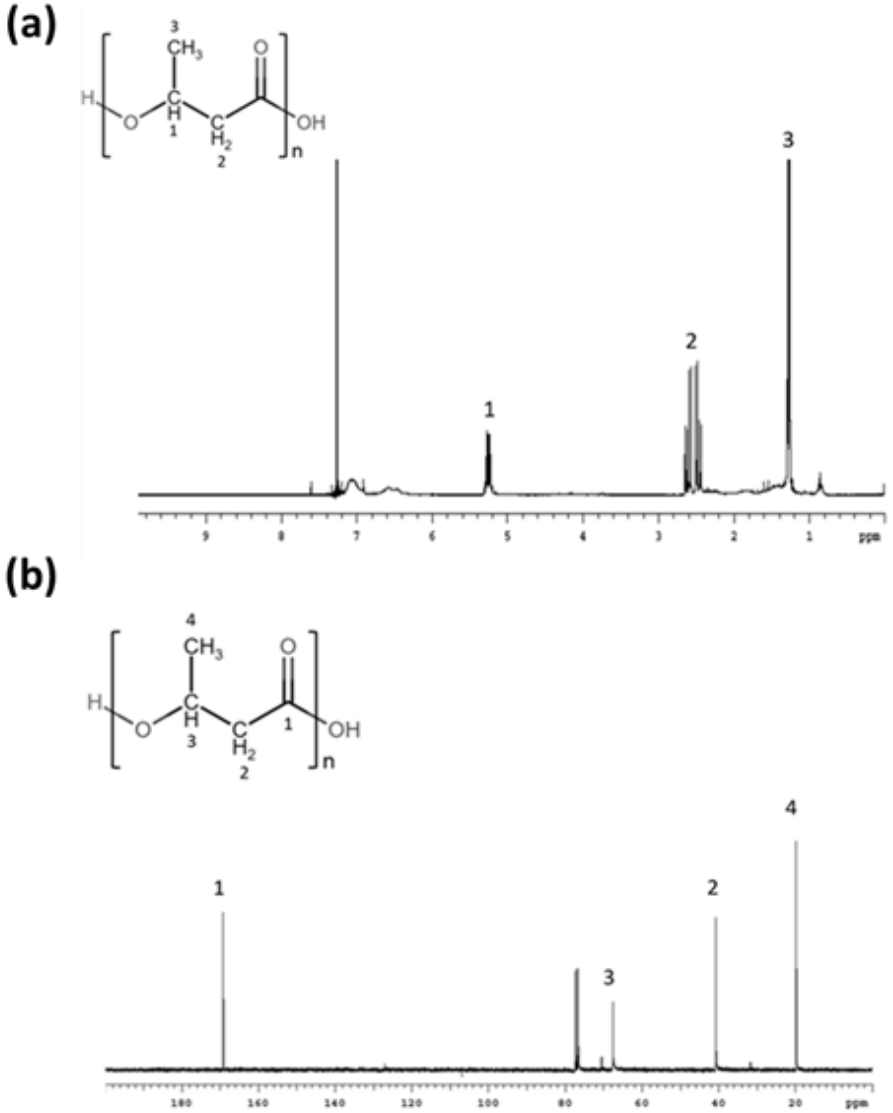

Fig. 4. ^{1}H-NMR (a) and ^{13}C-NMR (b) analysis of PHB produced by *Bacillus* sp

CONCLUSIONS

1. It was found that polyhydroxybutyrate (PHB) can be produced by the selected bacterial strain *Bacillus* sp. utilizing fruit waste as a cheap carbon source. The fruit waste, with its high sugar content, can be fermented into PHAs without expensive pretreatments.
2. In particular, *Bacillus* sp. from industrial wastewater exhibited a rapid growth rate, and PHAs can be produced through non-sterilized fermentation.
3. The process developed in this study not only reduces the overall production costs of PHAs but also offers a promising strategy for economical PHB production.

REFERENCES CITED

Atarés, L., Chiralt, A., González-Martínez, C., and Vargas, M. (2024). "Production of polyhydroxyalkanoates for biodegradable food packaging applications using *Haloferax mediterranei* and agrifood wastes," *Foods* 13(6), article 950.

Choi, J.-I., and Lee, S. Y. (1997). "Process analysis and economic evaluation for poly(3-hydroxybutyrate) production by fermentation," *Bioprocess Engineering* 17, 335-342. DOI: 10.1007/s004490050394

Chou, H. C., Chen, C. H., Huang, C. M., Wang, H. J., Hsiung, Y. C., Liang, C. H., Ou, C. M., and Guo, G. L. (2023). "Screening potential polyhydroxyalkanoate-producing bacteria from wastewater sludge," *Archives of Microbiology* 205(4), 120. DOI:10.1007/s00203-023-03446-7

Crutchik, D., Franchi, O., Caminos, L., Jeison, D., Belmonte, M., Pedrouso, A., Val del Rio, A., Mosquera-Corral, A., and Campos, J. L. (2020). "Polyhydroxyalkanoates (PHAs) production: A feasible economic option for the treatment of sewage sludge in municipal wastewater treatment plants?" *Water* 12(4). DOI: 10.3390/W12041118

Guo, W., Yang, K., Qin, X., Luo, L., Wang, H., and Huang, R.K. (2022). "Polyhydroxyalkanoates in tissue repair and regeneration," *Engineered Regeneration* 3(1), 24-40. DOI:10.1016/j.engreg.2022.01.003

Koller, M., Bona, R., Braunegg, G., Hermann, C., Horvat, P., Kroutil, M., Martinz, J., Neto, J., Pereira, L., and Varila, P. (2008). "Articles from ISBP 2004," *Time* 6, 561-565.

Kourmentza, C., Plácido, J., Venetsaneas, N., Burniol-Figols, A., Varrone, C., Gavala, H. N., and Reis, M. A. M. (2017). "Recent advances and challenges towards sustainable polyhydroxyalkanoate (PHA) production," *Bioengineering (Basel)* 4(2), article 55. DOI: 10.3390/bioengineering4020055

Lee, S.Y. (1996). "Bacterial polyhydroxyalkanoates," *Biotechnology and Bioengineering* 49, 1-14. DOI: 10.1002/(SICI)1097-0290(19960105)49:1<1::AID-BIT1>3.0.CO;2-P

Li, Z., Yang, J., and Loh, X. J. (2016). "Polyhydroxyalkanoates: Opening doors for a sustainable future," *NPG Asia Materials* 8(4), article e265. DOI:10.1038/am.2016.48

Obruca, S., Benesova, P., Marsalek, L., and Marova, I. (2015). "Use of lignocellulosic materials for PHA production," *Chemical and Biochemical Engineering Quarterly* 29(2), 135-144. DOI:10.15255/CABEQ.2014.2253

Pereira, J. R., Araújo, D., Freitas, P., Marques, A. C., Alves, V. D., Sevrin, C., Grandfils, C., Fortunato, E., Reis, M. A. M., and Freitas, F. (2021). "Production of medium-chain-length polyhydroxyalkanoates by *Pseudomonas chlororaphis* subsp. aurantiaca:

Cultivation on fruit pulp waste and polymer characterization," *International Journal of Biological Macromolecules* 167, 85-92. DOI: 10.1016/j.ijbiomac.2020.11.162

Rayasam, V., Chavan, P., and Kumar, T. (2020). "Polyhydroxyalkanoate synthesis by bacteria isolated from landfill and ETP with pomegranate peels as carbon source," *Archives of Microbiology* 202(10), 2799-2808. DOI: 10.1007/s00203-020-01995-9

Rodriguez-Perez, S., Serrano, A., Pantión, A. A., and Alonso-Fariñas, B. (2018). "Challenges of scaling-up PHA production from waste streams. A review," *Journal of Environmental Management* 205, 215-230. DOI: 10.1016/j.jenvman.2017.09.083

Song, J. H., Jeon, C. O., Choi, M. H., Yoon, S. C., and Park, W. (2008). "Polyhydroxyalkanoate (PHA) production using waste vegetable oil by *Pseudomonas* sp. strain DR2," *Journal of Microbiology and Biotechnology* 18(8), 1408-1415.

Suwannasing, W., Imai, T., and Kaewkannetra, P. (2015). "Cost-effective defined medium for the production of polyhydroxyalkanoates using agricultural raw materials," *Bioresource Technology* 194, 67-74. DOI: 10.1016/j.biortech.2015.06.087

Zinn, M., Witholt, B., and Egli, T. (2001). "Occurrence, synthesis and medical application of bacterial polyhydroxyalkanoate," *Advanced Drug Delivery Reviews* 53(1), 5-21. DOI: 10.1016/S0169-409X(01)00218-6

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